The Miscellaneous Mystery of Esophageal Cancer:

New pathogenetic and clinical insights

Brechtje A. Grotenhuis

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CONTENTS

Introduction

General introduction

Esophageal cancer is the 8th most common type of malignancy and the 6th most common cause of cancer mortality in the world.[1] Worldwide more than 400,000 patients are newly diagnosed with esophageal cancer each year. The majority of patients (>90%) is diagnosed with the two most common histological subtypes: squamous cell carcinoma or adenocarcinoma of the esophagus. Esophageal squamous cell carcinoma arises from dysplastic squamous epithelium, usually as a result of chronic irritation. Substantial alcohol intake, especially in combination with smoking, greatly increases the risk of squamous cell carcinoma and accounts for more than 90 percent of all cases of squamous cell carcinoma of the esophagus in the developed world.[2] Patients with recurring symptoms of reflux have an eightfold increase in the risk of developing esophageal adenocarcinoma.[3] Ongoing gastroesophageal reflux results in the replacement of normal squamous epithelium by a columnar-lined esophagus, which is characterized by the presence of intestinal metaplasia.[4, 5] This so-called Barrett's esophagus is the precursor lesion of esophageal adenocarcinoma, that develops through a metaplasia – dysplasia – carcinoma sequence.[6]

The incidence of esophageal cancer is rising in the Western world, mainly due to the increase in adenocarcinomas in the distal esophagus and around the gastroesophageal junction over the past decades.[7] In The Netherlands, approximately 1,500 new patients are diagnosed with esophageal cancer annually. Frequently, patients are seen at an advanced stage of the disease. Symptoms such as dysphagia and odynophagia develop when the tumor is large and obstructing the esophageal lumen. Moreover, esophageal cancer is an aggressive disease with early lymphatic and hematogenous dissemination. Hence, less than half of the newly diagnosed patients are eligible for curative therapy due to tumor invasion into adjacent organs such as heart and major airways, or due to the presence of distant metastases.[8, 9]

After having established the diagnosis esophageal cancer, an assessment of patient's fitness for major surgery and tumor staging need to be performed. The staging of esophageal cancer is generally based on the tumor-node-metastasis (TNM)

classification developed by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC).[10] The T-stage represents the depth of tumor infiltration through the esophageal wall, the N-stage describes the absence (N0) or presence (N1) of locoregional lymph node metastases, and the M-stage reflects potential distant metastases. The T-stages are shown in Figure 1. Endoscopic ultrasonography (EUS) has shown to be superior to other imaging modalities in staging patient's preoperative depth of tumor growth (T-stage) and involvement of lymph nodes (N-stage).[11-14] CT-scanning of chest and abdomen as well as external ultrasound of the neck are used for the detection of distant metastases, which often comprise involved supraclavicular and cervical lymph nodes and liver metastases.[11]

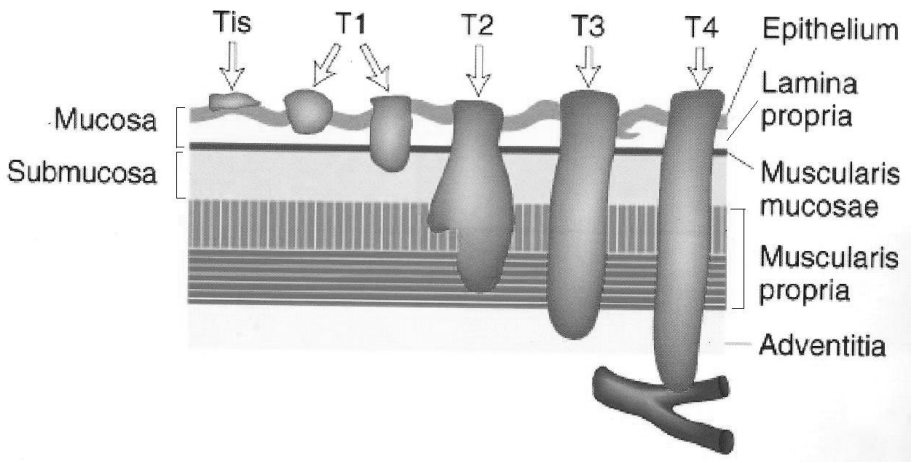

Figure 1. T-stages of esophageal cancer according to the TNM-classification. A T1-tumor is limited to the mucosa (T1a) or submucosa (T1b). In case the tumor infiltrates into the muscularis propria or through the muscle layer, the lesion is called a T2 or a T3 tumor, respectively. When an adjacent organ is invaded, the tumor is staged as T4.

The treatment of patients with esophageal cancer is highly complex and requires an interdisciplinary approach with surgeons, gastroenterologists, medical and radiation oncologists, and pathologists.[8] In the past decade this treatment became more tailored to the individual patient.[15] In patients with early esophageal cancer, recent

advances in endoscopy have changed the indication for surgery. These patients with lesions limited to the mucosa (T1a tumors) can now be treated with endoscopic mucosal resection (EMR) in experienced centers.[16-18] In patients with locally advanced esophageal cancer, the role of surgery has also evolved. Surgical therapy varies from transthoracic esophagectomy (TTE) with extended lymphadenectomy to less invasive surgical techniques such as transhiatal esophagectomy (THE) and minimally invasive esophagectomy (MIE). A large randomized controlled trial (RCT) indicated that patients with adenocarcinoma of the (distal) esophagus may have a survival benefit after TTE, whereas in patients with tumors located at the gastroesophageal junction a THE may be sufficient.[19, 20]

Esophagectomy is associated with a high operative risk.[20, 21] Although operative mortality is below 5% in high volume centers[20, 22], esophageal resection is still accompanied by substantial morbidity. Early postoperative complication rates vary between 40% and 80%, depending on the applied criteria and on the extent of resection.[20, 23, 24] Predicting the severity of complications after esophagectomy may supply important information for both patient and surgeon. The impact of postoperative complications on quality of life plays an important role in decision making whether to proceed with an operation in an individual patient with esophageal cancer. Furthermore, the prediction of severity of complications in the preoperative phase may help in informing patients and in choosing the extent of the operation. It is now widely recognized that certain high-risk surgical procedures have lower mortality and morbidity rates when performed in high-volume centers, which is also true for esophageal cancer surgery.[25, 26] For continuous improvement of esophagectomy outcome, an optimal treatment strategy for the individual patient should be based on proper patient selection by means of preoperative risk assessment as well as accurate staging of patient's TNM-stage and tumor location.

Nevertheless, the prognosis for patients with esophageal cancer who undergo esophagectomy with a curative intent rarely exceeds 40%.[27, 28] In order to improve long-term survival, the role of neoadjuvant chemoradiotherapy has been investigated recently in a large multicenter RCT[29], of which the long-term results have not been published yet.

Outline of the thesis

This thesis includes studies that address various aspects of esophageal cancer: etiology, diagnosis, staging and treatment. The thesis is divided into three parts: part A – pathogenesis of Barrett's adenocarcinoma, part B – optimization of staging and part C – preoperative risk assessment and surgical treatment.

Part A – Pathogenesis of Barrett's adenocarcinoma

Over the last decade increasing evidence has indicated that not all cells within a tumor have the same proliferative and tumorigenic ability, but only a (small) subset of the population, named cancer stem cells (CSCs). This cancer stem cell model assumes that only this subpopulation of cells has the capacity to sustain tumor growth. In **chapter 1** an overview is given of the current evidence for the existence of CSCs in malignancies, and its potential implications for treatment of solid tumors. Several markers have been employed to isolate CSCs from various malignancies, though not from esophageal adenocarcinoma. We tested whether Barrett's esophagus and esophageal adenocarcinoma might serve as a model for the CSC concept. The results of this study are described in **chapter 2**. In **chapter 3** an overview is given of the pathogenesis of Barrett's metaplasia and its progression towards esophageal adenocarcinoma. The risk factors for the development of Barrett's esophagus are reviewed as well as the different theories concerning the cell of origin of Barrett's metaplasia. Also, a summary is given of the tumorigenic steps that are involved in the development of esophageal adenocarcinoma. **Chapter 4** comprises a short report in which the reproducibility of a non-invasive mouse model of Barrett's esophagus has been investigated.

Part B – Optimization of staging

Accurate determination of patient's TNM-stage and tumor location is essential in the preoperative phase in order to determine a treatment strategy for the individual patient, in particular therapeutic decisions with regard to the optimal surgical approach and possible application of neoadjuvant therapy. In **chapter 5** the results are shown of the study in which the accuracy for determining the location of the primary

tumor according to the Siewert classification as well as the lymph nodal status per station were investigated prospectively. The preoperative assessment by endoscopy/ EUS and CT-scanning are compared with the histopathologic findings in the resection specimen (gold standard). In **chapter 6** a study on the feasibility of application of the sentinel node procedure in adenocarcinomas of the distal esophagus and GEJ is presented, with a focus on its value in the clinical setting.
In 2001 the Japanese Society of Esophageal Disease introduced its classification in which early cancers are subdivided in six successive layers of the mucosa (m1, m2, m3) or submucosa (sm1, sm2, sm3). Subdivision in six, rather than two categories has been shown helpful in directing patients to the most optimal treatment strategy. In general, current treatment options for early esophageal cancer vary from endoscopic mucosal resection (EMR) in mucosal lesions to surgical resection with extended or regional lymphadenectomy in submucosal tumors. In **chapter 7** we attempt to address the question whether the presence of occult metastases in patients with m3 and sm1 adenocarcinomas will influence the decision to undertake an endoscopic or surgical resection. In **chapter 8**, we evaluate the reproducibility of the Japanese classification of early esophageal cancer. The inter- and intraobserver variation among gastrointestinal pathologists in grading early oesophageal adenocarcinoma according to the criteria proposed by the Japanese Society for Esophageal Disease is analyzed on surgical resection specimens.

Part C – Preoperative risk assessment and surgical treatment

Individual preoperative risk assessment is essential for a proper patient selection in the preoperative phase. In **chapter 9** patients' preoperative risk assessment and the potential risk factors that can be taken care of both preoperatively and intraoperatively in order to prevent postoperative complications in patients with esophageal cancer are reviewed. Predictive factors such as age, pulmonary and cardiovascular condition, nutritional status, and neoadjuvant chemo(radio)therapy are discussed. In **chapter 10 and 11** the prognostic value of body mass index and of delay in diagnostic work-up on short-term and long-term outcome after resection of esophageal cancer are studied, respectively. Furthermore, a recently developed nomogram predicting the occurrence and severity of complications in esophagectomy patients is validated

externally in a large cohort of patients who underwent esophagectomy for cancer in an independent high-volume center. These results are shown in **chapter 12**. Finally, an international study is reported in **chapter 13**, which focuses on the best surgical treatment for early esophageal cancer with submucosal tumor infiltration (T1b). The short-term and long-term outcome of patients who underwent esophagectomy for T1b cancer through a transthoracic approach with extended lymphadenectomy is compared retrospectively to those of patients in whom a transhiatal esophagectomy was performed with regional lymph node dissection only.

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A

Pathogenesis of Barrett's adenocarcinoma

1

Cancer stem cells and their potential implications for the treatment of solid tumors

Brechtje A Grotenhuis
Bas PL Wijnhoven
J Jan B van Lanschot
Riccardo Fodde

Submitted for publication

Abstract

There is increasing evidence that a variety of human cancers is maintained by a subset of cells, the so-called cancer stem cells (CSCs), which sustain tumor growth, underlie its malignant behavior, and possibly initiate distant metastases. Notwithstanding the ongoing debate on the operational definition of CSCs, there is little doubt that at least some tumors share the hierarchical organization characteristic of normal tissues with multipotential progenitors as well as more differentiated cell types. Likewise, CSCs are characterized by the two main properties of normal stem cells: self-renewal (*i.e.* the capacity to retain their stem cell identity after each cell division) and the ability to differentiate into more specialized cell lineages. Also, CSCs seem to be intrinsicly resistant to DNA damaging agents, which make them resistant to chemo- and radiotherapy. As such, the very existence of CSCs has vast clinical implications and opens new avenues with regard to cancer treatment. The development of tailor-made CSC-targeted therapies may entail great promises: direct eradication by means of therapies directed at CSC-specific surface markers, inhibition of self-renewal signaling pathways that are crucial for CSCs, reversal of the intrinsic resistance of CSCs to chemo- and radiotherapy, and induction of terminal differentiation.

Here, we review and evaluate the current evidence for the existence of CSCs and the implications on the present management and treatment of cancer, including surgical therapy.

Introduction

Until recently, the knowledge of tumor development favored the consecutive accumulation of genetic and epigenetic[a] events at tumor suppressor genes and oncogenes in cells leading to the malignant phenotype. A number of cellular changes such as resistance to programmed cell death (apoptosis), insensitivity to growth-inhibitory signals, limitless replication and cell division, and growth induction of new blood vessels (angiogenesis), provide growth advantage to tumor cells.[1] In this 'textbook' model all cells within a certain tumor share these selective advantages and as such have similar tumor-propagating capacities. However, this model does not take into account the morphological and functional heterogeneity that characterizes most cancers. Over the last decade increasing evidence has indicated that only a (small) subset of tumor cells, the so-called cancer stem cells (CSCs), has tumorigenic properties together with the capacity to self-renew and differentiate.[2-4] Hence, CSCs may represent the driving force behind tumor growth, malignant behavior, and dissemination.

These alleged characteristics of CSCs have attracted interest from outside the scientific and medical community mainly because of the prospect that they might provide a unique target for cancer treatment. However, many questions need yet to be answered concerning the very existence of CSCs, their operational definition, and their applicability in the treatment of cancer.

Cancer stem cells: definition and controversies

The first evidence for the concept of CSCs came from hematologic malignancies. In 1997, John Dick and colleagues identified a subpopulation of human leukemia cells that was enriched for tumor initiating abilities: only a small subset of cells (earmarked by expression of the cell-surface markers CD34^{+}CD38^{-} [b]) was able to recapitulate human leukemia when injected into immunodeficient mice (NOD-SCID

a Epigenetics is the study of inherited changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence (mutations).

b CD-markers ('cluster-of-differentiation'-markers) are cell-surface markers. The marker expression CD34+CD38- refers to the presence of marker CD34 and the absence of marker CD38 on the surface of the cell.

mice[c]), in contrast to other tumor cells.[5] In specific, as few as 5,000 $CD34^+CD38^-$ cells were able to initiate tumor growth, whereas as many as 500,000 cells of the remaining tumor cell population failed to engraft. Furthermore, analysis of the engrafted mice demonstrated that $CD34^+CD38^-$ cells could expand and differentiate into a heterogeneous leukemic cell population reminiscent of the patient's primary disease.[6] In other words, tumor cells with expression of cell-surface markers other than $CD34^+CD38^-$ were again detected in the transplanted tumors, in ratios similar to those observed among leukemic cells directly isolated from the patient. $CD34^+CD38^-$ cells were then isolated a second time (now from the tumor engrafted in NOD-SCID mice) and injected into secondary recipient animals. Once again, only the newly isolated $CD34^+CD38^-$ cells were enriched in tumor propagating capacities when compared to other tumor cells. These studies provided experimental support for the capacity of this subset of cancer cells to self-renew and differentiate: two intrinsic features of stem cells in general. Accordingly, the term *cancer stem cells* (CSCs) is often employed. Alternatively, the name *tumor initiating cells* (TICs) has also been used although it only reflects their engrafting potential and does not encompass the ability of these cells to recapitulate the diversity of the patient's primary tumor (differentiation). As the latter has been shown to vary considerably depending on the experimental set up and the type of recipient animal employed[4, 7], we will here use the CSC acronym as it appropriately refers to the stem-like characteristics (self-renewal and differentiation) essential for tumor maintenance and growth.

Definition of cancer stem cells

CSCs have been defined as a distinct subpopulation of tumor cells able to propagate the primary malignancy when engrafted in immunodeficient mice at low multiplicities.[2, 8] The transplanted tumors recapitulate the complexity and heterogeneity of the patient's primary tumor. As illustrated by the original study by John Dick, these cells are thought to encompass the two main properties of normal tissue stem cells: self-renewal and differentiation.[2, 8] Self-renewal refers to the capacity of a stem cell

c NOD-SCID mice are non-obese diabetic severe combined immunodeficient mice, which lack both B- and T-cell activity. Immunodeficient mice are required for these experiments as the transplantation of human cells into mice with an intact immune system will cause a rejection of the human cells.

to divide while still retaining its ability to generate multiple cell lineages. This can be achieved by symmetric and asymmetric cell divisions. When a stem cell divides asymmetrically two different daughter cells are produced: one retains the stem cell properties (self-renewal) while the second is committed to become a specialized cell (differentiation).[9] Alternatively, symmetric cell divisions will result into two identical daughter stem cells. In both scenarios, stem cells retain their identity after each cell division thus preventing their own exhaustion. While symmetric and asymmetric cell divisions are finely tuned and balanced within a stem cell niche[d] to control tissue homeostasis, CSCs seem to have lost some of the control mechanisms thus resulting in a partially heterogeneous tumor mass when transplanted in immunodeficient recipient animals. Self-renewal and differentiation in CSCs are best assayed by serial transplantations (Figure 1): after isolation of the CSC-enriched cell population (earmarked by expression of a specific combination of CD-markers) from the patient's tumor and its injection into immunodeficent mice, a tumor is formed that recapitulates the diversity of the primary lesion. From the transplanted tumor, CSC isolation and transplantation can be serially repeated for multiple rounds with similar results ('serial transplantability'). At a consensus meeting, CSCs have indeed been defined as those cells within a tumor that possess the capacity to self-renew and to generate the heterogeneous cell population that comprises the primary tumor.[8] Hence, to date, the gold standard and operational definition of CSCs is the transplantation assay that assesses their ability to establish tumor growth when injected at low multiplicities into immunodeficient mice.[5, 6]

Recently, it has been shown that intestinal cancer is triggered only when specific mutations occur in normal stem cells but not in progenitor (transient-amplifying) cells.[10] However, the cell of origin[e] of the tumor is a distinct issue than the cancer stem cell concept.[11] CSCs are the cells that maintain the tumor mass but do not necessarily originate from the transformation of normal stem cells, and may also arise from progenitors or more differentiated cells that have acquired self-renewing capacity.[12]

d A stem cell niche is the microenvironment in which stem cells are found, which interacts with stem cells to regulate stem cell fate.

e The cell of origin is the cell that undergoes the first and rate-limiting mutation event (first hit) and thus initiates tumor formation.

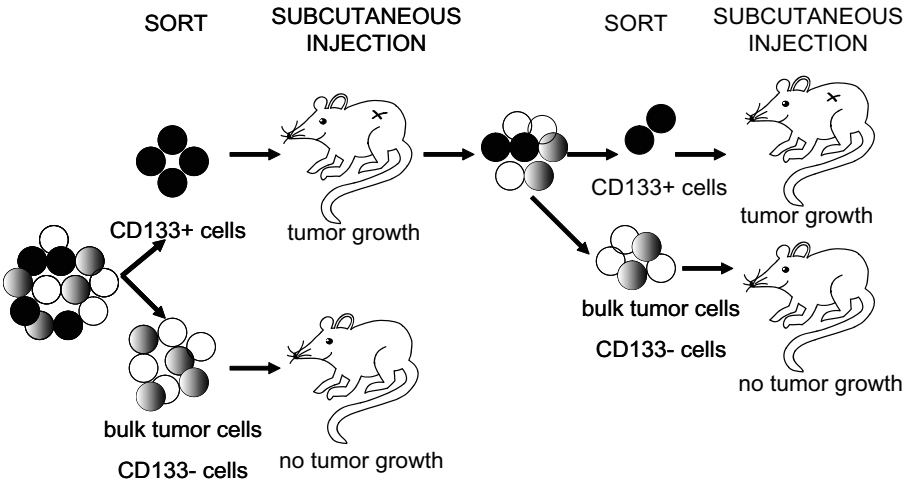

Figure 1. Schematic presentation of the transplantation assay applied in the study of cancer stem cells and the concept of serial transplantability.

Identification of cancer stem cells in solid tumors

Breast cancer represents the first solid malignancy from which CSCs were identified and prospectively isolated. A $CD44^{+}CD24^{-/low}$ cell population (comprising 1-10% of the bulk of the tumor) was identified to be significantly enriched for tumor propagating abilities. As few as 200 $CD44^{+}CD24^{-/low}$ breast cancer cells were capable of forming tumors when implanted into NOD-SCID animals.[13] In contrast, 20,000 cells that did not express these markers were unable to initiate tumor growth. Consistent with the CSC model, the $CD44^{+}CD24^{-/low}$ cell population was able to generate tumors that recapitulated the phenotypic heterogeneity of the primary cancer. Therefore, this study reported for the first time the identification of a CSC population in a solid tumor that was able to self-renew, proliferate and differentiate in order to regenerate the phenotypically heterogeneous primary tumor when injected into immunodeficient mice.

By following similar transplantation approaches, cell-surface antigens that earmark tumorigenic populations capable of transferring and recapitulating human disease into immunodeficient animals have been characterized in various other human malignancies. These include predominantly CD-markers that have proved useful for

the prospective isolation of subsets of live tumor cells enriched in CSCs (Table 1). Some of these cell-surface markers (*e.g.* CD133 or CD44) have been found to identify CSCs in a broad spectrum of cancers, though it is yet not known whether they also play a functional role in regulating CSCs.

Table 1. Cell-surface phenotype of CSCs identified in solid tumors.

Tumor type	CSC marker	Reference(s)
Breast	CD44+/CD24-/low	13
Brain	CD133+	55
Colon	CD133+	17, 18
	EpCAMhigh/CD44+/CD166+	56
Pancreas	CD44+/CD24+/EpCAM+	57
	CD133+	41
Lung	CD133+	58
Liver	CD90+CD45-	59
Melanoma	ABCB5+	50

Transplantation assay - a guide for skeptics?

To date, the transplantation assay[f] in immunodeficient mice represents the only operational definition of CSCs. As mentioned above, in this assay tumors are surgically resected from patients and then reduced into single cell suspensions by enzymatic digestion and mechanic dissociation. These suspensions are analyzed by means of flow cytometry (Fluorescence Activated Cell Sorting – FACS) to evaluate the expression of specific cell-surface antigens, and to sort the different tumor cell subpopulations to allow *in vitro* (cell culture) and *in vivo* (transplantation) analysis. Transplantations can either be performed subcutaneously or orthotopically[g].

Notwithstanding the merits of the operational definition by transplantation assays, it is important to recognize that CSC identification efforts that rely exclusively on tumorigenicity assays of sorted cancer cell subpopulations have clear limitations. An important question is whether the use of immunodeficient mice is a reliable model for studying human cancer. This method does not take into account the extent to which tumor growth might be positively or negatively affected by the macro- and

f The transplantation assay is the experimental standard for identification of CSCs: a distinct subpopulation of tumor cells can be identified that has the ability to propagate and recapitulate the complexity and heterogeneity of the primary tumor when engrafted in immunodeficient mice.

g An orthotopic injection is an injection of tumor cells in the tissue/organ of origin; *e.g.* injection of a breast cancer-cells into the mammary fat-pad of the mouse.

micro-environment provided by the host animal. The presence of residual immune function in the recipient animals can significantly influence the efficiency of human cell engraftment. For example, NOD-SCID mice showed improved engraftment rates with leukemia cells when compared to SCID mice, which also lack B- and T-cell activity though to a lesser extent.[5, 6] NOD-SCID mice still retain natural killer (NK) cell activity, which is thought to reject most transplanted human cells.[14] Accordingly, it was shown that the lack of NK-, B- and T-cell activity (as established in the so-called NOD-SCID-IL2Rγ-null mice) has dramatic effects on tumor engraftment rates and on the estimated frequency of tumor initiating cells: approximately one in a million melanoma cells form tumors in 'conventional' NOD-SCID mice compared to one in four in NOD-SCID-IL2Rγ-null mice.[7] Hence, depending on the type of immunodeficient recipient animals employed, transplantation assays can lead to large variations in the estimate of the frequency of tumorigenic cancer cells.

Another factor that affects tumor initiating cell estimates is represented by the transplantation site. Striking differences have been observed when tumor cells were transplanted into different microenvironments: intracranial injection of putative CSCs of glioblastoma consistently induced neoplastic growth, while significantly reduced rates of tumor formation were observed when the same cells were injected subcutaneously.[15] In the case of colon CSCs (where orthotopic transplantation experiments, *i.e*. by injection in the intestinal wall, have been described[16] but are relatively difficult to implement), marked differences in the tumor initiating frequency of cells injected in the kidney capsule compared to the subcutaneous technique were reported.[17, 18]

The variable frequencies of tumor initiating cells estimated in different malignancies depending on the experimental set-up have raised doubts on the validity of the CSC model.[7, 19] However, as emphasized by John Dick and colleagues[20], the fundamental concept underlying the CSC model is independent of the absolute tumor initiating frequency of these cells. Instead, the model proposes that the functional heterogeneity within tumors reflects a hierarchical organization with a distinct population of cells that can initiate malignant growth *in vivo* while the remaining cells cannot. Nevertheless, it cannot be *a priori* assumed that all types of malignancies, with no exception, comply with the CSC model.

In conclusion, notwithstanding its reported limitations, transplantation into immunodeficient mice is the only available method to operationally define whether human CSCs have the potential to form tumors. At present, the main alternative is represented by *in vitro* cell culture methods that select for the stem cell capacity of growing in suspension in an undifferentiated state (*spheroids)*. These non-adherent spheres were shown to be enriched in both stem- and progenitor cells and have been established from a broad spectrum of tumor types (*e.g.* neurospheres from glioblastomas[21], mammospheres from breast cancer[22], colospheres from colorectal cancer[18]). Primary sphere-derived cells are able to initiate secondary spheres as well as differentiate to give rise to various specialized cell lineages, thereby again demonstrating the two fundamental properties of stem cells, *i.e.* self-renewal and differentiation. Although promising, these *in vitro* suspension cultures of CSCs still rely on transplantation in immunodeficient mice to assay their *in vivo* differentiation potential.

Cancer stem cells and dissemination

Only a minority of cancer cells has the ability to detach from the primary tumor mass, invade the surrounding tissues and disseminate to distant organ sites to form metastases. Metastatic potential depends on multiple factors that determine overall tumor growth, angiogenesis and invasion. Among epithelial malignancies (carcinomas), the so-called epithelial-to-mesenchymal transition[h] (EMT) represents a crucial event in invasion and metastasis. In adult tissues, EMT is involved in processes such as wound healing, tissue regeneration and organ fibrosis.[23] In cancer, EMT is thought to endow tumor cells the capacity to detach from the primary tumor mass by loosing cell adhesive properties and acquiring motile features, thus enabling local invasion, intravasation into blood or lymph vessels, extravasation and the recapitulation of the primary tumor at distant sites through the reverse process of mesenchymal-epithelial transition (MET).[23, 24]

h The term epithelial-mesenchymal transition (EMT) refers to a process where epithelial cells undergo loss-of-cell-adhesion features and acquire increased cell mobility.

In colon cancer, constitutive activation of the Wnt signaling pathway[i] is preferentially observed at the invasive front of the primary lesion where it earmarks cells undergoing EMT and detaching from the primary tumor into the stromal microenvironment.[25, 26] Cells undergoing EMT may also develop resistance to anticancer agents. In fact, EMT can be induced by stress conditions such as neoadjuvant chemo- or radiotherapy.[27] These observations have led to the concept of 'migrating CSCs' where EMT cells have acquired stem cell features that give them the plasticity to adapt to different environments and eventually metastasize to distant organ sites.[25]

Indeed, recent evidence suggests that there may be a direct link between EMT and acquisition of CSC properties. Induction of EMT in a mammary epithelial cell line resulted in enrichment in cells with CSC characteristics such as tumor-seeding ability and the expression of cell-surface markers reminiscent of breast CSCs.[12, 28] This so-called phenotypic plasticity[j] suggests that a dynamic equilibrium may exist between CSCs and non-CSCs within tumors possibly regulated by the microenvironment, the CSC niche, similar to normal stem cell niches controlling stem cell number and proliferation.[29, 30] A more recent study has demonstrated that the stemness of colon cancer cells is at least in part regulated by the microenvironment.[31]

The studies showing that CSCs and non-CSCs are mutually exchangeable have been interpreted as the denial of the very CSC model: cancer stemness is not a cell autonomous state and in theory each tumor cell can be converted into a CSC upon exposure to specific environmental cues. Nevertheless, the possibility of a bidirectional exchange between stem-like and more committed cancer cells does not in itself undermine the essence of the CSC concept according to which tumors encompass distinct cell identities earmarked by specific phenotypic and functional characteristics.[32] Moreover, the CSCs' intrinsic capacity to undergo EMT may provide an explanation for the variation in frequency of CSCs between different malignancies[7] and perhaps even between patients. Individual and tissue-specific variations in the expression of microenvironmental signals are likely to affect relative numbers and malignant behavior of CSCs among different tumors and patients. Nevertheless, it

i The Wnt signaling pathway refers to a sequence of biochemical events capable of transducing an extracellular signal to the nucleus by modulating expression of specific target genes. Wnt plays an important role in the regulation of different stem cell niches and its constitutive (*i.e.* uncontrolled) activation represents the main cause of colon cancer.

j The phenotypic plasticity of a cell is the ability to change its phenotype in response to changes in the environment.

should be noted that thus far no direct experimental evidence has been provided showing that CSCs indeed underlie the process of dissemination and metastasis.

Therapeutic implications

The identification of CSCs has potentially vast implications: not only for cancer research but also (and more importantly) for the clinical management of cancer in terms of the development of future targeted therapies. For example, in genome-wide expression analyses of whole tumor preparation (in which CSCs often represent a minor subpopulation), CSCs are poorly represented in the resulting gene signatures meant to predict prognosis of the cancer patient. It will be interesting to assess whether CSCs signatures will be more accurate predictors of survival and of response to treatment. Here, we will focus on the consequences and implications of the CSC model for neoadjuvant therapy and surgical intervention.

Implications for neoadjuvant therapy and surgery

Together with surgery, neoadjuvant chemo- and/or radiotherapy are widely used in the treatment of specific stages of various solid malignancies such as breast and colorectal cancer. At present, neoadjuvant treatment targets the proliferative potential of the tumors by killing rapidly dividing cells within the bulk of the tumor. However, even a therapy that successfully affects the vast majority of tumor cells and is considered to be highly efficient based on the initial shrinkage of the primary mass, will most likely leave CSCs unaffected which will eventually underlie relapse. This hypothesis is plausible in view of several characteristics of CSCs, which make them resistant to DNA damage-induced cell death. An intrinsic or acquired resistance to cytotoxic agents or radiation is indeed likely to play a role.[33] The underlying mechanisms include increased DNA damage recognition and repair, impairment of tumor apoptotic pathways, and reduced accumulation of chemotherapeutic agents through enhanced drug efflux (toxic agents are pumped out by the CSCs).[33] Furthermore, it has been shown that, at least in some tumor types (*e.g.* leukemia and ovarian cancer), CSCs may encompass a slow-cycling subpopulation[34-36] (the so-called quiescent or dormant CSCs), which may escape conventional chemotherapeutic regimens that target actively cycling cells.

Evidence for preferential resistance of CSCs to neoadjuvant therapy has been provided. In glioblastoma the fraction of CD133+ CSC population was found to be enriched after radiation.[37] Also, compared to their CD133- counterparts, CD133+ glioblastoma cells exhibited lower rates of apoptosis in response to chemotherapy.[38] In breast cancer, significantly increased levels of cells expressing the CSC-markers have been reported in residual tumor cell populations in patients who had received conventional chemotherapy compared to the tumors evaluated prior to therapy.[39, 40] Also, in pancreatic cancer CD133+ cells showed increased resistance to chemotherapeutic agents.[41] The cellular and molecular mechanisms underlying CSCs' innate resistance to chemo- and radiotherapy include expression of specific interleukins[42, 43], lower levels of reactive oxygen species (ROS)[44], and ATP-dependent drug transporters[33]. Overall, these data point to the clinical relevance of the CSC model by providing experimental evidence for increased resistance of CSCs to conventional neoadjuvant therapies directed at bulk populations of tumor cells.

The CSC model predicts that complete cure can not be achieved unless all CSCs are removed, either surgically or in combination with neoadjuvant chemo- or radiotherapy. Accordingly, it has been hypothesized that preoperative labeling of CSCs can give valuable information on the best surgical approach with regard to tumor invasion into adjacent structures and lymph node dissection, but also on the presence of potential circulating tumor cells (CTCs).[45] In fact, significantly less CTCs were detected in portal blood after tumor mobilization by laparoscopic surgery compared to open surgery in colorectal cancer patients[46], thus further supporting the reduced tumor cell dissemination with a no-touch operation technique.

Therapeutic opportunities

The implications for neoadjuvant and surgical therapy highlight the therapeutic promise of CSC-directed treatment strategies. Potential therapies which are selective to CSCs and not toxic to normal stem cells hold great promise for the effective and potentially curative impact on many human malignancies. CSC-targeted strategies (Figure 2) may include (a) direct targeting of CSCs through antibodies directed at CSC-surface markers, (b) blocking essential self-renewal signaling pathways, (c) reversal of CSCs' innate resistance to chemo- and radiotherapy, and (d) induction of CSC terminal differentiation.[47, 48]

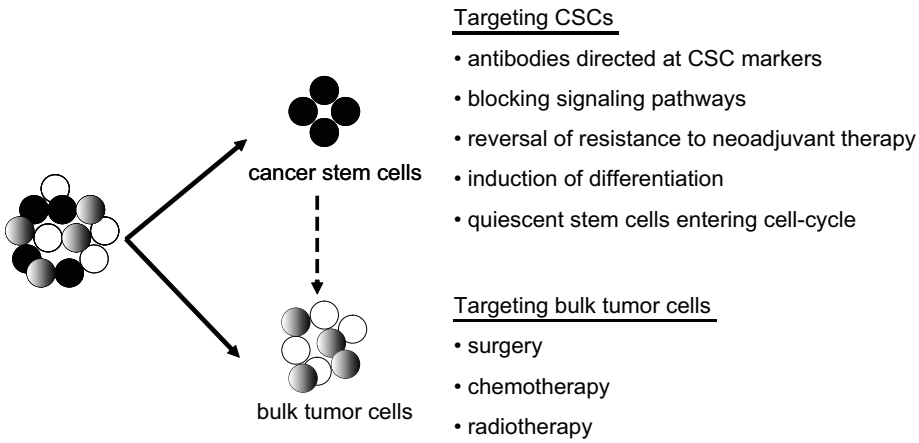

Figure 2. Therapeutic strategies for cancer treatment directed at cancer stem cells and bulk tumor cells.

Direct targeting of CSCs through their CSC-surface markers seems an obvious yet not straightforward solution as most of the CD markers identified to isolate tumor cell subpopulations enriched in CSCs are also expressed in normal cells. Therefore, the definition of an appropriate 'therapeutic window' will represent a major challenge for the clinical implementation of this CSC-targeted strategy. Nevertheless, selective killing by means of administration of an anti-CD44 and anti-ABCB5 antibodies in acute myeloid leukemia (AML)[49] and melanoma[50], respectively, was sufficient to inhibit tumor growth, thereby supplying the initial proof of principle for the potential therapeutic applications of this approach.

Blocking essential self-renewal signaling pathways such as Wnt, Hedgehog and Notch (known to be activated in CSCs) also represents a promising therapeutic strategy. In colon cancer, small-molecules inhibiting the Wnt- and Notch pathway have recently been identified.[51] Nevertheless, these pathways play equally important roles in the maintenance of normal stem cells, which again points to the potential cytotoxic side-effects of such small molecules on normal tissues. Specific targeting of signals that regulate CSC resistance to chemo- and/or radiotherapy, may also represent a promising approach for adjuvant therapy to enhance the efficacy of conventional chemo- and radiotherapy regimes. Inhibition of the enhanced drug

efflux characteristic of CSCs has been achieved by antibodies directed against ABC-transporters[k] in melanoma.[52] In colon cancer, further support for the potential utility of CSC chemosensitizing agents was provided: pretreatment of $CD133^+$ colon CSCs with an interleukin-4-specific neutralizing antibody[l] enhanced apoptosis mediated by chemotherapy both *in vitro* and *in vivo*.[43]

Finally, differentiation therapy might induce CSCs to differentiate into more mature tumor cells thus limiting their tumorigenic and invasive potential. For example, salinomycin has been described as the first compound that is able to decrease the proportion of CSC-phenotypic breast cancer cells and to eradicate selectively the tumor by inducing terminal epithelial differentiaton.[53] The alleged existence of quiescent[m] CSCs and their established intrinsic resistance to DNA-damaging agents which rely on active cell division also opens novel therapeutic avenues. In a mouse model for AML, quiescent stem cells can be induced to enter the cell cycle by treatment with growth factors (e.g. G-CSF) thus rendering them more susceptible to conventional chemotherapy. In combination with cell cycle-dependent chemotherapy, G-CSF treatment was indeed shown to significantly enhance induction of apoptosis and elimination of human primary AML stem cells *in vivo*[54].

k ABC-transporters (ATP-binding cassette transporters) are transmembrane proteins that utilize the energy of ATP hydrolysis to carry out a broad spectrum of biological processes including the transport of various substrates across cell membranes, *e.g.* metabolic products, lipids and drugs.

l Interleukin-4 (IL-4) is a cytokine that stimulates the immune system. It has been previously shown that IL-4 plays a central role in resistance to induction of cell death (apoptosis). By neutralizing its effect by means of an antibody, apoptosis caused by chemotherapy can be increased. In other words, cells are sensitized to chemotherapy.

m Quiescence is the state of a cell when it is not dividing; it refers to the G_0 phase of a cell in the cell cycle.

Conclusion

The growing evidence indicating that CSCs drive and maintain various types of human malignancies has important implications for the treatment of patients. However, over the years the development of CSC-targeted therapies has faced a number of potential hurdles, including normal stem cell toxicity and treatment resistance. These must be considered carefully in order to maximize the chance that such therapies will be successful. Following the more recent findings suggesting the interaction between EMT and CSCs and the regulation of the stemness of cancer cells by the tumor microenvironment (indicating that the distinction between CSCs and non-CSC-tumor cells may not be the clear dichotomy as it was proposed originally), cancer therapy should ideally include agents targeting both CSCs and non-CSCs (Figure 2).

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Barrett's esophageal adenocarcinoma encompasses tumor-initiating cells that do not express common cancer stem cell markers

Brechtje A Grotenhuis
Winand NM Dinjens
Bas PL Wijnhoven
Petra Sonneveld
Andrea Sacchetti
Patrick F Franken
Herman van Dekken
Hugo W Tilanus
J Jan B van Lanschot
Riccardo Fodde

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Abstract

Accumulating evidence has suggested that tumors have a hierarchical organization where only the cancer stem cells (CSCs) have tumor-initiating properties. Several surface antigens have been employed to isolate CSCs from various malignancies, though not from esophageal adenocarcinoma (EA). We tested whether Barrett's esophagus (BE) and EA might serve as a model for the CSC concept.

In vivo assays were performed by transplantation of serially diluted bulk EA cells into NOD-SCID mice to establish the presence and frequency of tumor-initiating cells. These were found to be present in approximately 1:64,000 cells. The transplanted tumors fully recapitulated the primary lesions. Subsequently, a panel of previously established CSC markers was employed for immunohistochemistry. CD24, CD29 and CD44 showed heterogeneous staining in EA. Nuclear ß-catenin accumulation increased during progression from metaplasia to dysplasia, and was often observed in the basal compartment with CD24 and CD29 staining. However, the overall staining patterns were not such to clearly point out specific candidate markers. Accordingly, all markers were employed to sort the corresponding subpopulations of cancer cells and transplant them at low multiplicities in NOD-SCID mice. No increased tumor-initiating capacity of sorted EA cells was observed upon transplantation.

These results indicate that tumor-initiating cells are present in EA, thus reflecting a hierarchal organization. However, antibodies directed against novel surface antigens are needed to detect subpopulations enriched for CSCs in EA by transplantation assays.

Introduction

Esophageal exposure to refluxed gastric contents (gastro-esophageal reflux disease, GERD) represents the major risk factor in the replacement of squamous epithelial cells lining the esophagus by an intestinal columnar epithelium, a condition known as Barrett's esophagus (BE)[1]. The intestinal metaplasia characteristic of BE features mucous glands and goblet cells[2,3], and is recognized as a premalignant condition[4, 5]. It is generally accepted that the development of esophageal adenocarcinoma (EA) follows a metaplasia (BE) → dysplasia → carcinoma sequence characterized by specific genetic and epigenetic changes[6]. However, the cell of origin involved in the transition from normal squamous epithelium into intestinal metaplasia has not been identified yet. Transdifferentiation of esophagus-specific cell types into intestinal-like columnar epithelial cells as the result of GERD may explain the observed histological changes[7, 8]. On the other hand, it is also plausible that resident stem cells are involved in this process. In this alternative scenario, BE develops when GERD damages the superficial layers of the esophageal squamous epithelium, thereby exposing the basal epithelial layers (where stem cells are thought to reside[9]) to tissue-damaging agents thus triggering abnormal differentiation programs and metaplastic changes[6, 7]. Attempts towards the isolation and characterization of stem cells of the normal esophageal epithelium have not been successful thus far, although more recently CD34 has been shown to represent a stem cell marker in the mouse esophagus[10].

Patients with BE have a significantly higher risk of developing EA when compared to the general population[11, 12]. The overall estimate of cancer incidence in BE varies between 6 and 7 cases per 1,000 person-years (0.6 – 0.7% per year)[13, 14]. Currently, high-grade dysplasia represents the most reliable predictor of progression to EA. Surgery (by means of radical esophagectomy) is the best curative option for adenocarcinoma of the distal esophagus or gastro-esophageal junction, provided that neither tumor infiltration in adjacent organs nor distant metastases are present. Nevertheless, even in this selected patient group, five-year survival rarely exceeds 40%[15-17].

In recent years, accumulating experimental evidence has suggested that tumors have a hierarchal organization where a minority of cells, the cancer stem cells (CSCs),

is characterized by tumor-initiating properties when transplanted into immuno-incompetent recipient animals[18-21]. The CSC concept has immediate therapeutic consequences: if cancer growth is sustained by CSCs, then curative therapy will require targeting this specific subpopulation[22, 23]. Combinations of cell-surface antigen markers are currently being employed for the prospective isolation of these tumor-initiating cells and their subsequent *in vitro* and *in vivo* functional analysis[24-26]. During the past years, specific combinations of these surface antigens, the so-called Cluster of Differentiation (CD) markers, have been shown to enrich for CSCs in different tumor types, among which colorectal adenocarcinoma[27-29], but not in EA of Barrett's origin.

Because of their etiology and natural history, BE and EA are likely to represent a unique model to study the cellular and molecular mechanisms underlying the onset and malignant behavior of CSCs. Here, we present an initial set of experiments aimed at the identification of tumor-initiating cells in EA as well as the evaluation of the expression pattern of CSC markers previously reported in other tumor types during the metaplasia → dysplasia → carcinoma sequence.

Material and methods

In vivo transplantation assays

To establish the presence and frequency of tumor-initiating cells in EA, esophagectomy resection specimens from patients with lesions located in the distal esophagus with or without infiltrative growth at the gastro-esophageal junction were employed. Only tumors of patients in whom no neo-adjuvant chemo- and/or radiation therapy was applied were considered appropriate for the transplantation experiments. In the period between January 2008 and February 2009, a total number of 17 esophagectomy resection specimens were obtained. Tumor samples were obtained from the resection specimens immediately after arrival at the Department of Pathology, according to the code for adequate secondary use of tissue (code of conduct "Proper Secondary Use of Human Tissue") established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

In general, tumor tissue was minced and dissociated to a single cell suspension after a 3 hours digestion with DNAse-I (50 µl/ml, Roche, Basel, Switzerland) and collagenase A (3 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) at 37˚C. Samples were then passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA, USA) and subsequently incubated with antibodies at 4˚C for 30 minutes. Dead cell discrimination was performed with Hoechst33258 (1:10,000 Invitrogen, Carlsbad, CA, USA). Fluorescence-activated cell sorting (FACS) was performed with a FACSAria™ cell sorter (BD Biosciences). Cells were sorted into RPMI medium supplemented with 5% fetal calf serum and penicillin-streptomycin. After sorting, cells were resuspended in a 1:1 mixture of this RPMI-based medium and Matrigel™ (BD Biosciences) in a total volume of 100µl and injected subcutaneously into NOD-SCID mice not older than 8 weeks. A maximum of four injection sites for each recipient mouse was applied. The relative frequency of tumor-initiating cells in EA was estimated by limiting dilution analysis[30] (analogous to that employed in the identification of stem cells in other tissues). Limiting dilution analysis calculations were done by using the L-Calc™ Software (StemCell Technologies) (http://www.stemcell.com/product_catalog/product_catalog_index.aspx?type=catalog_item&id=618).

First, in order to purify bulk tumor cells from contaminating cells present in the surgical specimens, we employed antibodies raised against well-characterized endothelial (CD31-APC, dilution 1:50, EMELCA Bioscience, Breda, The Netherlands), and hematopoetic (CD45-APC, dilution 1:200, BD Biosciences, and CD235A-APC, dilution 1:1000, BD Biosciences) antigens and depleted these so-called lineage-positive (Lin^+) cells by gating the $CD31^-/CD45^-/CD235A^-$ population[24]. The resulting Lin^- tumor cells (i.e. enriched for epithelial cells) were injected subcutaneously at different multiplicities, from 10^5 to 10^3 ('limiting dilution'), into NOD-SCID mice.

In a second set of transplantation assays, surface antigen markers that have previously been reported as (cancer) stem cell markers were employed to sort subpopulations of EA cells by FACS (CD24[31], CD29[31], CD34[10], CD44[29], CD133[27, 28], CD166[29], EpCAM[29], β-catenin[32]). To this aim, the tumor-derived single cell suspensions were incubated with the following antibodies: CD24-PE (dilution 1:10, BD Biosciences), CD29-FITC (dilution 1:25, Bioconnect, Huissen, The Netherlands), CD34 (dilution 1:500, EMELCA Bioscience), CD44-FITC (dilution 1:1000, BD Biosciences), CD133-PE (dilution 1:100,

Miltenyi Biotec, Auburn, CA, USA), CD166-PE (dilution 1:100, R&D Systems, Abingdon, UK), and EpCAM-PerCP (dilution 1:100, BD Biosciences). Although most of the above Ab's are directly labelled which bypass the use of isotypic controls, their staining specificity was verified against isotypic controls at the same concentration. Also, the staining pattern of individual tumors is per se indicative of specific staining for all the markers here employed.

Subpopulations of Lin^- cells positive for the different cancer stem cell markers were then sorted by FACS and injected subcutaneously at a fixed multiplicity (n = 5,000) in NOD-SCID mice. The viability of sorted cells was estimated in an independent set of experiments to be ~80-90% (data not shown). Injections of 5,000 Lin^- cells were employed as controls. This multiplicity (5,000) was chosen assuming an enrichment factor of at least 10 fold when compared with the bulk Lin^- cancer cells. The recipient mice were scheduled for an observation period of 6 months following transplantation, unless discomfort following tumor growth indicated earlier termination of the experiment.

In addition to the above mentioned panel of (cancer) stem cell markers, we also attempted the prospective isolation of ALDH1-positive tumor cells from esophageal adenocarcinomas (data not shown). As for ALDH1 as a potential CSC marker[33], preliminary experiments showed that it is technically feasible to sort Aldefluor–positive and –negative cells, (data not shown). Subsequently, we injected these subpopulations from 4 individual tumor samples. However, no tumor growth could be observed after 6 months. Eventually, also in view of the lack of ALDH1 IHC analysis and of the appropriate positive controls (i.e. injection of 100,000 Lin- cells) for the 4 tumors FACSorted according to ALDH1 expression, we decided to omit these data from the present mansucript.

Immunohistochemistry (IHC)

A panel of previously established (cancer) stem cell markers (CD24, CD29, CD34, CD44, CD133, CD166, EpCAM, β-catenin) was employed for IHC analysis. To this aim, we used resection specimens from 20 BE patients who underwent esophagectomy for either high-grade dysplasia or EA. The surgical specimens were transported to the Department of Pathology, where an experienced gastro-intestinal pathologist

(HvD) selected separate tissue samples indicative for Barrett's metaplasia and EA. Barrett's mucosa was diagnosed by both macro- and microscopy when epithelium of the intestinal type was demonstrable in the tubular esophagus[34], including the presence of goblet cells, characteristic for intestinal metaplasia (mucin-filled cytoplasm that stains positively with Alcian blue)[35]. In this series of 20 formalin-fixed paraffin-embedded resection specimens, a total of 15 areas of metaplasia, 12 areas of low-grade dysplasia (LGD), 18 areas of high-grade dysplasia (HGD) and 28 areas of adenocarcinoma were identified in hematoxylin-eosin (H&E) stained sections. From each formalin-fixed, paraffin-embedded tissue block, 14 consecutive 2 µm sections were cut. Subsequently, the first 13 of 14 consecutive sections were stained by IHC in the specific sequence of CD24, ß-catenin, CD29, ß-catenin, CD34, ß-catenin, CD44, ß-catenin, CD133, ß-catenin, CD166, ß-catenin and EpCAM, respectively. The last section of the series was employed as a negative control. Multi-tissue sections containing pancreas-, liver-, stomach-, colon-, and tonsil-samples were used as positive controls to test antibody specificity.

Sections were mounted on aminoacetylsilane-coated slides (Starfrost, Berlin, Germany) and IHC was performed using the Envision system (DAKO, Glostrup, Denmark). In brief, the sections were de-waxed in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was inhibited by incubating the sections in methanol with 3% hydrogen peroxide for 20 minutes. For all antibodies, microwave pretreatment (700 W) in Tris-EDTA (pH 9) was performed for 15 minutes, except for CD29, CD44 and EpCAM, for which pretreatment in citrate buffer (pH 6) was used. All primary antibodies, i.e. against CD24 (clone CLB-gran-B-Ly/1-1B5, dilution 1:200, RDI, Concord, MA, USA), CD29 (clone 4B7R, dilution 1:100, Abcam, Cambridge, UK), CD34 (clone QBEEnd/10, dilution 1:100, Thermo Scientific, Fremont, CA, USA), CD133 (clone AC133, dilution 1:100, Miltenyi Biotec), CD166 (clone MOG/07, dilution 1:80, Abcam), EpCAM (clone Ber-EP4, dilution 1:00, Abcam) and ß-catenin (clone 14, dilution 1:100, BD Biosciences) were incubated on the sections for one hour at room temperature, except the CD44H antibody (clone IM7, dilution 1:100, BD Biosciences) that was incubated overnight at 4°C. Negative controls were performed by omitting the primary antibody. This was followed by the secondary incubation step of the Envision system. Diaminobenzidene tetrachloride

from the Envison kit, prepared according to the manufacturer's instructions, was employed for visualization. Tissues were counterstained with hematoxylin. Finally, the slides were dehydrated through a graded ethanol series, cleared in xylene and mounted in Malinol (Chroma-Gesellschaft, Köngen, Germany).

The specific areas of metaplasia, LGD, HGD and adenocarcinoma identified by the pathologist (HvD) in the H&E stained sections, were compared with their counterparts in the 14 consecutive sections, stained with the above mentioned antibodies. The immunoreactivity patterns were evaluated independently by three investigators (BAG, HvD and WNMD). Discrepant results were re-evaluated by all three investigators to reach consensus. All 15 sections from each tissue block (i.e. the H&E section and the 14 consecutively stained sections) were microscopically evaluated at both high and low magnifications and scored according to multiple parameters: percentage of positive cells in the defined areas (>25% of cells as ++, 1-25% of cells as +, and no cells as 0), their location, a potentially increased staining during progression in the metaplasia → dysplasia → carcinoma sequence, and potential overlaps in the staining patterns.

Results

Tumor-initiating cells are present, though rare, in EA

In order to assess whether tumor-initiating cells are present in EA, limiting dilution transplantation assays were performed with a consecutive series of surgically resected tumors. Lin^- (bulk) EA cells from seven patients were transplanted subcutaneously in NOD-SCID mice at different multiplicities, ranging from 10^5 to 10^3 cells. Out of the 7 primary tumors employed, four were of moderate and three of poor differentiation grade.

Tumor growth was almost invariably observed when 10^5 bulk EA cells were injected, with the only exception of a poorly differentiated adenocarcinoma (tumor #5 in Table 1). Transplantation with 10^4 Lin^- cells also resulted in frequent tumor growth though not in all cases (4/7) and with lower incidence (1 out of 3-4 injections). No tumor growth was observed when 1,000 Lin^- cells were injected (Table 1).

Table 1. Limiting dilution assay: observed tumor growth after transplantation of Lineage-negative bulk esophageal adenocarcinoma cells into NOD-SCID mice.

TUMOR	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Differentiation grade	Moderate	Moderate	Moderate	Poor	Poor	Moderate	Poor
No. injected cells							
- 100,000	4/4	3/3	2/4	3/3	0/2	2/2	1/1
- 10,000	1/4	1/3	1/4	0/3	0/4	0/4	1/4
- 1,000	0/4	0/3	0/4	0/3	ND	ND	0/4

ND = not done

By performing limiting dilution analysis (L-Calc™ Sotware, StemCell Technologies) it was estimated that the frequency of tumor-initiating cells in the Lin^- population of EA (n=7) was 1 in 64,287 cells (s.e. 18,049). The histology of all the tumors obtained by transplantation of bulk EA cells in NOD-SCID mice fully resembled the differentiation grade of the primary tumors (Figure 1).

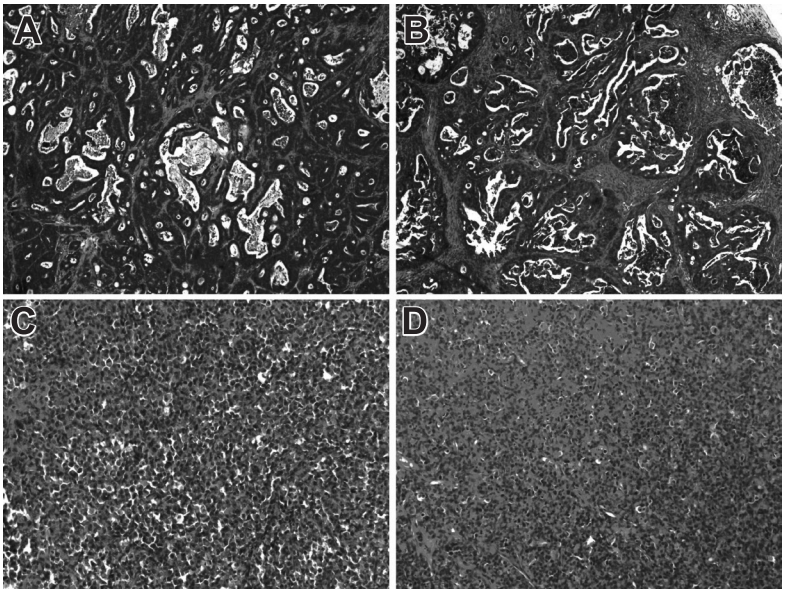

Figure 1A-D. Histology of tumors derived from the limiting dilution experiment.

A-B. Histology showing a moderately differentiated tumor in both the primary tumor (A) and the mouse-tumor (B) derived from injected Lineage-negative cells.

C-D. Histology showing a poorly differentiated tumor in both the primary tumor (C) and the mouse-tumor (D) derived from injected Lineage-negative cells.

Expression analysis of CSC markers in BE and EA by IHC

Subsequently, we performed IHC analysis of resection specimens from 20 unrelated BE/EA patients with a panel of previously established (cancer) stem cell markers (CD24[31], CD29[31], CD34[10], CD44[29], CD133[27, 28], CD166[29], EpCAM[29], β-catenin[32]). The IHC analysis was here meant to explore whether the expression pattern and subcellular localization of the above markers may be suggestive of their usefulness as CSCs markers in esophageal adenocarcinomas and possibly to select a subset of them to perform the further FACSorting and transplantation assayThe overall results of the IHC analysis including the percentage of cells positive for specific CSC markers in the different histological lesions are summarized in Table 2. In a unique tissue block derived from the resection specimen of a single patient, defined areas of all progression stages (normal squamous epithelium → metaplasia → LGD → HGD → adenocarcinoma) could be identified, which were here employed as representative of the staining patterns observed throughout the cohort (Figure 2).

CD24 and CD29 positive cells were mainly located at the basal compartment of the epithelium in metaplastic and dysplastic areas (Figures 2 and 3A-B).

The CD24 and CD29 staining did not increase with advanced stage, but a patchier staining pattern was observed within EA (Figure 4A-B). The endothelial cell marker CD34 predominantly labeled blood vessels in the stroma, but not epithelial cells (Figure 2). CD44 staining showed positive stromal cells in the metaplastic and dysplastic lesions (Figure 2), and a heterogeneous membranous staining pattern in tumor cells in more than half of all adenocarcinomas examined (15/28, 54%, Figure 4C). CD133 IHC did not reveal any staining in the normal squamous epithelium or in the pathological specimens (data not shown). CD166 IHC revealed extensive staining in almost all epithelial cells, with a homogeneous staining pattern in EA (Figure 2). Likewise, EpCAM appeared to be a good indicator for epithelial cells with homogeneous staining in metaplastic and dysplastic tissue as well as in adenocarcinoma (Figure 2).

Table 2. Outcome of immunohistochemical analysis of individual potential (cancer) stem cell markers along the metaplasia – dysplasia – carcinoma sequence.

		METAPLASIA N=15	LGD N=12	HGD N=18	EA N=28
1. CD24	++	0/15	0/12	0/18	5/28
	+	9/15	9/12	13/18	15/28
	0	6/15	3/12	5/18	8/28
2. CD29	++	0/15	0/12	0/18	2/28
	+	8/15	7/12	9/18	11/28
	0	7/15	5/12	9/18	15/28
3. CD34	++	0/15	0/12	0/18	0/28
	+	0/15	0/12	0/18	0/28
	0	15/15	12/12	18/18	28/28
4. CD44	++	0/15	0/12	2/18	1/28
	+	0/15	0/12	2/18	14/28
	0	15/15	12/12	14/18	13/28
5. CD133	++	0/15	0/12	0/18	0/28
	+	0/15	0/12	0/18	0/28
	0	15/15	12/12	18/18	28/28
6. CD166	++	15/15	12/12	15/18	25/28
	+	0/15	0/12	0/18	0/28
	0	0/15	0/12	3/18	3/28
7. EpCAM	++	5/15	5/12	10/18	20/28
	+	3/15	0/12	1/18	0/28
	0	7/15	7/12	7/18	8/28
8. β-catenin*	++	15/15 (3/15)	12/12 (4/12)	17/18 (7/18)	27/28 (6/28)
	+	0/15	0/12	0/18	0/28
	0	0/15	0/12	1/18	1/28

Notes. The categories indicated as ++, +, and 0 indicate the presence of >25%, 1-25%, or no positive cells, respectively. LGD = low-grade dysplasia; HGD = high-grade dysplasia; EA = esophageal adenocarcinoma. *number of sections positive for membranous and/or cytoplasmic β-catenin staining; the number of sections with nuclear β-catenin is indicated in brackets.

IHC analysis of β-catenin expression and subcellular localization also showed preferential staining of cells located in the basal compartment, with a decreasing gradient of intensity along the basal-luminal axis (Figure 5A). ß-catenin was mainly observed in the cytoplasm of all progression stages, with >25% cells staining positively (Figure 3C and 5A-B). An increase in the percentage of cells with nuclear ß-catenin accumulation was seen from metaplasia and LGD lesions (3/15, 20.0%; 4/12, 25.0%, respectively) to HGD (7/18, 38.9%), but not in EA (6/28, 21.4%). However, groups of cells with heterogeneous nuclear ß-catenin staining were detected at the invasive front of the majority of the tumors (Figure 5C). Hence, it appeared that nuclear accumulation of ß-catenin partially overlapped with the staining pattern observed for the cell-surface antigens CD24 and CD29 (Figure 3A-C).

Overall, although the IHC analysis of resection specimens from BE/EA patients with previously established (cancer) stem cell markers did not unequivocally point to a specific candidate cell surface antigen, it provided some interesting clues on the basal compartment (CD24 and CD29) and the invasive front (β-catenin) as alleged locations of subpopulation of cancer cells with distinct biological properties. Nevertheless, the IHC results were not such to justify the selection of a subset of CD markers for the further sorting and transplantation assays. Therefore, we decided to employ all of the above (cancer) stem cell markers for the sorting and transplantation of subpopulations of EA cells and the evaluation of their putative tumor-initiating capacities.

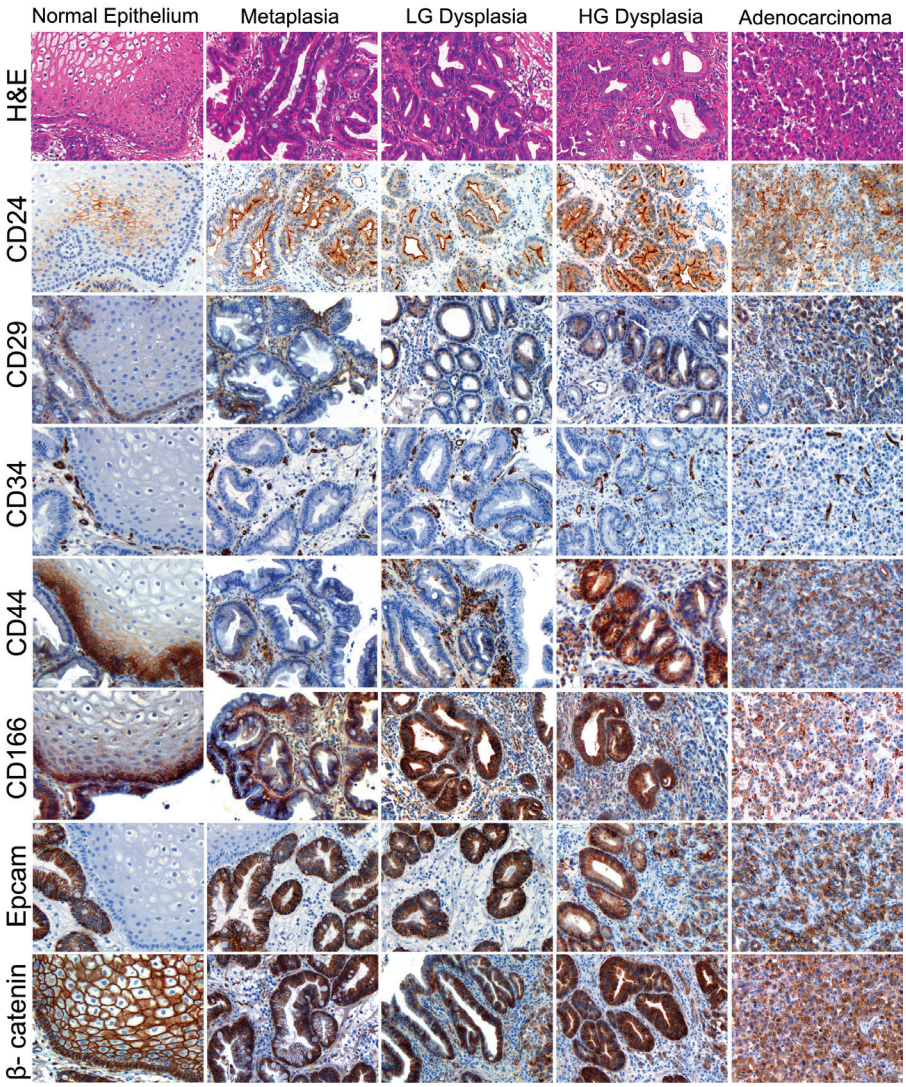

Figure 2. In one unique tissue block of the present series, defined areas of all stages towards malignant progression (normal squamous epithelium → metaplasia → LGD → HGD → EA) could be identified and were employed as representatives of the staining patterns for all potential CSC markers on IHC.

Common CSC markers do not enrich for EA initiating cells

The above demonstration of the existence of a minority of tumor-initiating cells in EA (frequency of 1 in 64,287 cells) and the heterogeneous expression pattern of some CSC markers are of good auspices for the isolation of EA initiating cells. EA cell suspensions derived from 13 resection specimens were stained with antibodies directed against the same cell-surface antigens tested by IHC, analyzed and sorted by FACS, and transplanted in NOD-SCID mice. In this way, we were able to assess whether any of the resulting subpopulations encompassed an increased fraction of tumor-initiating cells when compared to the bulk tumor cells. The percentages of Lin- tumor cells positive for the employed CD markers upon FACS analysis greatly varied among individual patients (Table 3). Supplementary Figure 1 illustrates some examples of the observed FACS profiles.

Sorted cells were transplanted subcutaneously at a fixed multiplicity (N=5,000). For each CD marker, 8-20 transplantations were performed. Also, subpopulations of cells positive for a combination of these markers (*i.e.* CD24/29, CD44/166) were transplanted. Following transplantation, the recipient mice were observed for a period of 4-6 months. However, no tumor growth could be detected in any of the tumor cell subpopulations out of a total of 180 transplantation assays (Supplementary Table 1).

Table 3. Percentages of Lin- primary esophageal adenocarcinoma cells from 13 unrelated patients positive for the employed CD (cancer) stem cell markers.

Primary tumor	1	2	3	4	5	6	7	8	9	10	11	12	13
Lin- CD24+ CD29+	0.9	19.2	6.9	5.0	2.0	24.4	6.5	1.4	NA	NA	NA	0.8	NA
Lin- CD44+	69.8	6.9	3.8	39.5	6.9	16.9	12.2	3.8	NA	NA	NA	0.6	NA
Lin- CD166+	59.5	33.8	21.8	39.8	50.5	24.1	30.2	1.8	NA	NA	NA	14.9	NA
Lin- CD44+CD166+	52.0	5.8	2.6	29.6	5.8	14.0	6.1	0.6	NA	NA	NA	0.6	NA
Lin- CD133+	0.1	13.1	18.7	6.6	6.9	10.6	13.3	0.2	NA	NA	NA	17.9	NA
Lin- EpCAM+	NA	NA	NA	NA	NA	NA	NA	NA	33.4	44.8	26.5	20.7	41.7
Lin- CD34+	NA	NA	NA	NA	NA	NA	NA	NA	0.3	17.8	11.6	0.3	1.2

NA = not available.

Discussion

In this study, we tested whether BE and EA might serve as a disease model for the cancer stem cell concept. Based on the *in vivo* limiting dilution experiments, we can now state that tumor-initiating cells are present in EA at an average frequency of 1 in 64,287 cells (s.e. 18,049). The histology of the transplanted tumors resembles that of the primary tumors thus confirming the capacity of these tumor-initiating cells of recapitulating the heterogeneous composition of these lesions. IHC analysis of several (cancer) stem cell markers in BE and EA indicated that, based on their focal staining pattern and co-staining with ß-catenin, CD24 and CD29 may represent potentially interesting markers for the enrichment of CSCs in EA. The somewhat patchy CD44 expression pattern is also of interest and may point to a specific subpopulation of tumor cells within EA. The negative or homogeneously positive expression observed with the markers CD133, CD166 and EpCAM is less likely to be of any significance for the isolation of CSCs in EA.
However, the overall IHC results were not such to clearly point to one or few specific cell surface antigens as potential CSC markers in esophageal adenocarcinoma. Moreover, it is known that the specificity of the antibodies employed for IHC and FACS analysis may vary. Hence, although the negative or homogeneously positive expression patterns of markers like CD133 and CD166 may suggest their unlikely role as tools to enrich for CSCs in EA, one could be easily misled as the corresponding antibody employed for FACSorting recognizes different epitopes and/or isoforms of the same protein. Therefore, we proceeded by testing all of the above markers for the FACS and transplantation assays. Unfortunately, as shown by the results of the *in vivo* transplantation assay, none of the above cell-surface antigens, previously established as CSC markers in a broad spectrum of malignancies, resulted in a significant enrichment of EA initiating cells.

The evolution from the normal squamous esophagus to pre-malignant metaplasia (BE) and EA represents a unique model system to study the underlying environmental, genetic, and epigenetic events. From this perspective, the availability of biopsies from the different stages allows the definition of the temporal order of causative molecular events as in the classical case of colorectal cancer.

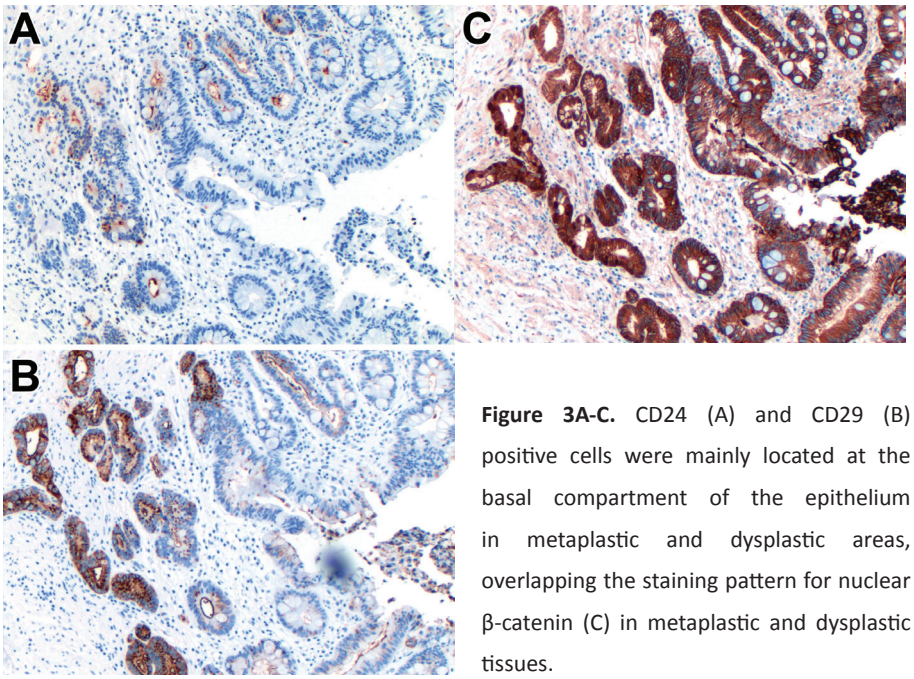

Figure 3A-C. CD24 (A) and CD29 (B) positive cells were mainly located at the basal compartment of the epithelium in metaplastic and dysplastic areas, overlapping the staining pattern for nuclear β-catenin (C) in metaplastic and dysplastic tissues.

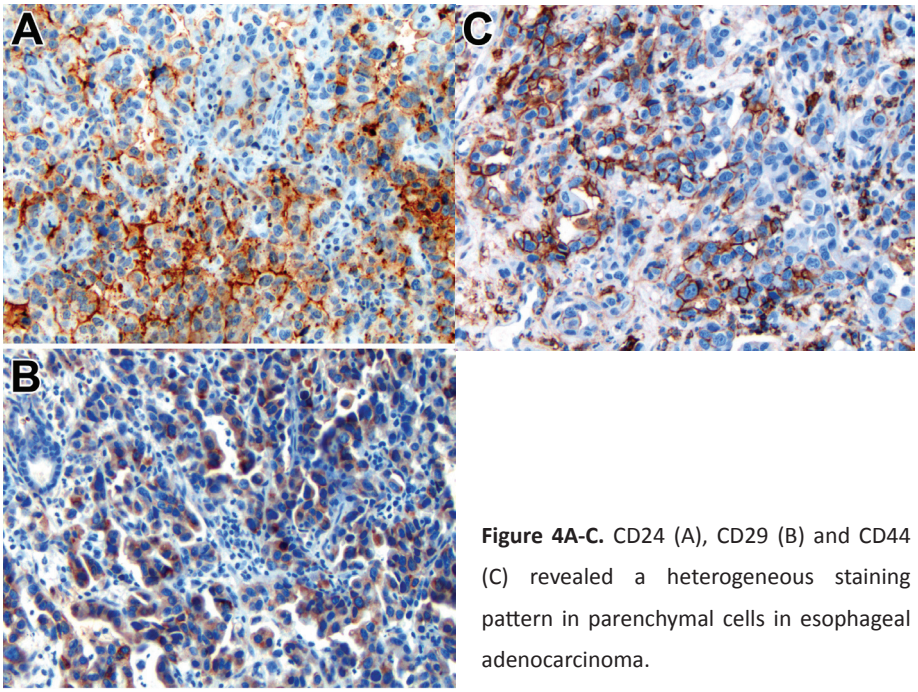

Figure 4A-C. CD24 (A), CD29 (B) and CD44 (C) revealed a heterogeneous staining pattern in parenchymal cells in esophageal adenocarcinoma.

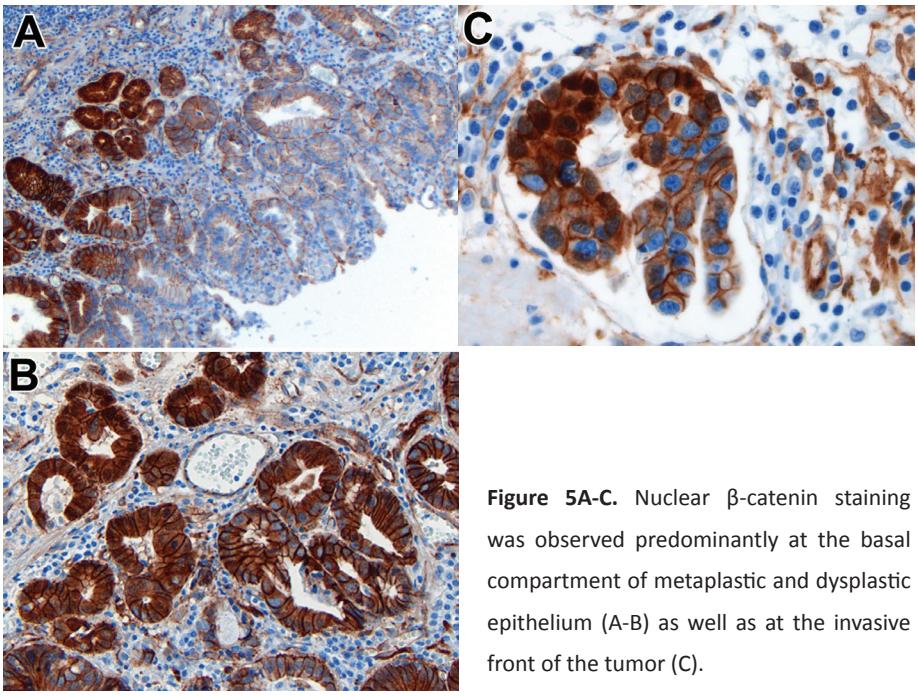

Figure 5A-C. Nuclear β-catenin staining was observed predominantly at the basal compartment of metaplastic and dysplastic epithelium (A-B) as well as at the invasive front of the tumor (C).

Among the different molecular alterations identified in BE and EA, several members of the Wnt/β-catenin signal transduction pathways appear to be affected by a broad spectrum of different genetic and epigenetic mechanisms[36,37], thus pointing to a functional role for the constitutive activation of this pathway in esophageal metaplasia and neoplasia[38]. Nuclear β-catenin accumulation is generally regarded as the hallmark of constitutive activation of the Wnt pathway and is thought to earmark CSCs in several malignancies, among which colorectal cancer[32,39]. Notably, intracellular β-catenin accumulation is observed in the majority of EA, often along the invasive front, while less frequently in Barrett's metaplasia[40-42]. We confirmed these observations by showing an increase in nuclear ß-catenin staining during the progression from metaplasia (5/14, 36%) into LGD (5/12, 42%) and HGD (10/16, 63%). Moreover, β-catenin was often observed in the nucleus of EA cells located at the invasive front, and its general pattern of intracellular accumulation appears to overlap with the expression of CD24 and CD29, two cell-surface antigens known to earmark normal stem cells and CSCs in tissues such as the mammary gland[31].

CD24 functions as a cell adhesion molecule that is also involved in regulation of proliferation[43, 44]. CD24 is also known to be expressed in a broad spectrum of human cancers where it is thought to contribute to the acceleration of tumor growth, the shedding of tumor cells from the primary mass, and their transmigration and invasion across endothelial cells, thereby promoting the dissemination of cancer cells[43, 44]. CD29 (more commonly known as ß1-integrin) comprises the largest subgroup of the integrin family of transmembrane receptors, which enables cell adhesion as well as transduction of signals into cells[45, 46]. However, although the results indicate an overlap between the two staining patterns, co-localization of CD24 and CD29 with β-catenin should be validated by double staining of the same histological section. Neoplastic cells tend to lose the integrins that secure their adhesion to the basement membrane, and maintain or over-express integrins that contribute to survival, proliferation and migration during tumor invasion and dissemination[46]. Notably, a recent study reported that the CD24-mediated increase in cell migration depends on the ß1-integrin subunit[47]. Also, as mentioned before, $CD24^{+}CD29^{hi}$ epithelial cells have been shown to represent the stem cells of the mouse mammary gland capable of reconstituting a complete and functional gland *in vivo*[31]. Besides CD24 and CD29, CD44 appeared to be an interesting marker based on the IHC results, as its expression pointed to a subpopulation of tumor cells within EA. The CD44 transmembrane glycoproteins mediate cell-matrix adhesion and cell migration. Up-regulation of specific CD44 isoforms has been associated with poor prognosis in Barrett's adenocarcinoma[48, 49]. Furthermore, it has been shown that CD44 is a robust CSC marker in colorectal cancer[29].

Despite the above-mentioned IHC results, we were not able to demonstrate that surface-antigens previously established as CSC markers in other malignancies enrich for tumor-initiating cells in EA. However, several points may be considered with regard to the applied methods in the present study. To date, CSCs are only operationally defined by the transplantation assay in immuno-deficient mice. Subpopulations of tumor cells enriched for CSCs are thought to result in tumor formation even when injected at low mulitiplicities, in contrast with bulk cancer cells that give rise to tumors when injected at higher numbers only. Previous experimental evidence for the existence of CSCs has been provided by incorporating a selection step prior to

injection into mice. In contrast to the direct transplantation method employed in the present study (primary tumor cells injected directly into the recipient mice), enrichment for CSCs was facilitated by means of xenografting or culturing suspension spheres before transplantation experiments were carried out[29, 50, 51]. Furthermore, transplantation into NOD-SCID mice has shown to underestimate the frequency of human cancer cells with tumorigenic potential in some malignancies[52]. Modified transplantation conditions, e.g. by employing more intensely immunocompromised mice (for example the NOD-SCID IL2Rγ^{null} mice that lack the interleukin-2 gamma receptor and therefore have a decreased natural killer cell activity) may allow for more efficient engrafting of CSCs. Also, instead of subcutaneous grafting, one might also consider orthotopic transplantations (i.e. in the esophageal epithelial lining of the recipient animals), though these are technically challenging and yet unreported assays[21].

In conclusion, our results indicate the presence of a subpopulation of EA cells with tumor-initiating capacities. However, FACS sorting of EA cells by surface antigen markers that have previously been established to enrich for CSCs in other types of epithelial malignancies (*e.g.* colorectal cancer), did not increase the relative frequency of tumor-initiating cells. Future studies should focus on the search for novel surface antigens and on the modification of the experimental set-up, for example by employing NOD-SCID IL2Rγ^{null} mice instead of the conventional NOD-SCID mice, and by pre-selection of the tumor cell subpopulations *in vitro* (establishment of sphere cultures *i.e.* esospheres) or *in vivo* (use of xenografts rather than primary resection specimens). Notwithstanding the above-mentioned technical obstacles, the natural history and etiology of BE and EA still represent an ideal setting for the study of how modification of the cellular environment may lead to reprogrammed resident stem cells which may result in the establishment of CSCs underlying tumor growth and invasive behaviour[53].

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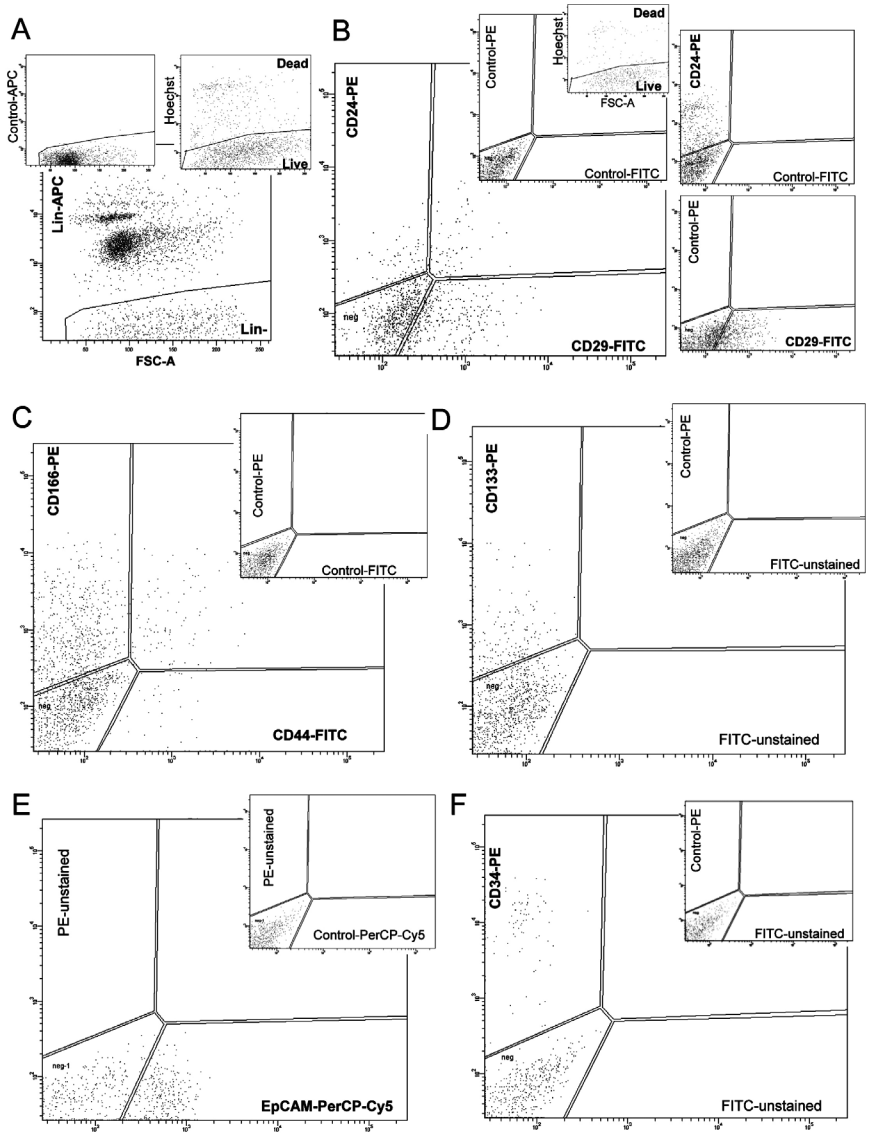
Supplementary Table 1. Tumor growth of subpopulations of Lineage-negative cells transplanted in recipient NOD-SCID mice at a multiplicity of 5,000 cells.

Primary tumor	1	2	3	4	5	6	7	8	9	10	11	12	13
Lin- CD24+ CD29+		0/4	0/4			0/4	0/4	0/4					
Lin- CD24+ CD29-		0/4	0/4			0/4							
Lin- CD24- CD29+		0/4	0/4			0/4							
Lin- CD24- CD29-		0/4	0/4			0/4							
Lin- CD44+				0/4	0/4	0/4							
Lin- CD166+				0/4	0/4	0/4							
Lin- CD44+CD166+	0/4			0/4	0/4	0/4							
Lin- CD44+CD166-	0/4												
Lin- CD44-CD166+	0/4												
Lin- CD44- CD166-	0/4												
Lin- CD133+		0/2	0/2		0/4	0/4							
Lin- CD133-		0/2	0/2		0/4	0/4							
Lin- EpCAM+									0/4	0/4	0/4	0/4	0/4
Lin- EpCAM-									0/4	0/4	0/4	0/4	0/4
Lin- CD34+											0/4	0/4	
Lin- CD34-											0/4	0/4	



Supplementary Figure 1. FACS profiles representative of the staining of the different cell-surface markers employed in the present study. (A) Staining for the lineage markers in the APC channel. Left upper panel: isotypic control antibodies. Right upper panel: cell viability (=78%) after Lin- sorting; (B) Right panel: double staining of CD24 (PE channel) and CD29 (FITC channel). Lower inset: isotypic control antibodies. Upper inset: viability (=87%) after sorting of $Lin^{-}CD24^{+}CD29^{+}$ tumor cells. Left panel: staining with the single CD markers indicative of the quality of the compensations applied throughout the present work; (C) Staining of CD44 (FITC channel) and CD166 (PE channel) with relative isotypic controls (inset); (D-F) staining of CD133 (PE channel), EpCAM (PerCP-Cy5 channel), and CD34 (PE channel) along with their respective isotypic controls (insets).

3

The pathogenesis of Barrett's metaplasia and the progression to esophageal adenocarcinoma

Brechtje A Grotenhuis
J Jan B van Lanschot
Winand NM Dinjens
BPL Wijnhoven

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Abstract

The most important risk factor for the development of Barrett's esophagus is reflux of both gastric and duodenal contents into the esophagus. Why Barrett's metaplasia develops only in a minority of patients suffering from gastroesophageal reflux disease remains unknown. It is also unclear what the exact mechanism is behind the transition of normal squamous epithelium into specialized columnar epithelium. It is likely that stem cells are involved in this metaplastic change, as they are the only permanent residents of the epithelium. Several tumorigenic steps have been described that lead to the underlying genetic instability which is indispensable in the progression from columnar metaplasia to esophageal adenocarcinoma. This review outlines the process of pathogenesis of Barrett's metaplasia and its progression to esophageal adenocarcinoma.

1. Introduction

Over the past 50 years more insight has been gained into the pathophysiology and molecular pathways associated with the development of Barrett's esophagus and esophageal adenocarcinoma. Gastroesophageal reflux disease (GERD) has now been recognized as the most important risk factor for the onset of Barrett's metaplasia and esophageal adenocarcinoma. Environmental, dietary and genetic factors are also likely to play an important role. However, the exact mechanism underlying the transition from normal squamous epithelium towards metaplastic epithelium has not been elucidated yet. The identification of stem cells in the normal squamous esophageal epithelium has lead to speculations about the contribution of these cells in the metaplastic process, as these cells are the only permanent residents of the epithelium. Recently, some studies have been published that have shed new light on the molecular and cellular basis of Barrett's esophagus.

This review gives an overview of the pathogenesis of Barrett's metaplasia and its progression towards esophageal adenocarcinoma. The risk factors for the development of Barrett's esophagus are reviewed as well as the different theories concerning the cell of origin of Barrett's metaplasia. Finally, a summary is given of the tumorigenic steps that are involved in the development of esophageal adenocarcinoma.

2. Normal esophageal epithelium

The luminal surface of the normal esophagus is lined by stratified squamous epithelium of the non-keratinizing type.[1, 2] This epithelium can histologically be divided into two zones: (1) a luminal "differentiated zone" consisting of progressively flattened, terminally differentiated keratinocytes, and (2) a basal "generative" zone. Within the latter, a basal layer (single layer of cells next to the basal membrane) and several epibasal layers (variable number of cell layers above the basal layer) can be distinguished (Figure 1). At regular intervals along the epithelium the lamina propria

invaginates and forms papillary structures within the epithelium. Subsequently, in the basal layer two components can be distinguished: the interpapillary basal layer (IBL) covering the interpapillary zone and a papillary basal layer (PBL) overlying the papillae (Figure 1).[2] Shedding of epithelial cells occurs when cells have migrated from the basal zone towards the esophageal lumen.[3]

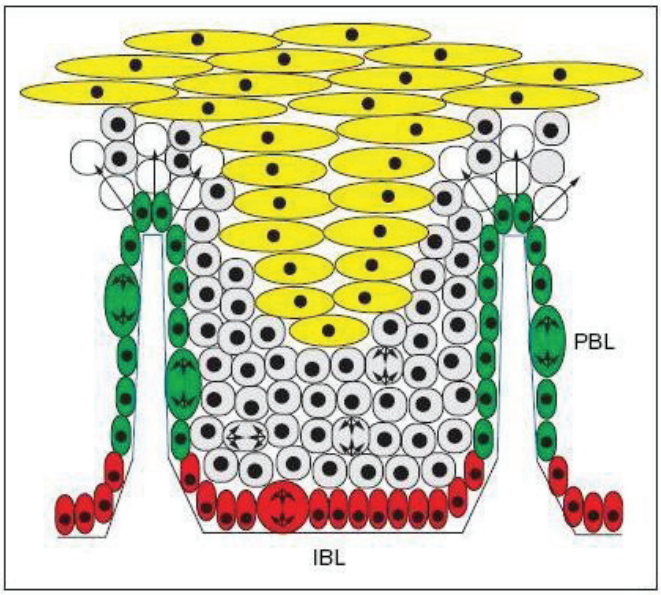

Figure 1. Schematic representation of the organization of the human esophageal epithelium. The interpapillary basal layer (IBL) cells constitute the stem cell compartment (red). Transit amplifying cells are proposed to reside in the papillary basal layer (PBL in green) and epibasal layers (grey). Suprabasal cells that can no longer divide and are undergoing terminal differentiation are shown in yellow. Arrows indicate the direction of cell movement. Re-printed from Seery and Watt (2000)[5] with permission.

To maintain epithelial integrity, the rapidly proliferating esophageal mucosa is repopulated by a limited number of stem cells present in the generative basal zone. These stem cells divide, replace the stem cell compartment itself and generate transit amplifying cells (differentiating daughter cells that enter the epibasal layer).[4] It has been observed that the process of low proliferation and asymmetric cell division (giving rise to one stem cell and one transit amplifying cell) specifically characterizes the IBL.[5]

It is hypothesized that another stem cell population might also account for reconstitution of the surface epithelium. This population is thought to reside in the tubuloalveolar glands that are present in the submucosal layer of the esophageal epithelium.[2] Although there is no direct evidence for the existence of these stem cells, an analogy can be drawn with the epidermis in which stem cells are located not only in the interfollicular epithelium, but also in the bulge region of the hair follicle.[6, 7]

3. Pathogenesis of Barrett's metaplasia

3.1 Development of Barrett's esophagus: congenital versus acquired

Columnar epithelium in the intrathoracic part of the gastrointestinal tract in combination with an ulcer and esophagitis was first described in 1950 by Norman Barrett, a British surgeon.[8] However, he misinterpreted the condition as a tubular intrathoracic stomach. Also, he was convinced that this was due to a congenitally short esophagus.[8] Johns hypothesized that this condition might be due to a premature cessation of the physiologic replacement of the columnar ciliated epithelium (which lines the esophagus during embryogenesis) by stratified squamous epithelium, starting around 22 weeks of gestation.[8, 9] In his opinion this was a congenital disorder. However, arguments against this congenital theory include the fact that the squamous replacement of the fetal columnar epithelium begins in the mid esophagus and progresses towards each end.[9] The cervical region appears to be the last to lose its embryonic lining, which is contradictory to the fact that the columnar epithelium in a Barrett's esophagus is always found in the lower esophagus.[10] In 1953, Allison and

Johnstone demonstrated that the columnar epithelium was located proximal to the lower esophageal sphincter, and thus it was recognized definitively as an abnormality of the esophageal mucosa.[11] Furthermore, the association between columnar lined epithelium and GERD was recognized and led to the concept of an acquired condition. Moersch *et al*. and Hayward were the first to suggest that the columnar lining might be an acquired condition due to reflux esophagitis that destroys the squamous epithelium.[12, 13] This concept was broadly accepted when Bremner *et al*. showed columnar cell regeneration in the distal esophagus in an experimental model of chronic gastroesophageal reflux.[14]

3.2 Definition of Barrett's metaplasia

Three histological types of columnar epithelium in the esophagus have been described: a gastric fundic type composed of chief and parietal cells; a junctional type composed of mucous glands without parietal cells; and a specialized type with intestinal characteristics including mucous glands and goblet cells.[15] These three types are nowadays referred to as oxyntocardiac mucosa, cardiac mucosa and intestinal metaplasia, respectively.[16] It is only intestinal metaplasia that has been recognized as a premalignant condition, which is included in the definition of a Barrett's esophagus: a condition in which the normal squamous epithelium of the distal esophagus is replaced by specialized columnar epithelium, which is characterized by the presence of intestinal metaplasia.[17, 18] Metaplasia refers to the conversion of one cell type to another during postnatal life and might be the effect of conversion of tissue-specific stem cells.[19] Goblet cells are characteristic for intestinal metaplasia, which are barrel-shaped and have a distended, mucin-filled cytoplasm that stains positively with Alcian blue.[20, 21]

3.3 Gastroesophageal reflux disease

There are many risk factors associated with the development of a Barrett's esophagus. GERD is considered to be the key risk factor.[22, 23] Chronic GERD is characterized by various conditions, including nonerosive and erosive esophagitis, ulceration and strictures of the esophagus. It has been reported that approximately 10 % of patients with GERD-symptoms develop a Barrett's esophagus.[24-26] Furthermore, increased

age[27, 28], male sex[29, 30] and Caucasian race[31] are general risk factors for Barrett's esophagus as described in several epidemiologic studies.

3.3.1 Pathophysiology

Esophageal exposure to refluxed gastric contents is considered to be the major factor in the development of reflux esophagitis and Barrett's esophagus. Animal models have demonstrated that gastric acids are involved in injuring the esophageal mucosa.[14, 32] In humans, patients with a Barrett's esophagus typically have greater esophageal acid exposure based on 24-hour pH monitoring when compared to patients with esophagitis or normal subjects.[33-36] A direct relationship between the severity of esophageal mucosal injury and the degree and frequency of refluxed acid exposure has been reported.[33] Furthermore, it has been found that patients with Barrett's esophagus have a significantly longer exposure time to a pH lower than 4 than patients with esophagitis.[37] Interestingly, there is a clear correlation between length of the columnar lined esophagus and the severity of reflux.[38] However, one study showed that in a group of patients with a Barrett's esophagus that was followed-up for more than 7 years, the length of the Barrett's segment did not change.[27] It has been hypothesized that the transformation of squamous epithelium into columnar metaplasia does not occur after intestinal metaplasia has developed.[39] In other words, the occurrence of intestinal metaplasia in cardiac mucosa might act as a break for further columnar transformation of squamous epithelium by reflux. Nevertheless, this hypothesis is still unproven, and further research is needed in this field.

Acid injury involves the ability of H^+ ions to enter the cytoplasm of the esophageal epithelial cell with subsequent cell death. In the normal situation, the apical membrane of the epithelial cells is not permeable to acid.[40] When luminal acidity is sufficiently high, intercellular junctions are damaged and widening of the intercellular spaces is observed.[41, 42] Subsequently, H^+ ions are able to penetrate into the cell through the basolateral membrane. The intracellular acids lead to cell death and, finally, to ulceration once the necrosis affects a large area.

Pepsin is a digestive enzyme, secreted as pepsinogen and activated into pepsin by gastric acid. Pepsin is considered to be harmful as it may cause erosive esophagitis in an acidic environment by increasing the cell permeability to H^+ ions.[3, 43] However, the exact role of pepsin in damaging the esophageal mucosa has not been explored extensively thus far.

Besides the effect of gastric acids and pepsin, also excessive reflux of duodenal contents into the esophagus contributes to the development of Barrett's metaplasia. Bile reflux or alkaline reflux are terms that are often used to describe the reflux of duodenal contents, which consists of conjugated and unconjugated bile salts, lysolecithin, and pancreatic enzymes such as trypsin. The term 'alkaline reflux' suggest a pH > 7, although it has been reported that the majority of esophageal bilirubin exposure occurs when the pH is between 4 and 7.[44] Therefore, the term duodenogastroesophageal reflux (DGER) may be more appropriate, referring to the retrograde reflux of duodenal contents (bile and pancreatic fluid) into the stomach as well as the esophagus. It is believed that both pancreatic enzymes and bile salts are able to induce severe esophagitis.[45, 46]

Trypsin is a pancreatic enzyme that is responsible for lysis of proteins. It is thought to affect intercellular substances causing shedding of epithelial cells.[47] Trypsin can cause substantial injury to the esophageal mucosa at alkaline pH. The role of lysolectihin, another component of duodenal juice, is less understood.
Bile salts are conjugated with either taurine or glycine when secreted by the liver. The conjugation process makes bile acids more soluble in an acidic environment (range pH 2-7) by lowering the Pk_a dissociation constant[48, 49]; an environment in which synergistic damaging effects have been described from gastric acids and conjugated bile salts.[50, 51] However, acidification of bile salts to a pH of less than 2 leads to irreversible precipitation and inactivation of the bile salts. At neutral or alkaline pH, conjugated bile salts cause only minimal injury. However, this is in contrast with trypsin and unconjugated bile salts, which have the greatest potential to damage the esophageal mucosa under alkaline circumstances.[52]

It has been suggested that in a moderately acidic gastric environment (range pH 2-7), as can occur with the use of acid-suppression medication, bile salts are partially soluble and are potentially harmful to mucosal cells.[53] For conjugated bile salts to remain completely harmless in a patient with GERD taking acid-suppression medication, a gastric pH of at least 7 must be aimed for during day and night.[53] Hence, incomplete acid suppression may allow esophageal mucosal damage to occur while the patient is asymptomatic.[44]

Several studies using combined pH and bile-reflux monitoring in non-operated GERD patients, suggest increasing amounts of both acid reflux and DGER with increasing severity of esophageal lesions.[44, 50, 54-57] Two consecutive studies showed that the highest bilirubin concentration and percentage of time with pH < 4, were seen in patients with Barrett's esophagus, followed by patients with esophagitis and controls.[37, 50] The results of these studies are supportive of a synergistic activity of acid and bile reflux. Moreover, simultaneous esophageal exposure to both acid and DGER was the most prevalent reflux pattern (95%) in patients with a Barrett's esophagus.[37] Reports on esophageal aspirates have shown conflicting results with regard to the role of DGER: some studies could detect an increased amount of bile acids in patients with Barrett's esophagus, whereas other studies could not confirm this.[58] However, aspiration techniques have been criticized because of the short aspiration periods and intrinsic limitations of these techniques.[50] The overall results of animal studies, esophageal monitoring and aspiration studies[58, 59] suggest a synergistic role for gastric and bile acids in the etiology of a Barrett's esophagus.

3.3.2 Role of inflammation and oxidative stress

Increased exposure of the normal esophageal mucosa to (duodeno)gastroesophageal reflux results in mucosal damage and tissue inflammation. The mucosal inflammatory response is characterized by specific cytokine and chemokine profiles. The nuclear factor-kappa B (NF-kB) pathway is thought to play a pivotal role in this response: NF-kB comprises a family of transcription factors that regulates the host inflammatory and immune responses by increasing the expression of many genes that are involved in the inflammatory reaction.[60] Subsequently, increased levels of cytokines including

TNFα, interleukin (IL)-1β, IL-6 and IL-8, can also directly activate the NF-kB pathway, thus establishing a positive autoregulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation.[60] Inappropriate activation of NF-kB has been linked to a variety of inflammatory and neoplastic conditions.[60, 61]
The cytokines that are released in response to GERD may thus contribute to the activation of the NF-kB pathway in these patients. Moreover, NF-kB was found to be up regulated in Barrett's epithelium.[62, 63]

Gastric acid and bile salts can activate the arachidonic acid pathway, which controls inflammation. Cyclooxygenase-2 (COX-2) is a key enzyme of this pathway, and catalyzes the conversion of arachidonic acids into prostaglandins. COX-2 is usually not detectable in normal tissues, but can be induced in processes like inflammation and carcinogenesis.[64] Also, it has been shown that activation of NF-kB can lead to an increase in COX-2.[65] An *in-vitro* study showed that COX-2 is functionally active in Barrett's esophagus: treatment with a COX-2 inhibitor diminished cell growth, whereas proliferation could be restored by treatment with prostaglandin.[66] A gradually increased COX-2 expression has been reported from normal squamous epithelium towards Barrett's metaplasia and esophageal adenocarcinoma.[67-69] Moreover, an increased COX-2 expression seems to be related with a reduced survival in patients with esophageal adenocarcinoma.[70, 71]
Chronic inflammation can also induce the production of reactive oxygen species: highly reactive free radicals that are generated as products of oxygen degradation during injury. These free radicals have an important role in the inflammation process. They may damage DNA by causing mutations, induce the production of proinflammatory cytokines, and produce growth factors for epithelial cells.[72] Under normal conditions cells are protected from reactive radicals by antioxidant defense systems. When oxidative stress (an imbalance between oxidant production and the antioxidant capacity of the cell) arises, these defense systems promote the expression of antioxidants.[73] In patients with reflux esophagitis and Barrett's esophagus, it has been demonstrated that mucosal damage is associated with increased oxidative stress, characterized by an enhanced free radical proportion and

decreased antioxidant activity.[74, 75] Also, one study suggested that the lower levels of the antioxidant vitamin C found in patients with a Barrett's esophagus, supporting the hypothesis of oxidative stress being of importance in the pathogenesis of metaplastic epithelium.[76]

3.3.3 GERD-related factors

Since not all patients with GERD develop a Barrett's esophagus, this implicates that additional risk factors play an important role. As we will discuss below, genetic predisposition, presence of a hiatal hernia, a low esophageal sphincter pressure, obesity and dietary patterns have been described to contribute to this risk. It should be kept in mind that most of these factors are related to the severity of GERD and still cannot fully explain why only the minority of patients with GERD will develop Barrett's esophagus. Indirect evidence for a possible genetic susceptibility comes from a study that has reported an increased prevalence of GERD among family members of patients with Barrett's esophagus or esophageal adenocarcinoma.[77] It is hypothesized that an unknown susceptibility gene is inherited in an autosomal dominant fashion with incomplete penetrance, as not all individuals in these families develop Barrett's esophagus or esophageal adenocarcinoma.[78] It has been suggested that a tumor suppressor gene is involved.[79] In this model, germline mutations in the gene predispose to neoplasia, and once the second allele is lost or mutated (*i.e.* a 'second hit' caused by environmental factors like chronic GERD), cancer may develop. Furthermore, polymorphisms (specific variant alleles) of different genes have been described that may be associated with an altered esophageal cancer risk.[80] For example, polymorphisms in genes involved in carcinogen metabolism, DNA repair and cell cycle control have been correlated with the presence of Barrett's esophagus and esophageal adenocarcinoma.[80]

Interference with the physiologic function of the esophago-gastric junction can occur in two conditions: dysfunction of the lower esophageal sphincter (LES) and the presence of a hiatal hernia.[81]

A defective LES causes an increased acid exposure in the distal esophagus and can be found in over 95% of patients with a Barrett's esophagus.[82] In the presence of

an incompetent LES, ineffective clearance function due to motility disorders of the esophageal body further prolongs the time the esophagus is exposed to gastric contents.[83]
A hiatal hernia may contribute to GERD by a variety of mechanisms.[84] First, clearance of acid from the esophagus is impaired; gastric acid may be trapped in the hernial sac and can subsequently be refluxed in the esophagus during a swallow-induced relaxation.[85] Secondly, esophageal emptying can be impaired when an irreducible large hiatal hernia is present.[86] Finally, a large hiatal hernia causes a widening of the esophageal hiatus that may impair the ability of the crural diaphragm to function as an external sphincter.[87] It has been reported that the presence of a hiatal hernia is a risk factor in the development of a Barrett's esophagus[88] and metaplastic epithelium has been observed more often in patients with a hiatal hernia than in those without.[89]

Obesity could increase the risk for the development of a hiatal hernia and provoke reflux through an elevated intra-abdominal pressure. Increased body mass index (BMI) is known to be a risk factor for GERD[90], but it remains unclear whether the increased risk for Barrett's esophagus associated with BMI is mediated by GERD directly or whether there is a higher risk regardless of reflux. A recently published meta-analysis provided evidence that increasing BMI does not present an increased risk of Barrett's esophagus above what would be expected from GERD alone.[91] However, it was commented that the BMI does not take into account the distribution of fat within the body.[92] Markers of central obesity (visceral fat) like the waist-hip ratio could be more reliable in the determination of a possible independent relationship between obesity and the development of a Barrett's esophagus.[93]

Diet is a modifiable risk factor that may influence cancer risk through several mechanisms. Studies of fruit and vegetable intake are consistent with a protective role for anti-oxidants against the development of a Barrett's esophagus. A case-control study revealed that diets rich in fruits, vegetables and fish were inversely associated with Barrett's esophagus, whereas this risk in persons following Western dietary patterns (high in fast food and meat) may be adversely associated.[94] But again, it cannot be excluded that the association between diet and Barrett's esophagus is mediated by GERD.

Dietary nitrate is another component that may promote the development of Barrett's esophagus as a consequence of GERD. Nitrate is secreted by the salivary glands (that derive nitrate from the entero-salivary recirculation of dietary nitrate), and is converted into nitrite by oral bacteria. When swallowed saliva enters the acidic gastric environment, the nitrite is converted into nitrous acid and nitrosating species, which can form potentially carcinogenic compounds (see Figure 2). However, this process is inhibited by the vitamin ascorbic acid, which is actively secreted in gastric juice[95], thereby reducing these compounds to nitric oxide. Although this action of ascorbic acid inhibits the luminal generation of the potentially carcinogenic nitrosating species, it has been shown that nitric oxide can also rapidly diffuse into the adjacent epithelium, resulting in nitrosative stress in the epithelial cells (Figure 2).[96] It has been reported that in the case of severe GERD this process occurs in the esophageal lumen rather than the cardia, as saliva encounters gastric acids at a more proximal location.[97] However, the clinical significance of this nitrosative chemistry in the distal esophagus during acid reflux remains unclear. It has been suggested that high concentrations of nitric oxide causes oxidative stress, which may contribute to carcinogenesis.[98] Moreover, the same authors hypothesized that the increase in the incidence of adenocarcinoma of the distal esophagus and gastric cardia might be related to the increased dietary content of nitrates.[99]

Helicobacter pylori infection causes a chronic gastritis that is associated with the development of intestinal metaplasia and cancer.[100] *H. pylori* does not infect the esophagus, and its presence is not associated with an increased risk of Barrett's esophagus. In fact, some data suggest that gastric *H. pylori* infection may protect the esophagus from the effects of acid reflux by decreasing gastric acidity due to gastric atrophy.[101, 102] In fact, *H. pylori* infection might be associated with an increased risk of esophageal adenocarcinoma in patients in whom it causes high acid secretion secondary to an antrum-predominant, non-atrophic gastritis, but it might be associated with a reduced risk when the infection induces gastric atrophy.[102] Therefore, the pattern of gastric colonization induced by *H. pylori* infection may be the determinant of the effects of the infection on reflux disease.

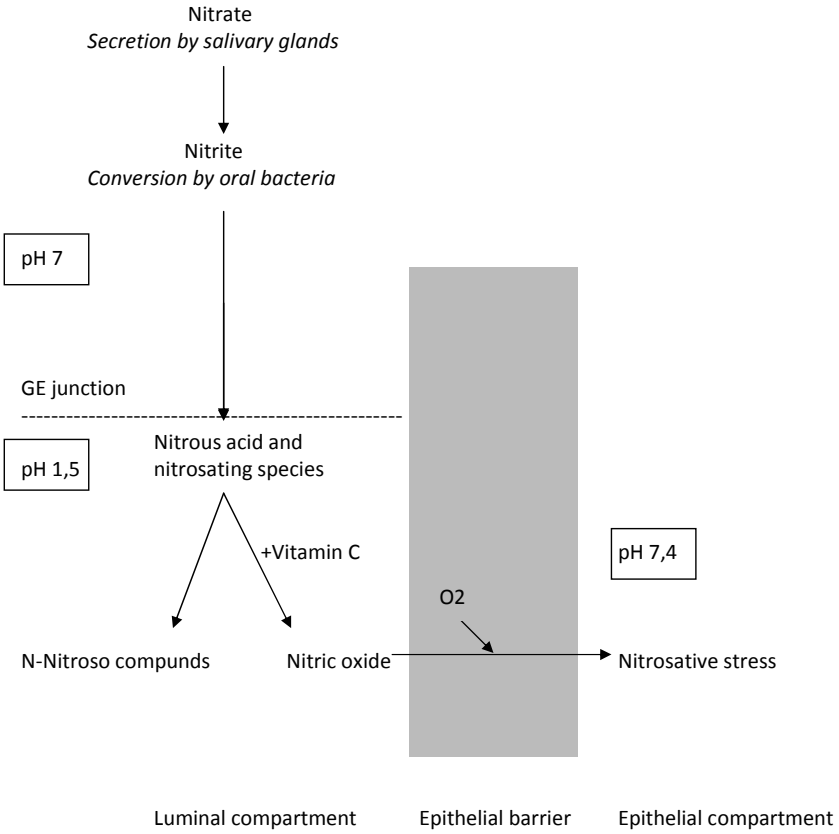

Figure 2. Chemical reaction occurring at the gastroesophageal junction when nitrite in saliva encounters acidic gastric juice. Adapted from Iijima *et al* (2003)[96].

3.4 Cell of origin of Barrett's metaplasia

It has now been generally accepted that Barrett's esophagus is considered to be an acquired condition as a consequence of GERD. Although the process of GERD and its contributing risk factors are well described, the exact mechanism underlying the transition from normal squamous epithelium into metaplastic columnar epithelium has not been identified yet. However, there are several theories with regard to the cell of origin that gives rise to the metaplastic change of the epithelium.

3.4.1 Upward migration of gastric epithelium

Initially, upward cell migration from the gastric epithelium into the distal esophagus to reconstitute the reflux-damaged squamous epithelium was favored.[11, 14] However, it was demonstrated in animal studies that development of a columnar esophagus is not hindered when there is a mucosal defect separating the distal esophagus from the transitional zone at the gastro-esophageal junction.[32, 103]

Furthermore, Barrett's metaplasia may include a variety of epithelial cells (including goblet and neuroendocrine cells) that are not found in the proximal stomach. Therefore, it was hypothesized that the cell giving rise to the columnar mucosa is intrinsic to the esophagus itself.

3.4.2 Transdifferentiation

Another possibility is the direct conversion of differentiated cells into another cell type in the absence of cell proliferation, a process called ´transdifferentiation´. It is based on the normal developmental process whereby the esophagus undergoes a columnar to squamous cell transition at 18 weeks of gestation.[104] Furthermore, it has been shown that during the development of the mouse esophagus, squamous cells arise directly from columnar cells independently of cell division and apoptosis.[105] It is assumed that the reverse transdifferentation (from squamous to columnar epithelium) could account for the generation of Barrett´s metaplasia in the context of GERD. However, this extrapolation of data may not be valid, as the embryological maturation of the esophagus may be quite different from the pathological development of metaplastic epithelium.

3.4.3 Transitional zone theory

The transitional zone theory states that cells at the gastro-esophageal junction undergo cellular migration and colonize the gastric cardia or distal esophagus in response to damaging luminal agents during reflux. This theory was based on the identification of a cell with features of both squamous and columnar epithelium that had been identified with scanning electron microscopy at the transitional zone between the normal squamous esophageal epithelium and the columnar epithelium of Barrett's esophagus.[106, 107] These newly colonized cells can express either a columnar

or a squamous phenotype depending on their location (esophagus or cardia)[108] and can maintain a growth advantage due their resistance to the luminal components. Furthermore, cells have been identified at the squamo-columnar junction that express both squamous and columnar cytokeratin markers.[109] Similarities exist between the structure of the GEJ and transitional zones in other areas of the body such as the cervix uteri, which shows cells of high plasticity in the transitional zone.[110]

3.4.4 De-novo metaplasia

More than twenty years ago, it has been hypothesized already that GERD induces esophagitis with destruction of squamous epithelium and ulceration, and that the ulcer is reepithelialized by multipotential undifferentiated stem cells.[111] The prevailing hypothesis today is that Barrett's esophagus develops when GERD damages the superficial layers of the esophageal squamous epithelium, thereby exposing stem cells in the basal layers of the epithelium to toxic agents that stimulate an abnormal differentiation.[112] As a result of chronic epithelial damage possibly induced by bile reflux and inflammatory conditions, the stem cells undergo a phenotypic or metaplastic change that will lead to Barrett´s stem cells eventually. It has been reported that a similar change can be observed during the process of mucinosis in the squamous mucosa of the vagina, which can be seen in atrophic vaginal epithelium in post-menopausal women.[113, 114] At this location there is little known about the exact cause and the clinical significance of these metaplastic cells.

3.4.5 Duct cell metaplasia

Columnar cells covering a Barrett's esophagus may also originate from ductal cells of esophageal submucosal glands.[32, 115] It has been suggested that stem cells exist in the glandular neck region of esophageal submucosal gland ducts similar to those found within the bulge region of the hair follicle.[116] Therefore, it is believed that after ulceration or damage, stem cells may grow out to form a new gland, giving rise to a duct by which the glandular cells are carried to the surface. The basis for this mechanism is the ulcer associated cell lineage: the development of a new cell lineage from mucosal stem cells that occurs adjacent to ulceration in the gastrointestinal tract.[117] Peptides related to the maintenance of mucosal integrity (*i.e.* the two trefoil

peptides pS2 and human spasmolytic polypeptide that contain three-fold shaped ('trefoil') cysteine-rich domains) are associated with this process and their expression was also reported in the metaplastic epithelium of Barrett's esophagus.[118] However, in rats in which no glandular structures are located in the esophageal epithelium, reflux can still trigger a similar transition into a Barrett's like metaplastic epithelium.[119]

3.4.6 Bone marrow stem cells

Apart from tissue-specific stem cells, it is now known that bone-marrow derived stem cells that circulate in the blood have such a degree of plasticity that they can also give rise to diverse epithelial cells.[120] Recently, a study reported on the contribution of bone marrow stem cells to the development of Barrett's esophagus in an animal model. Female rats were given a high dose of irradiation, followed by reconstitution of their bone marrow and immune systems through bone marrow transplants of male rats. Furthermore, both severe esophagitis and intestinal metaplasia were induced by esophagojejunostomy. The study revealed that after 8 weeks the male adult progenitor cells of bone marrow origin could be detected in the esophageal epithelial cells, thereby contributing to the esophageal regeneration and metaplasia in this model of Barrett's esophagus.[121] However, the authors have already pointed out that the possibility of fusion of the donor's bone marrow cells with the host's epithelial cells instead of the transdifferentiation into esophageal epithelial cells cannot be excluded.[121]

Overall, it can be concluded that the exact origin of the cells involved in the transition from a normal squamous epithelium into a metaplastic Barrett's epithelium has not been identified yet. However, it is most plausible that stem cells are involved in this process, as they are the only permanent residents of the epithelium.

3.5 Transformation into a columnar epithelium

To date only few studies have reported on the transformation of normal squamous esophageal cells into columnar epithelial cells from a molecular point of view. A recent study investigated the role of the bone morphogenetic protein (BMP) pathway in the metaplastic transformation process both *in vivo* and *ex vivo*.[122]

The study was based on the finding of the same group that the BMP-4 gene was abundantly expressed in Barrett's esophagus and esophagitis as a result of GERD.[123] BMP-4 is a protein belonging to the transforming growth factor (TGF)-β family that is involved in controlling cellular differentiation, migration and proliferation. In general, BMPs are induced during inflammation and injury. The BMP-pathway proved to be over-activated in esophagitis and Barrett's esophagus when compared to controls. Moreover, in *ex vivo* experiments it was shown that the differentiation of normal squamous cells toward a columnar cell type was induced by BMP-4, which was particularly illustrated by changes in cytokeratin expression patterns. Therefore, it was suggested that the BMP-pathway could play a role in the transdifferentiation of normal squamous esophageal cells into columnar cells.[122]

Another study investigated the role of retinoic acid in the transition between squamous and columnar cell types.[124] Retinoic acid (RA) is a powerful inducer of differentiation during embryogenesis and activates a number of cell-signaling pathways that are involved in determining the fate of embryonic cells.[125, 126] Indeed, one of the target genes of RA is the homeobox gene Cdx2 (encoding for a so-called homeodomain transcription factor that is specifically involved in the regulation of patterns of development, the morphogenesis), which is likely to induce a change in cell differentiation status.[127] In the esophagus, Cdx2 expression is observed in the areas of specialized intestinal metaplasia and this expression seems to be enhanced after exposure to various bile acids.[127-131] Interestingly, one of the components of the bile refluxate (lithocholic acid) has been demonstrated to influence the efficiency of retinoic acid.[132] In this study it was shown that *ex vivo* exposure of squamous biopsy specimens to both retinoic acid and lithocholic acid caused columnar differentiation. Conversely, an *ex vivo* Barrett's esophagus biopsy specimen could be transformed into a squamous-appearing epithelium through the inhibition of retinoic acid.[124] These observations implicate a retinoic acid-induced transformation to metaplastic epithelium. However, follow-up is needed by *in vivo* experiments.

3.6 Clonal expansion

Barrett's esophagus has been described as a clonal proliferative disorder: clonal fields of abnormal cells populate the metaplastic epithelium, with each field having potential clonal alterations in DNA content (ploidy), mutations or deletions.[133] After the initiation of a metaplastic stem cell, a stage of clonal expansion takes place, which may lead to rapid colonization of the adjacent mucosa.[134] Under conditions such as ongoing GERD, it is thought that this stem cell divides to produce two metaplastic stem cells instead of one stem cell and one differentiating transit amplifying cell.[134] Gland bifurcation is thought to be the consequence of this mechanism[135] (see Figure 3). These bifurcating glands will divide again thereby producing a large group of epithelial cells with a common genotype (clonal expansion). This process has also been shown to occur in the colon, thereby offering support to this theory.[136]

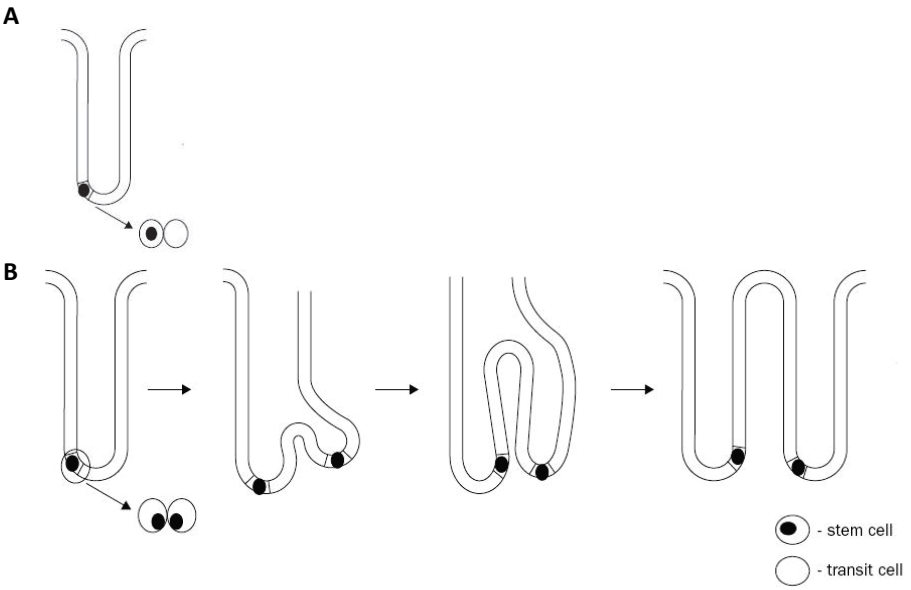

Figure 3A-B. Role of stem cell number in controlling glandular phenotype. Adapted from Jankowski *et al* (2000)[200].

3A. Stem cell division results in one transit cell and one stem cell, which causes gland homeostasis.

3B. Stem cell division results in two stem cells, which causes gland bifurcation.

4. Progression to esophageal adenocarcinoma

Patients with a Barrett's esophagus have a higher risk of developing esophageal adenocarcinoma when compared to the general population.[25, 137] Two meta-analyses showed that the overall estimate of cancer incidence in Barrett's esophagus varies between 6 and 7 cases per 1,000 person-years (0.6 – 0.7% per year).[138, 139] Another recently published systemic review focused on the incidence of esophageal adenocarcinoma in patients with histologically proven high-grade dysplasia who were undergoing surveillance. An average incidence rate of 6.6 per 100 patient years (range 2.3 – 10.3) was found.[140] However, one study showed an inverse relationship between study size and reported cancer risk in the setting of Barrett's esophagus, with small studies reporting much higher risks of cancer than larger studies.[141] This finding suggests publication bias, which may have lead to an overestimated cancer risk in patients with Barrett's esophagus in the literature.

It is generally accepted that the development of esophageal adenocarcinoma follows a metaplasia – dysplasia – carcinoma sequence, which is characterized by a number of genetic and epigenetic changes.[112] Currently, the histologic finding of high-grade dysplasia remains the most reliable predictor of progression to esophageal adenocarcinoma. Also, genetic changes linked to this progression may be used as biomarkers.[142, 143]

4.1 Hallmarks of cancer progression

In the development of an invasive carcinoma, six essential steps have been described: self-sufficiency in growth, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and ability for invasion and dissemination.[144] In Barrett's carcinogenesis there is clear documentation for all of these biological characteristics, which has been summarized previously.[145-147] Here, an overview is given of the most important molecular changes during the progression from metaplasia to dysplasia and, ultimately, invasive carcinoma.

4.1.1 Self-sufficiency in growth

The cell cylce is divided into G1 (first gap), S (DNA synthesis), G2 (second gap), and M (mitosis) phase. In G1, cells reach a key restriction point at which they either enter the S phase and complete the cell cycle, or exit the cycle and become quiescent (G0).[148] Growth signals are required for cells to leave the G0 phase and progress through the restriction point. Growth-signaling molecules bind to receptors on the cell surface, thereby activating intracellular pathways involving activation of growth regulatory molecules, including cyclins D1 and E. Cyclin D1 is a key regulator of cell-cycle progression, particularly at the transition from G1- to S-phase.[149] Expression of cyclins D1 and E has increased in neoplastic cells in Barrett's esophagus.[150-153]

Several growth factors have been associated with the metaplasia – dysplasia – carcinoma sequence in esophageal adenocarcinoma. The epidermal growth factor (EGF) as well as transforming growth factor-α (TGF-α) bind to the EGF receptor to stimulate cell proliferation.[154] Overexpression of the EGF receptor has been reported to correlate with tumor progression and a poor differentiation grade.[155-158]

Besides the EGF receptor, it has been shown that the hepatocyte growth factor (HGF) receptor (also known as Met) is overexpressed in both dysplastic epithelium and esophageal adenocarcinoma.[159] Activation of Met causes decreased apoptosis and enhanced proliferation, angiogenesis and invasion.[159, 160] Another study has identified Met expression as an independent prognostic risk factor in patients with esophageal adenocarcinoma: patients with high Met expression had a reduced survival and were more likely to develop distant metastases and local recurrences compared to patients with low Met expression.[161] Interestingly, inhibition of COX-2 has been shown to downregulate Met expression both *in vitro* and *in vivo.*[159, 162]

4.1.2 Insensitivity to anti-growth signals

In normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. These growth-inhibitory signals are received by cell surface receptors linked to intracellular signaling pathways. Proliferation can be blocked by two distinct mechanisms: cells may be forced out of the active cell

cycle into the G0 phase or cells may be pushed towards a permanent growth arrest characterized by differentiation. The Retinoblastoma (Rb)-pathway plays an important role in this process. Changes in genes that normally block Rb-phosphorylation (*i.e.* p16 and p53) have been identified. Loss of heterozygosity (LOH), mutations or promoter hypermethylation of the p16 gene have been reported in up to 80% of patients with a Barrett's esophagus.[163] Furthermore, p16 alterations are recognized as early molecular lesions associated with clonal proliferation within metaplastic epithelium.[164]

The adenomatous polyposis coli (APC) gene is a tumor suppressor gene that blocks cell proliferation by binding cellular signal proteins and by inducing differentiation. The prevalence of mutations in the APC gene is low in esophageal adenocarcinoma compared with colon cancer[165]; on the other hand, LOH on chromosome 5q (where the APC gene is located) frequently occurs.[166, 167]

Cell cycle progression of normal epithelial cells is inhibited by transforming growth factor-β (acting as a negative growth factor) whereas malignant epithelial cells are often insensitive to the growth inhibitory effects of TGF-β. Indeed, TGF-β responsiveness is reduced during all stages of the metaplasia – dysplasia – carcinoma sequence, resulting in an impaired TGF-β signaling. Loss of expression of the functional receptor for TGF-β is associated with adenocarcinoma of the esophagus.[168, 169] During subsequent tumor progression, TGF-β can be overexpressed, and may contribute to tumor invasion and systemic tumor spread. In esophageal adenocarcinoma TGF-β overexpression is associated with advanced tumor stage.[170]

4.1.3 Evading apoptosis

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation, but also by the rate of cell apoptosis (programmed cell death). Apoptosis can be regulated through several pathways that are activated by DNA damage. The protein p53 activates one of these pathways: DNA damage results in accumulation of p53 which stops the progression of the cell cycle until the genetic damage has been repaired or apoptosis has been induced.[171-173] Mutations,

LOH and deletions of the p53 gene have been reported in the majority of patients with esophageal adenocarcinomas.[174-176] Moreover, p53 mutations were associated with poor tumor differentiation grade, reduced disease-free survival and reduced overall survival.[177-180]

4.1.4 Limitless replicative potential

In normal cells the replicative potential is limited by the length of telomeres (ends of chromosomes). During each cell cycle a loss of 50-100 base-pair telomeric DNA of each chromosome is noted. After a certain number of divisions, the telomeres are too short to protect chromosomes from degradation, and the cell is triggered to exit from G1 into a permanent growth-arrested G0-state. To reach a state of unlimited replication, tumor cells must stabilize the length of their telomeres. In 85-95% of human cancers, stabilization of telomeres is achieved by reactivation of telomerase (which can impede telomere degradation).[181] Increasing levels of telomerase are observed along the metaplasia – dyplasia – carcinoma sequence.[182] Furthermore, it has been presumed that telomere dysfunction contributes to genomic instability in human cancer.[183]

4.1.5 Sustained angiogenesis

Angiogenesis is required to maintain tumor growth as oxygen and nutrients supplied by the vasculature are crucial for the development and progression of a malignant tumor. Tumor angiogenesis is a multi-step process. The initial step requires the release of angiogenic factors that stimulate endothelial cell proliferation and migration. The most potent angiogenic molecules belong to the vascular endothelial growth factor (VEGF) family, and are secreted by almost all solid cancers. Several groups have reported that, compared with the normal squamous epithelium of the esophagus, a higher level of expression of VEGF-A can already be observed in non-neoplastic Barrett's epithelium, with a further increase in high-grade intra-epithelial neoplasia and superficial cancer.[184-186] The switch towards an angiogenic state appears to be an early event in the progression towards esophageal adenocarcinoma. However, no prognostic role of increased expression of VEGF in patients with invasive esophageal cancer has been established yet.

4.1.6 Tissue invasion and dissemination

Abnormalities in cell-cell adhesion molecules play an important role in the process of invasion and dissemination of tumor cells. The principle functions of these molecules are to hold cells together and mediate cell-cell interactions. For example, E-cadherin on the surface of all epithelial cells, is linked to the actin cytoskeleton through interactions with catenins in the cytoplasm (especially β-catenin), and is able to form bridges with other cells. In epithelial cancers, a disrupted cell-cell adhesion might lead to metastases.[187, 188] A significant reduction of E-cadherin expression has been shown as the Barrett's metaplasia – dysplasia – carcinoma sequence progresses.[189] Furthermore, it has been reported that a reduced expression of both E-cadherin and β-catenin correlates with decreased patient survival in esophageal adenocarcinoma.[190, 191]

Loss in epithelial cell-cell contact is thought to play a pivotal role in the process by which epithelial cells acquire motile properties that are required for invasion. This process is called the epithelial to mesenchymal transition (EMT).[192] During EMT, epithelial cell-cell contact is decreased by the down-regulation of cytoskeletal components and the cell morphology becomes more fibroblast-like with up-regulation of mesenchymal markers.[193] It has been shown that EMT promotes cellular motility and invasion in a range of tumor cells *in vitro*. TGF-β is an important inducer of EMT,[193] and one immunohistochemical study confirmed its role in EMT in patients with esophageal adenocarcinoma.[194] However, more evidence is needed to support these limited data.

4.2 Genetic instability

The tendency towards these six tumorigenic steps to occur is increased by a general underlying phenomenon of genetic instability. Exposure to (duodeno-) gastroesophageal reflux has been shown to cause non-specific DNA damage,[195] and the most prominent gene abnormality that promotes mutagenesis in response to DNA damage is the loss of the p53 tumor suppressor protein. Epigenetic changes (*i.e.* hypo- or hypermethylation of DNA) may also result in genetic instability, which has been reported in the development of esophageal adenocarcinoma.[196] Aneuploidy

(abnormal nuclear DNA content) does not correlate with any single mutation, but reflects widespread DNA changes due to genomic instability.[146] Several studies showed that aneuploidy in Barrett's epithelium is associated with risk for progression to malignancy, and that the prevalence of aneuploidy increases with the degree of dysplasia.[197-199]

5. Summary

Barrett's esophagus is an acquired condition in which the normal squamous epithelium of the esophagus has been replaced by specialized (intestinal-type) columnar epithelium. Reflux of both duodenal and gastric contents is thought to be the causative factor. Several factors have been described that promote duodenogastroesophageal reflux, including dysfunction of the lower esophageal sphincter, the presence of hiatal hernia, obesity, dietary patterns and *Helicobacter pylori* infection. The refluxate induces several changes in the esophageal epithelium at the cellular and molecular level. Why only a minority of patients suffering from GERD develops Barrett's epithelium remains unknown. Despite recent progress in our understanding of some pathophysiologic observations in Barrett's esophagus, we have not been successful in identifying the key steps in cellular transformation. It is most plausible that stem cells are involved in this process, as they are the only permanent residents of the epithelium. Obviously, further research in this field is required that should focus on revealing the stem cells involved in the development of Barrett's esophagus, in order to achieve better understanding of this complex process.

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4

Early morbidity encountered in the dietary-related mouse model of Barrett's esophagus: A question of zinc?

Brechtje A Grotenhuis
Patrick F Franken
Willem JC Swinkels
Andre Boonstra
Martin A van der Valk
J Jan B van Lanschot
Riccardo Fodde

Diseases of the Esophagus – in press

Abstract

Introduction: Recently, a mouse model for Barrett's esophagus based on a zinc-deficient diet supplemented with deoxycholic bile acids has been published. The aim of this study was to attempt to reproduce these data and extend them by employing genetically modified mice and intraperitoneal iron supplementation.

Methods: The study design encompassed 6 experimental groups (wild type, *Apc*-mutant and *Smad4*-mutant mice, with or without iron injections), with all animals fed with the zinc-deficient diet supplemented with deoxycholic bile acids. All treatments were started at 3-5 weeks of age (the majority (78%) at 5 weeks). Animals were scheduled for euthanasia at two distinct time points, namely at 3 and 6 months of age.

Results: All mice showed signs of considerable distress already 4 weeks after the start of the modified diets, and had to be euthanized before the first evaluation time point (mean age 9.3 weeks, range 5-15 weeks). No differences were observed between wild type and genetically modified mice, or between animals with or without iron supplementation. On histological examination, we could not detect any lesions (Barrett's esophagus-like or tumors) other than esophagitis.

Conclusion: In the currently presented experimental settings, we were not able to reproduce the mouse model according to which Barrett's like lesions could be detected in animals fed with the zinc-deficient diet supplemented with deoxycholic bile acids.

Short report

Introduction

Gastroesophageal reflux disease is thought to trigger the development of Barrett's esophagus (BE), a condition where specialized intestinal cells replace the normal squamous epithelium of the distal esophagus. These metaplastic changes progress to dysplasia and esophageal adenocarcinoma (EA) with an annual conversion rate of approximately 0.5-1%. The molecular and cellular mechanisms underlying this metaplasia → dysplasia → carcinoma sequence are as yet poorly understood, partly due to the lack of a convenient and clinically relevant animal model. To date, the only *in vivo* model for BE is based on surgical manipulation of the rat's upper gastrointestinal (GI) tract, namely esophago(gastro)duodenal anastomosis leading to chronic reflux of both gastric and duodenal contents into the distal esophagus.[1] Although lesions resembling BE and/or EA do occasionally develop, a number of limitations make this model rather unpractical for research purposes, *e.g.* considerable animal suffering and death, low incidence of BE/EA lesions among surviving animals, uncertainty about the true nature of the lesions, and the limited possibilities of genetic manipulation in the rat. More recently, a new mouse model of esophagitis and BE has been described by feeding C57Bl6/J inbred mice with a zinc-deficient diet supplemented with deoxycholic bile acids.[2] All animals fed with the modified diet developed esophagitis and 63% presented with a Barrett's-like lesion after 88 to 152 days. However, no malignant EA-like lesions were observed.[2]

In an attempt to reproduce and expand on the results by Guy et al. [2], we fed the zinc-deficient diet supplemented with deoxycholic bile acids to wild type as well as *Apc*- (*Apc*$^{1638N/+}$)[3] and *Smad4*- (*Smad4*$^{E6sad/+}$)[4] mutant mice (on the inbred C57Bl6/J genetic background), the latter in view of their intrinsic predisposition to GI tract tumors. Furthermore, we tested the effects of intraperitoneal iron supplementation, previously shown to enhance tumor formation in the surgery-based model.[5] We hypothesized that the combination of these risk factors (zinc-deficient diet with supplemented bile acids, germline *Apc* and *Smad4* mutations, and iron supplement) would lay the basis for the generation of a novel non-invasive mouse model for BE-related EA.

Material and Methods

The study design encompassed 6 experimental groups (wild type, *Apc*-mutant and *Smad4*-mutant mice, with or without iron injections; see Table 1), with all animals fed with the zinc-deficient diet supplemented with deoxycholic bile acids.[2] The wild type mice without iron injections served as a control group for the study by Guy et al.[2] All treatments were started at 3-5 weeks of age. The majority of the animals (78%) received the zinc-deficient diet at 5 weeks of age after having been fed a standard mouse diet (zinc content 86.59 mg/kg, Special Diet Services, Essex, United Kingdom) from weaning. The remaining mice started were fed the zinc-deficient diet immediately from weaning. Animals were scheduled for euthanasia at two distinct time points, namely at 3 and 6 months of age.

Table 1. Study design: a total of 72 C57Bl6/J mice were allocated to 6 experimental groups according to the scheme here depicted. Note: to limit experimental variability, both the modified diet and the 0.2% deoxycholic acid supplements (Sigma D6750) were obtained from the same manufacturer (Harlan Teklad, Madison, WI, USA) as described in the original study.[2] Treatments were started at weaning and continued until the first signs of discomfort were observed.

	zinc-deficient diet + bile acids[2]	zinc-deficient diet + bile acids[2], supplemented by Fe^{2+} IP injections
wild type C57BL6/J	12	12
Apc$^{1638N/+}$ C57BL6/J	12	12
Smad4$^{E6sad/+}$ C57BL6/J	12	12

Results

All mice, regardless of the experimental group, showed signs of discomfort already 4 weeks after the start of the modified diets, with diarrhea and gradual weight loss. In view of the considerable distress, all animals had to be euthanized before the first evaluation time point scheduled at 3 months (mean age 9.3 weeks, range 5-15 weeks). No differences were observed between wild type and genetically modified

mice, or between animals with or without iron supplementation. Upon macroscopic examination, the intestines of all mice appeared severely inflamed (the colon being predominantly affected), and a distended coecum was found in the majority of the mice. On histological examination by H&E and Alcian Blue staining, we could not detect any lesions (BE-like or tumors) other than esophagitis (Figure 1).

To exclude that the observed lack of reproducibility resulted from an incorrect formulation of the zinc-deficient diet supplemented with deoxycholic bile acids, an independent batch was ordered from the same manufacturer and fed to wild type C57BL6/J mice (N=42). As for the larger study, similar results in terms of early signs of discomfort, esophagitis (Figure 1), and absence of BE-like lesions were observed.

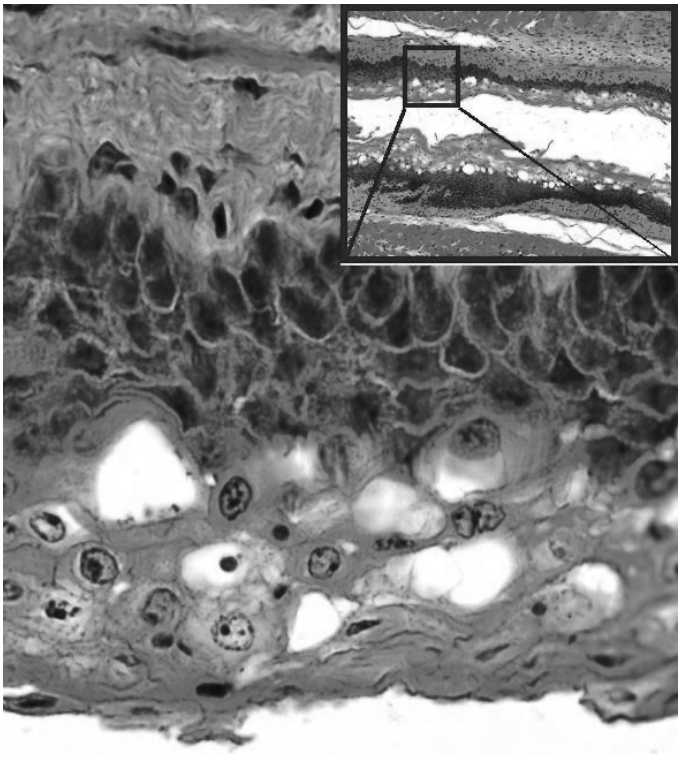

Figure 1. *Example of esophagitis as observed in wild type C57BL6/J mice fed with the zinc-deficient diet supplemented with bile acids, as described by Guy et al.[2] General hyperplasia of the squamous epithelium, including acanthosis, keratosis and degenerative vacuolization can be observed.*

Discussion

In this study we attempted but were not able to reproduce the results by Guy et al. according to which Barrett's like lesions could be detected after 88 to 152 days in 69% of the animals fed with the zinc-deficient diet supplemented with deoxycholic bile acids.[2] Several explanations can be envisaged for our results. First, the effect of zinc-deficiency on immunity and the possible presence of pathogens (*e.g. Helicobacter*) in the mouse facility. However, the above-described experiments were performed within a pathogen-free unit where no *Helicobacter* has been detected during the time our study was carried out.

Second, the early morbidity in the present study may be explained by the different time points at which administration of the modified diet was started: whereas in the study by Guy et al. all mice were kept on a regular dietary regime for 2 weeks after weaning before being fed the experimental diet (*i.e.* at 5 weeks of age), in our study the age of the animals upon start of the treatment was 3 to 5 weeks. However, only a minority of these animals were fed the diet immediately after weaning (*i.e.* at 3 weeks of age) whereas the vast majority (78%), in compliance with the Guy et al. study, were first administered a conventional chow for 2 weeks after which the zinc-deficient diet was provided. Notably, the zinc level present in regular chow employed in the Guy study was significantly higher than present in mouse milk.[6] As zinc levels build up in the tissues (when provided in the diet), it is plausible to assume that the mice started the zinc-deficient regime with a considerable zinc reserve. The zinc content of the regular chow administered in our study prior to the start of the experimental diet was 86.59 mg/kg, which is very similar to the zinc content (89.3 mg/kg) of standard mouse diet fed prior to administration of the zinc-deficient regime in the Guy et al. study.[2]

A third putative explanation for the contrasting results resides in the regular bedding of the mice cages the zinc content of which may represent a confounding factor. Bedding may indeed contain zinc, although it is not known how much it can affect zinc intake in rodents. According to the testing results of the bedding in our mouse facilities (Agrolab, Kiel, Germany), no zinc has been detected in the reports sent out during the period of time in which our experiments were carried out. No details have

been provided with regard to this issue in the primary report of Guy et al. Hence, it is possible that the presence of zinc in the cage bedding employed in the Guy et al. study is responsible for a protective effect to the early morbidity observed in our experimental setup.

Overall, although our results did not confirm the original report by Guy and collaborators[2] and failed to induce Barrett's-like lesions, the presence of inflammatory lesions (esophagitis) is of good auspices and it is still plausible to think that a different formulation or administration schedule of the diet, or the use of a different type of cage bedding will result in the modeling of the effects of gastroesophageal reflux on Barrett's metaplasia and esophageal adenocarcinoma. If confirmed, the diet approach will also be useful in addressing the role played by mutation in specific genes as here attempted with the *Apc* and *Smad4* tumor suppressor genes.

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B

Optimization of staging

5

Preoperative assessment of tumor location and station-specific lymph nodal status is inaccurate in patients with esophageal adenocarcinoma

Brechtje A Grotenhuis
Bas PL Wijnhoven
Jan Werner Poley
John J Hermans
Katharina Biermann
Manon CW Spaander
Marco J Bruno
Hugo W Tilanus
J Jan B van Lanschot

Submitted for publication

Abstract

Introduction: In patients with resectable adenocarcinoma of the gastroesophageal junction (GEJ) preoperative staging will determine the therapeutic strategy with regard to type of surgical procedure and use of neoadjuvant therapy. Tumor location and lymph node status play a pivotal role in this tailored strategy. The aim of this study was to prospectively evaluate the accuracy of preoperative assessment of tumor location according to the Siewert classification and lymph node status per station with endoscopy/endoscopic ultrasound (EUS) and CT.

Methods: In 50 esophagectomy patients with adenocarcinoma of the GEJ without application neo-adjuvant therapy, tumor location according to Siewert and N-stage per nodal station (paratracheal, aortopulmonary window, subcarinal, paraesophageal, lesser curvature, celiac trunk nodes) as determined preoperatively by endoscopy/EUS and CT were compared with the histopathologic findings in the resection specimen.

Results: Overall accuracy in predicting tumor location according to the Siewert classification was 70% for endoscopy/EUS and 72% for CT. Preoperative data could not be fully compared with the pathologic assessment in 11 patients (22%), as large tumors obscured the landmark of the gastric folds. The overall accuracy for predicting the N-stage in 250 lymph node stations was 66% for EUS and 68% for CT. The accuracy was good for those stations located high in the thorax, but poor for celiac trunk nodes.

Conclusion: Given the frequent discrepancy between the endoscopic and pathologic location of the GEJ and the common problem of advanced tumors obscuring the landmarks used in the assessment of the Siewert classification, its usefulness in the assessment of tumor location is limited. The overall accuracy for EUS and CT in predicting the N-stage per station was moderate. When the therapeutic approach depends on the status of a specific lymph node station, a more objective and reliable assessment of lymph nodal involvement (*e.g.* EUS-FNA) should be considered.

Introduction

In patients with resectable esophageal adenocarcinoma, surgical therapy is the treatment of choice. A complete removal of the primary tumor and its lymphatic drainage is the primary goal of any surgical approach. An individualized therapeutic strategy is thought to improve overall survival in patients with esophageal cancer.[1-3] Decisions on the surgical approach and possible application of neoadjuvant therapy play an important role in this tailored strategy, which relies largely on the location of the primary tumor and positive lymph nodes. Hence, accurate preoperative staging is a prerequisite for choosing the most optimal therapeutic strategy.

The 'Siewert classification' was introduced in 1997 for the classification of adenocarcinomas arising in the area of the gastroesophageal junction (GEJ).[4] This classification is based on the specific topographic characteristics, *i.e.* the location of the tumor center up to 5 cm above, at, or up to 5 cm below the gastric cardia (defined by the proximal end of the gastric folds). It is thought that three distinct tumor entities arise in this area (Figure 1): type I - adenocarcinomas of the distal esophagus with the center of the tumor more than one centimeter above the GEJ; these tumors generally arise from an area of intestinal metaplasia in the esophagus; type II - adenocarcinomas of the gastric cardia, arising from cardiac epithelium or a short segment of intestinal metaplasia, with the tumor center located within 1 cm proximally and 2 cm distally to the GEJ ('true' carcinomas of the cardia); type III - subcardial gastric carcinomas, with the tumor center more than 2 cm distal to the GEJ infiltrating the junction and the distal esophagus.

Lymph node status (N-stage) is divided into two categories according to the TNM-classification: N0 (no suspicious locoregional lymph nodes) and N1 (one or more locoregional suspicious lymph nodes).[5] Endoscopic ultrasonography (EUS) has been shown to be superior in the assessment of locoregional lymph node status in patients with esophageal cancer[6-9]: the reported diagnostic accuracy of EUS varies between 65% and 86%.[10-16] No subdivision or detailed assessment per nodal station is usually provided as N1-status is rarely considered a contra-indication for surgery. Recently, a revised classification has been proposed, in which the prognostic significance of

the location of the lymph nodes (in relation to the diaphragm) has already been emphasized.[17]

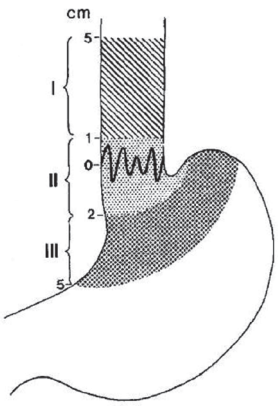

Figure 1. *Classification of adenocarcinomas of the esophagogastric junction according to Siewert: this classification is based on the topographic anatomic characteristics and the location of the tumor center up to 5 cm above (type I), at (type II), or up to 5 cm below (type III) the gastric cardia (as defined by the proximal end of the gastric folds). Reprinted with permission from Fein et al, Surgery, 1998.*[44]

For an individualized surgical treatment, the type of operation as well as the application of neoadjuvant therapy largely depend on the location of the tumor and its related pattern of dissemination.[1] The surgical strategy will be influenced by the preoperative assessment of tumor location according to the Siewert classification (esophagectomy versus gastrectomy) and nodal status (transthoracic versus transhiatal esophagectomy). Furthermore, a reliable classification with regard to tumor location is indispensable in case neoadjuvant therapy is considered, since patients with a cardia carcinoma benefit from (neo)adjuvant chemotherapy according to the MAGIC-scheme[18], whereas patients with esophageal cancer might benefit from neoadjuvant chemoradiation.[19] Accurate information on the location of positive lymph nodes is also required to determine the extent of the radiotherapeutic field (distance between tumor and involved lymph nodes) which can turn the scale whether neoadjuvant chemoradiation is feasible in the first place. Moreover, in patients with

gross involvement of celiac trunk lymph nodes, induction chemotherapy or palliation are the treatment options of choice rather than primary surgery.[20]

Hence, an accurate preoperative assessment of the location of the primary tumor and positive lymph nodes is required for making therapeutic decisions for the individual patient. Nowadays upper gastrointestinal endoscopy combined with EUS is considered the gold standard for these preoperative assessments. However, in the literature little evidence is available about the accuracy of EUS and CT for determining the location of the primary tumor and potential positive lymph nodes. The aim of the present study was to investigate prospectively the accuracy of endoscopy/EUS and CT in determining tumor location according to the Siewert classification and lymph node status per station by comparing the preoperative assessment with the histopathologic findings in the resection specimen (gold standard).

Patients and methods

Patients

Between April 2008 and December 2009, 104 patients underwent esophagectomy for cancer of the esophagus or GEJ in the Erasmus MC. Patients with esophageal cancer received neoadjuvant chemoradiotherapy in the setting of a randomized controlled trial (N=20).[19] (Neo)adjuvant chemotherapy was given to patients with a tumor located in the cardia.[18] Induction chemo- and/or radiotherapy was given in patients with either a cT4-tumor without distant metastases or in patients with gross involvement of celiac trunk lymph nodes (M1a), who were not considered eligible for primary surgical therapy (N=10). To avoid possible stage migration following chemo- and/or radiotherapy, patients receiving neoadjuvant therapy were excluded from this analysis. In five patients no surgical resection was performed as the tumor was irresectable intraoperatively. As the Siewert classification arranges adenocarcinomas in the vicinity of the GEJ, patients with a squamous cell carcinoma of the esophagus (N=4) were excluded from this analysis. In total, data of 50 patients were available in the present study. In 26 patients a transthoracic esophagectomy (TTE) with extended

lymphadenectomy was performed, whereas 24 patients underwent a transhiatal esophagectomy (THE) with locoregional lymphadenectomy only. The applied surgical techniques have been described previously.[21, 22]

Methods

All patients underwent the standard work-up including upper gastrointestinal endoscopy combined with EUS, CT of chest and abdomen and external ultrasound of the neck. Endoscopic procedures were performed by experienced gastroenterologists with a Q-endoscope (GIF-Q180; Olympus Europeholding, Hamburg, Germany) and an electronic radial echoendoscope (GF-UE160-AL5; Olympus Europeholding, Hamburg, Germany). Stenotic tumors were not dilated. The mechanical blind probe (MH-908) has not been available in our unit. The CT protocol consisted of a standardized timing of contrast in both arterial and venous phases, maximized esophageal and gastric distension by adding oral carbon dioxide-granules, and adjustment of the thickness of the (reconstruction) sections to a maximum of 5 mm.[23, 24]

Data were prospectively collected of all patients in whom a malignant tumor of the esophagus or GEJ was suspected and who underwent the routine staging procedures. The Medical Ethics Committee of the Erasmus MC approved the study; there was no need for patients' informed consent. Data-analysis was carried out with SPSS version 15.0 (SPSS, Chicago, IL, USA).

A. Accuracy of assessing tumor location according to the Siewert classification

Registration of data with regard to the exact tumor location on upper gastrointestinal endoscopy combined with EUS, and CT was completed by the endoscopist and radiologist, respectively. On endoscopy and EUS the upper and lower limit of the tumor as well as the position of the Z-line (squamocolumnar junction), GEJ (proximal end of the gastric folds) and diaphragm were determined (distance as measured from the incisors). The endoscopist scored patient's tumor type according to the Siewert classification by assessing the length and location of the tumor in relation to the proximal end of the gastric folds on conventional endoscopy. In case the tumor covered this landmark (thus prohibiting a proper assessment of its location), patient's

tumor type was assessed in relation to the diaphragm on EUS. The radiologist examined the length of the tumor and the position of the GEJ (above or below the diaphragm). Subsequently, the tumor was radiologically classified as a Siewert type I, II or III. The surgical resection specimen was analyzed by a dedicated gastrointestinal pathologist to determine patient's tumor type according to the Siewert classification by assessing the length and location of the tumor in relation to the proximal end of the gastric folds. Finally, a comparison was made between the endoscopic and radiologic classifications as established preoperatively (by endoscopy/EUS and CT) and the histopathologic classification as assessed postoperatively by the pathologist in the resection specimen. The latter was considered the gold standard.

B. Accuracy of assessing N-stage per lymph node station

Data were collected with regard to tumor stage (cTNM-stage) as predicted by EUS and CT. The most commonly involved lymph node stations (intrathoracic: upper paratracheal - station 2, lower paratracheal including the azygos vein nodes - station 4, aortopulmonary window - station 5, subcarinal - station 7, paraesophageal - station 8; intraabdominal: lesser curvature (including paracardiac nodes), celiac trunk) were evaluated separately.[25-27] In the vicinity of the celiac trunk the splenic and hepatic arteries were identified. Lymph nodes within a radius of 2 centimetres of its branching were classified as celiac trunk nodes. On EUS a lymph node was considered malignant based on morphological criteria as previously described.[28, 29] In case fine needle aspiration (FNA) was performed of lymph nodes, the initial endoscopic classification was not changed when the cytology results were disclosed. The radiologist identified suspected lymph nodes according to size (>6 mm for paraesophageal nodes and >10mm for all other lymph node stations) and the ratio between the longitudinal and transverse dimensions (L/T ratio<1.5) of the nodes.[24] During a transthoracic resection with an extended lymphadenectomy the surgeons sampled separately the intrathoracic lymph node stations (paratracheal, aortopulmonary window, subcarinal nodes) as well as the celiac trunk nodes. An experienced gastrointestinal pathologist identified the paraesophageal and the lesser curvature lymph nodes in the surgical resection specimen and analyzed these nodes separately. In case a transhiatal procedure was performed, only three lymph

node stations were assessed (paraesophageal, lesser curvature, celiac trunk nodes). The pathologist did not search for micrometastases or isolated tumor cells. Finally, a comparison was made between the preoperative N-stage per lymph node station (as evaluated by EUS and CT) and the histopathologic N-stage (as assessed by the pathologist in the surgical resection specimens). The latter was considered the gold standard.

Results

Clinicopathological characteristics of the current study population are shown in Table 1. The endoscopic preoperative evaluation was incomplete in 3 patients as the tumor could not be passed by a conventional or pediatric endoscope; in 7 patients the tumor was too stenotic on EUS to allow complete passage. In 6 patients radiological staging was incomplete since the tumor was not visible on the CT-scan. Four of these 6 patients were diagnosed with an early tumor stage (pT1). The median time period between the preoperative investigations and surgery was 6.0 weeks (± 2.4, range 3.0 – 12.0 weeks).

A. Accuracy of assessing tumor location according to the Siewert classification
In 11 patients (22%) the pathologist was not able to assign the exact location of the tumor to one of the 3 tumor types according to the Siewert classification. In 8 of these 11 patients the tumor had grown circumferentially in the distal esophagus and invaded the proximal part of the stomach, thereby covering the proximal end of the gastric folds and thus impeding a proper classification. These tumors were classified as Siewert type I/II. In another 3 patients the landmark of the gastric folds was visible, but the tumor invaded exactly one centimetre from the GEJ proximally into the esophagus and 4-5 cm distally into the stomach, without a clear tumor center at either side of the spectrum. Therefore, these three tumors were classified as Siewert type II/III.

Table 1. Clinicopathological characteristics of 50 patients in our study population who underwent esophagectomy for adenocarcinoma of the distal esophagus or gastroesophageal junction.

Age*	65 years (48-81)
Gender	39 (78%)
- *male*	11 (22%)
- *female*	
Type of operation	26 (52%)
- *transthoracic esophagectomy*	24 (48%)
- *transhiatal esophagectomy*	
Barrett's metaplasia	20 (40%)
- *yes*	30 (60%)
- *no*	
Tumor infiltration depth (pT)	4 (8%)
- *pT1*	9 (18%)
- *pT2*	37 (74%)
- *pT3*	
Lymph nodal status (pN)	14 (28%)
- *pN0*	36 (72%)
- *pN1*	
Differentiation grade	
- *G1 (good)*	4 (8%)
- *G2 (moderate)*	23 (46%)
- *G3 (poor)*	23 (46%)
Radicality of resection	36 (72%)
- *R0*	9 (18%)
- *R1a*	5 (10%)
- *R1b*	
Total number of harvested nodes*	25 (8-58)
Number of positive lymph nodes*	3 (0-34)
Lymph node ratio*	0.10 (0.00-1.00)

** Value presented as median (range in brackets)*

R0	= resection margin microscopically tumor-free, > 1mm
R1a	= resection margin microscopically tumor-free, but < 1mm
R1b	= resection margin microscopically not tumor-free
Lymph node ratio	= number of positive lymph nodes/total number of harvested nodes

The results of the preoperative assessment of tumor location by endoscopy/EUS are shown in Table 2A. Endoscopic accuracy was high when the tumor was (mainly) located in the distal esophagus (accuracy 100% in type I tumors). However, the accuracy of the type II tumors was 33% only; the majority of the type II lesions was preoperatively classified as a type I. In those patients in whom the tumor was allocated as a type I/II by the pathologist, the endoscopist had classified 6 out of 8 tumors either as a type I or a type II tumor. Finally, the overall accuracy of endoscopy in combination with EUS in determining the location of the tumor according to the Siewert classification was 70% (35/50) if allowing a type I or type II tumor on endoscopy to predict a type I/II tumor and a type II or type III tumor to predict a type II/III tumor.-

The results of the preoperative assessment of tumor location by CT are shown in Table 2B. The radiologist was able to correctly classify type II tumors more often than the endoscopist (53%), although the accuracy for type I tumors was lower (77%). The overall accuracy of CT in determining the location of the tumor according to the Siewert classification was 72% (36/50) if allowing a a type I or type II tumor on the CT-scan to predict a type I/II tumor and a type II or type II tumor to predict a type II/III tumor. In 7 patients (14%) in whom the endoscopist encountered a stenosis on endoscopy/EUS, the radiologist was able to correctly determine the location of the tumor on the CT-scan in 6 out of 7 patients (86%).

B. Accuracy of assessing N-stage per lymph node station

The endoscopist was not able to fully complete the preoperative evaluation in 7 patients (14%) as the tumor could not be passed by the echoendoscope. In 5 of these 7 patients no suspected nodes were seen proximally to the tumor (cNx=5, cN1=2). In 26 patients an extended lymphadenectomy was performed by means of TTE. In most of these patients high paratracheal (N=26), low paratracheal (N=24), aortopulmonary window (N=24) and subcarinal lymph node stations (N=26) were harvested intraoperatively. In all fifty patients paraesophageal and lesser curvature lymph nodes were identified by the pathologist in the resection specimen, whereas the surgeon had always sampled the celiac trunk nodes separately. Therefore, a total number of 250 lymph node stations was assessed in the current study.

Table 2A-B. Accuracy of preoperative staging of the location of the tumor according to the Siewert classification by upper gastrointestinal endoscopy in combination with EUS (2A) and CT (2B). The histopathologic findings (tumor location in the surgical resection specimen) were considered the gold standard.

2A.

Pathology	Endoscopy / EUS			
	Type I	Type II	Type III	*NA*
Type I (N=22)	22	0	0	*0*
Type II (N=15)	9	5	0	*1*
Type III (N=2)	0	1	1	*0*
Type I/II (N=8)	5	1	0	*2*
Type II/III (N=3)	2	1	0	*0*

NA = not available; as the tumor appeared to be stenotic on endoscopy (N=3), assessment of its location according to the Siewert classification was not feasible

2B.

Pathology	CT			
	Type I	Type II	Type III	*NA*
Type I (N=22)	17	2	0	*3*
Type II (N=15)	6	7	0	*2*
Type III (N=2)	0	0	2	*0*
Type I/II (N=8)	7	0	0	*1*
Type II/III (N=3)	1	2	0	*0*

NA = not available; tumor was not visible on CT (N=6), thereby impeding an evaluation of its location according to the Siewert classification.

When comparing the tumor infiltration depth and overall lymph nodal status as assessed preoperatively by EUS (cTNM-stage) with the histopathologic outcome as evaluated in the resection specimen (pTNM-stage), the overall accuracy of EUS was 79% for patient's T-stage and 66% for N-stage (N=43). The overall accuracy of CT was 52% for patient's N-stage (N=50). The overall accuracy for predicting patient's N-stage per station was 66% (166/250) for EUS and 68% (170/250) for CT. The accuracy for all stations as assessed preoperatively by EUS or CT and compared with the histopathologic outcome is shown in Table 3. The accuracy of EUS and CT in predicting the N-stage per lymph node station was good for those stations located

high in the thorax (paratracheal and aortopulmonary window nodes). The accuracy declined to 50-70% for the predominantly peritumoral lymph nodes (subcarinal, paraesophageal and lesser curvature nodes).

Table 3. Accuracy in the evaluation of the N-stage of 250 lymph node stations, when comparing the preoperative assessment by means of EUS and CT, respectively, with the postoperative histopathologic outcome.

Pathology	EUS	CT
PT-high (N=23)	20/23 (87%)	20/23 (87%)
PT-low (N=24)	22/24 (92%)	21/24 (88%)
AOP (N=24)	20/24 (83%)	22/24 (92%)
SC (N=26)	13/26 (50%)	15/26 (58%)
PE (N=50)	34/50 (68%)	31/50 (62%)
LC (N=50)	24/50 (48%)	26/50 (52%)
TR (N=50)	29/50 (58%)	35/50 (70%)

PT-high = high-paratracheal lymph nodes
PT-low = low-paratracheal lymph nodes
AOP = aortopulmonary window lymph nodes
SC = subcarinal lymph nodes
PE = paraesophageal lymph nodes
LC = lesser curvature lymph nodes
TR = celiac trunk lymph nodes

For staging celiac trunk nodes, EUS and CT showed an accuracy of 58% and 68%, respectively. This relatively low accuracy was mainly caused by understaging (false-negative rate 34% with EUS and 25% with CT). When the results of the 7 patients in whom the preoperative evaluation by EUS could not be completed due to a stenotic tumor are discarded, a total number of 202 lymph node stations was available for analysis (Table 4). EUS and CT showed similar results in terms of false-positive and false-negative staging. Notably, approximately one out of five lymph node stations had been classified with a false-negative status (23% for EUS and 22% for CT). For EUS, these data result in an overall sensitivity of 35% and a specificity of 81% in determining N-status per lymph nodal status with EUS, and for CT 34% and 86%, respectively.

Table 4. Staging the lymph nodal status of 202 lymph node stations, when comparing the preoperative assessment by means of EUS and CT with the postoperative histopathologic outcome: accurate, false-positive or false-negative prediction.

	Accurate prediction	False-positive prediction	False-negative prediction
EUS	137/202 (68%)	18/202 (9%)	47/202 (23%)
CT	138/202 (68%)	20/202 (10%)	44/202 (22%)

Discussion

In the present study the accuracy of preoperative assessment of primary tumor location according to the Siewert classification and lymph nodal status per station were evaluated prospectively in patients with adenocarcinoma of the GEJ. The predicted tumor location and N-stage per nodal station as determined by endoscopy/EUS and CT were compared with the histopathologic findings in the resection specimen (gold standard). First of all, the overall accuracy in predicting the location of the tumor according to the Siewert classification was 70% for endoscopy/EUS and 72% for CT. These results are in line with an other study that attempted to evaluate the Siewert classification by means of a retrospective comparison between endoscopic and pathologic results, in which an accuracy of 72.5% was achieved by examining the tumor location with endoscopy/EUS in 54 patients over a 15-year time period.[30] However, in the present study a proper comparison between the preoperative data and the pathologic outcome was not feasible in 11/50 patients (22%) as large tumors obscured the landmarks in such way that a clear assignment to one of the Siewert classes was impossible. Previous studies have also shown that determining the precise location of the GEJ can be difficult, reflecting a practical problem with regard to the implementation of the Siewert classification in daily clinical practice. Even pathologists sometimes struggle to correctly classify these tumors. Another illustration of this problem was shown in a randomized controlled trial on esophageal and GEJ adenocarcinomas, in which a substantial difference

was demonstrated between the endoscopic tumor classification and the pathologic tumor classification in the resection specimen.[22] The endoscopists tended to classify tumors at the GEJ as type I, whereas the pathologists decided in favor of type II relatively more often.[22] This shift from type I to type II tumors was also observed in the present study. An other study analyzed retrospectively the endoscopy reports of 613 patients in order to assign the location of the tumors according to the Siewert classification. However, in 96 cases (16%) the size of the tumor precluded a precise classification due to obscured landmarks.[31] In general, practical problems with the Siewert classification in case of a locally advanced tumor with invasion of the GEJ have been recognized before. Although not specified in the official classification, it has been described by Siewert and colleagues that in patients with locally advanced tumors, the tumor mass must be used as a guide for classification rather than the tumor center.[2] If more than 50% of the tumor mass is located within the tubular esophagus, the presence of a type I tumor can be assumed.[2] However, this does not solve the problem, because determination of the tumor location remains difficult in case the anatomical landmarks of the GEJ are invisible.

Secondly, the overall accuracy for predicting the N-stage per lymph node station was 66% (166/250) for EUS and 68% (170/250) for CT. The endoscopist was not able to fully complete the preoperative evaluation in 7/50 patients (14%) as the tumor could not be passed by the echoendoscope. The evaluation of stenotic tumors (a subgroup of up to 25% of patients with esophageal cancer[32]) is a well-recognized major limitation of EUS. Partial staging of the tumor (only up to the proximal margin of the stenosis) has a poor accuracy (<50%).[32] Smaller calibre probes have been introduced that can address this problem, as dilation of the tumor for staging is in general not advisable.[33] However, these mini-probes have no value in the staging of stenotic esophageal cancer since the penetration depth is very limited and acoustic coupling below the level of the stenosis is almost impossible. In the present study it appeared that the accuracy of EUS and CT in predicting the N-stage per lymph node station was good for those stations located high in the thorax, which play a large role in the decision making whether to perform an extended lymphadenectomy. The accuracy declined to 50-70% for the lymph node stations located peritumorally, although these results are without clinical consequences in patients with advanced

tumors. More disappointingly, EUS showed an accuracy in terms of predicting the N-stage of celiac trunk nodes of 58% (with a high false-negative rate), which is much lower than reported in literature (approximately 80%).[34, 35] Also, both EUS and CT tend to understage in our study, as one out of five lymph node stations was classified as false-negative by these imaging-modalities. When evaluating retrospectively the false-negative classified lymph nodes, it appeared that in most cases these nodes have not been seen on EUS (29/47) or CT (34/44), rather than misinterpretation has played a role. In many institutes EUS has a significant impact on deciding which treatment modality should be offered to an individual patient with esophageal cancer.[36, 37] However, based on the results of the current study, this should be done with caution in case the status of the celiac trunk nodes plays a large role in the clinical decision-making.

To optimize treatment for patients with adenocarcinoma of the GEJ, improvement in the preoperative assessment of the location of the primary tumor and positive lymph nodes is required. The Siewert classification is based on the anatomical location of the tumor center or, in patients with an advanced tumors, the location of the tumor mass.[4] Rather than focusing on whether the epicenter of the primary tumor or tumor mass is located in the esophagus or stomach, information on to what extent the other organ is involved (*e.g.* a distal esophageal tumor with invasion into the proximal stomach) will provide more useful information. After all, it is the presence and extent of tumor invasion across the GEJ (either from the proximal or distal side) that will influence the surgical approach. A total gastrectomy is usually not performed in case of proximal gastric cancer if substantial tumor invasion (>1-2 cm) of the distal esophagus is present: the risks of a positive proximal resection margin as well as lymphatic dissemination to the thoracic basins are too high. On the other hand, in case a distal esophageal tumor largely invades the cardia (>4-5 cm), it is questionable whether a resection with gastric tube reconstruction is oncologically safe. Information about tumor invasion across the GEJ and about total tumor length (which determines the extent of the radiotherapeutic field) can also be of importance when deciding whether a patient is eligible for neoadjuvant chemoradiation. The extent of tumor invasion at both sides of the GEJ can be assessed by means of conventional endoscopy with the proximal end of the gastric folds as landmark. In

case the GEJ is not visible due to advanced tumor growth, the diaphragm can be taken as landmark on EUS, although this will not supply accurate results in patients with a hiatus hernia.

The moderate overall accuracy for EUS and CT in predicting the N-stage per station will require a more objective and reliable assessment of lymph node involvement. EUS-guided fine needle aspiration (FNA) that allows a cytological diagnosis can play an important role in this field.[38] Indeed, EUS-FNA has proven to be more accurate in comparison to EUS alone and CT for nodal staging[16], although sampling error may occur in daily clinical practice. Furthermore, EUS-FNA is not feasible in case lymph nodes are located peritumorally and in stenotic tumors. Because of these practical problems, we have restricted the use of EUS-FNA to those lymph nodes of which the status will influence the therapeutic approach.[39, 40]

Furthermore, lymph node detection by magnetic resonance imaging has improved since a new contrast agent USPIO (ultrasmall superparamagnetic iron oxide) has been introduced. Early clinical studies have shown that USPIO–enhanced MRI improves sensitivity and specificity for detection of nodal metastases based on enhancement patterns.[41-43] Nevertheless, further evidence for a possible additional value of USPIO-enhanced MRI is lacking thus far.

In conclusion, we evaluated prospectively the accuracy of preoperative assessment of primary tumor location according to the Siewert classification and lymph nodal status per station. The overall accuracy in predicting the location of the tumor was moderate (EUS 70%, CT 72%). Moreover, given the frequent discrepancy between the endoscopic and pathologic location of the GEJ and the encountered problem with advanced tumors obscuring the commonly used landmarks, the usefulness of the Siewert classification in the assessment of primary tumor location in daily clinical practice is limited. Assessment of tumor invasion across the GEJ rather than focusing on the anatomical location of the GEJ and the epicenter of the tumor might be a useful alternative. Finally, the overall accuracy for EUS and CT in predicting the N-stage per lymph node station was moderate (EUS 66%, CT 68%). The accuracy was good for those stations located high in the thorax, but poor for the celiac trunk nodes. In case the status of a nodal station will influence the therapeutic approach, a more objective and reliable assessment of lymph nodal involvement (*e.g.* EUS-FNA) should be considered.

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6

The sentinel node concept in adenocarcinomas of the distal esophagus and gastroesophageal junction

Brechtje A Grotenhuis
Bas PL Wijnhoven
Ronald van Marion
Herman van Dekken
Wim C Hop
Hugo W Tilanus
J Jan B van Lanschot
Casper HJ van Eijck

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Abstract

Introduction: The sentinel node concept is of great value in the treatment of various malignancies. In this study we investigated whether the application of the sentinel node procedure is feasible in esophageal adenocarcinoma and whether it can tailor surgical treatment of the individual patient.

Methods: In 40 patients with an adenocarcinoma of the distal esophagus or gastroesophageal junction, blue dye was injected around the tumor intra-operatively. Sentinel nodes (blue-stained) as well as non-sentinel nodes were identified and dissected during transhiatal esophagectomy. In sentinel nodes negative for tumor cells on routine H&E examination, multi-level sectioning and immunohistochemical staining were performed to search for micrometastases.

Results: The sentinel node procedure was technically successful in 39/40 patients (98%). The median number of sentinel nodes identified was 4. Sentinel nodes were present in more than one nodal station in 8 patients (21%). In 6 patients in whom the sentinel node was negative for metastasis, non-sentinel nodes were positive for tumor cells (false-negative rate 6/39=15%). Micrometastases and isolated tumor cells were detected in 7/19 (37%) sentinel node-negative patients, but this finding did not affect the false-negative rate.

Conclusion: Detection of sentinel nodes is technically feasible during esophagectomy for cancer. However, given the relatively high false-negative rate of 15% and the high frequency of sentinel nodes in more than one nodal station, the clinical relevance of the sentinel node concept (through application of the blue dye technique) in the current treatment of patients with an adenocarcinoma of the distal esophagus or gastroesophageal junction seems limited.

Introduction

Most esophageal adenocarcinomas arise from the lower esophagus and the gastroesophageal junction (GEJ).[1,2] Prognosis after diagnosis is still poor as overall five-year survival rates rarely exceed 20%. One of the strongest predictors of long-term survival after radical (R0) esophagectomy is the presence of lymph node metastasis.[3-5] However, extended lymphadenectomy for the removal of all locoregional nodes can be at the cost of increased peri-operative morbidity.[6,7] Therefore this approach should ideally be restricted to those patients who are most likely to benefit.

A tailored surgical treatment for the individual patient may be applicable with help of the sentinel node concept. It states that the first lymph nodes (or a single lymph node) that receive the most direct lymph drainage from the primary tumor have the greatest potential to harbor metastatic disease when present.[8,9] On this basis, examination of the sentinel node can be used to predict overall lymph node status. Hence, applying the sentinel node concept to esophageal cancer could potentially have two important clinical implications. First, it may allow for the selection of patients who are not likely to benefit from an extended lymphadenectomy. If the sentinel node is not involved, then patients could be spared more extensive surgery by tailoring the extent of lymphadenectomy. Secondly, it may affect the process of pathological examination of the resected lymph nodes. Immunohistochemical detection of micrometastases and isolated tumor cells has been reported[10-12] and was found to be clinically relevant in one study.[12]

The sentinel node concept has not been studied extensively in esophageal cancer. We hypothesize that identification of the sentinel node(s) in esophageal adenocarcinoma can be achieved with a low false-negative rate and a high accuracy. In this study we determined the feasibility of application of the sentinel node procedure in adenocarcinomas of the distal esophagus and GEJ (with the false-negative rate as primary outcome parameter), and evaluated its value in the clinical setting.

Patients and methods

Patients

Over a 14-months period (May 2000 – June 2001), 101 patients underwent an esophageal resection and reconstruction for cancer of the distal esophagus or GEJ in our hospital. These patients were evaluated for inclusion in the study based on the following criteria: histologically proven adenocarcinoma of the distal esophagus or GEJ pre-operatively (Siewert type I or type II, respectively) and no application of neoadjuvant chemo- and/or radiotherapy. Induction chemo- and/or radiotherapy was given in patients with either a cT4-tumor without distant metastases or in patients with involvement of celiac trunk lymph nodes (M1a), who were not considered eligible for primary surgical therapy. There were 30 patients who received chemo- and/or radiotherapy before surgery; in 20 patients histological examination confirmed a squamous cell carcinoma; 2 patients underwent surgery for high-grade dysplasia and in one patient a double tumor of the esophagus lead to exclusion. Eight patients did not participate in the study due to logistic reasons. Consequently, 40 patients were included in this study and the sentinel node procedure was performed as described below. Informed consent was obtained from the patients before operation. The Medical Ethics Committee of the Erasmus MC approved the study.

Sentinel node mapping

Mapping of the sentinel nodes was carried out *in vivo* after opening the hiatus of the diaphragm and mobilization of the gastroesophageal junction under direct vision. Minimal dissection was performed in order to maintain intact lymph channels. At 3 different sites in the vicinity of the tumor, 1-2 cc Patent Blue V (Guerbet-Laboratories, Issy les Moulineaux, France) was injected in the submucosal layer. Within the next 5 minutes, the sentinel node(s) were identified by following the blue-stained lymphatic vessels. These nodes were tagged with a suture. Once the resection specimen was taken out, the sentinel nodes were harvested *ex vivo* and sent as a separate specimen to the Department of Pathology. The remaining non-blue-stained lymph nodes present in the resection specimen (non-sentinel nodes) were identified by the pathologist and categorized according to the location as para-esophageal, peri-gastric or celiac trunc nodes.

Surgery

All patients underwent a transhiatal esophagectomy. The primary tumor and its adjacent lymph nodes were dissected under direct vision through the widened hiatus of the diaphragm up to the level of the inferior pulmonary vein. Subsequently, a gastric tube was created. The left gastric artery was transected at its origin, with *en bloc* resection of celiac trunc lymph nodes. After mobilization and transection of the cervical esophagus, the intrathoracic esophagus was mobilized bluntly from the neck to the abdomen with a vein stripper. Esophagogastrostomy was performed in the neck, without a formal cervical lymphadenectomy.

Pathology

Pathologic evaluation of all lymph nodes consisted of conventional hematoxylin-eosin (H&E) staining. If no tumor cells were identified in the sentinel node(s), multi-level serial sectioning was performed. These lymph nodes were cut at 10 levels of approximately 100 µm (dependent on lymph nodes' size). Subsequently, sections were cut with a thickness of 4 µm which were examined for tumor cells with H&E staining as well as immunohistochemistry (IHC) to reveal micrometastases (metastatic lesions larger than 0.2 mm in dimension but smaller than 2.0 mm) and isolated tumor cells (metastatic lesions no larger than 0.2 mm in dimension).[13-14] The mouse-monoclonal antibody CAM 5.2 (NCL5D3, Novo Castra, Wetzlar, Germany) which is specific for intracellular cytokeratin-8 and –18, was used for this experiment.[15]

Statistics

To allow for intra-patient dependencies between outcomes of the investigated lymph nodes, the method of generalized estimating equations was used (SAS PROC GENMOD, SAS Institute Inc., Cary, NC, USA). Two-sided p-values <0.05 were considered to be significant.

Results

The sentinel node procedure was attempted in 40 patients who underwent a transhiatal esophagectomy. Patients' characteristics are described in Table 1. In 3 patients a large tumor covered both the distal esophagus and the gastroesophageal junction (type I/II), and no proper distinction between a type I or type II tumor could be made.

The sentinel node procedure was successful in identifying one or more sentinel nodes in 39 of 40 patients (98%). Technical failure occurred in one patient: the blue specimen that was presumed to be the sentinel node did not contain any lymphatic tissue when examined by the pathologist. Hence, the data of this patient are excluded from further analysis.

Table 1. Patients' characteristics.

Gender	Male	30 (75%)
	Female	10 (25%)
Age*	62 (range 41-80)	
pT-stadium	pT1	9 (23%)
	pT2	5 (13%)
	pT3	26 (65%)
Barrett's metaplasia	Yes	24 (60%)
	No	16 (40%)
Tumor location	Siewert I	20 (50%)
	Siewert II	17 (43%)
	Siewert I/II	3 (8%)

** Age is given as median*

A total of 424 lymph nodes were resected, comprising both sentinel nodes (N=143) and non-sentinel nodes (N=281). The median number of sentinel nodes identified per patient was 4 (range 1-9), whereas the number of non-sentinel nodes accounted for a median of 7 nodes per patient (range 1-22). The location of the identified sentinel nodes in relation to tumor site is shown in Table 2. The percentage of para-esophageal sentinel nodes was significantly higher in patients with a Siewert type I tumor than in patients with a Siewert type II tumor: 64% (45/70) versus 11%

(7/64), p<0.001. On the other hand, for sentinel nodes situated in the peri-gastric area, these percentages amounted 29% (20/70) for Siewert type I and 77% (49/64) for Siewert type II tumors, p=0.002. In one patient with a type I tumor no sentinel node could be identified during the abdominal phase of the operation; however, two blue nodes (5%) were detected coincidentally in the cervical region, implicating an upwards lymphatic drainage. Eight patients (21%) had sentinel nodes present in more than one nodal station. In the 39 patients in whom the sentinel node concept was applied successfully, the sentinel node was located adjacent to the tumor in 30 patients (77%).

Table 2. Relation between location of the sentinel node and tumor site.

	Sentinel Node Location				
Tumor site	Cervical	Paraesophageal	Perigastric	Celiac trunk	TOTAL
Siewert I (N=20)	2	45	20	3	70
Siewert II (N=16)	0	7	49	8	64
Siewert I/II (N=3)	0	2	7	0	9
TOTAL (N=39)	2	54	76	11	143

Of the total number of 424 lymph nodes examined by means of standard H&E staining, the sentinel nodes were more likely to contain tumor cells than the non-sentinel nodes: 40 of 143 (28%) sentinel nodes were positive *versus* 52 of 281 (19%) non-sentinel nodes (p=0.046, Table 3).

Table 3. Total number of lymph nodes derived from esophagectomy specimens of 39 patients: relation between (non-)sentinel nodes and tumor involvement as judged by H&E examination.

	Sentinel node	**Non-sentinel node**	**TOTAL**
Positive (N+)	40	52	92
Negative (N-)	103	229	332
TOTAL	143	281	424

In 20/39 (51%) patients the sentinel node contained tumor metastasis diagnosed on routine H&E examination. In 8 patients (21%) it was the sentinel node only that accounted for the N1-status of the patient; in 12 patients (31%) there were also non-

sentinel nodes in which a metastasis was found. In the remaining 19 patients (49%), the sentinel node was scored negative for tumor cells (Table 4A). However, in 6 of these 19 patients metastases were found in the non-sentinel nodes, which involved one patient with a pT1 tumor, one with a pT2 tumor and four patients in whom a pT3 tumor was diagnosed. These data correspond with a false-negative rate of 15% (6 of 39 patients), a negative predictive value of 68% (13/19), an accuracy of 85% (33/39), a sensitivity of 77% (20/26) and a specificity of 100% (13/13) of the sentinel node procedure in our study (see Table 5).

In patients in whom no metastasis was detected in the sentinel node by H&E staining (N=19), multi-level serial sectioning and immunohistochemical staining with CAM 5.2 was performed (in the sentinel nodes only). In one patient this revealed a macrometastasis that was not present in the conventional H&E section; a micrometastasis was identified in one patient and isolated tumor cells in five patients, resulting in upstaging of the histological diagnosis in seven patients (Table 4B). These results did not affect the false-negative rate of 15% (Table 5) because patients' lymph node status was not revised in any of the 6 patients in whom only non-sentinel nodes were scored positive (false-negatives in this study).

Table 4A and B. Nodal status - tumor positive (+) and negative (-) - of 39 patients after esophagectomy for cancer: relation between sentinel and non-sentinel nodes.

Table 4A. Before multi-level sectioning and immunohistochemistry.

	Non-sentinel Node +	Non-sentinel Node -	TOTAL
Sentinel Node +	12	8	20
Sentinel Node -	6	13	19
TOTAL	18	21	39

Table 4B. After multi-level sectioning and immunohistochemistry.

	Non-sentinel Node +	Non-sentinel Node -	TOTAL
Sentinel Node +	12	15	27
Sentinel Node -	6	6	12
TOTAL	18	21	39

Table 5. Definitions of diagnostic test parameters and corresponding values in 39 patients (before and after multi-level sectioning and immunohistochemistry).

	Definition	Value – before IHC	Value – after IHC
False-negative rate	Number of false-negative SN / Number of pts with an identified SN	6/39=15%	6/39=15%
Negative predictive value	Number of true node negative pts (SN + NSN) / Number of true node negative pts + false negative pts	13/19=68%	6/12=50%
Accuracy	Number of positive SN pts + number of pts with a true negative SN / Number of pts with an identified SN	33/39=85%	33/39=85%
Sensitivity	Number of true positive SN pts / Number of true positive SN pts + false negative pts	20/26=77%	27/33=82%
Specificity	Number of pts with a true negative SN / Number of true node negative pts + false positive pts	13/13=100%	6/6=100%

SN = sentinel node; NSN = non-sentinel node; pts = patients.

Discussion

In this study we investigated the application of the sentinel node procedure in the surgical treatment of patients with esophageal adenocarcinoma. The sentinel node procedure was technically successful in 39 of 40 patients (98%). The median number of sentinel nodes identified per patient was 4 (range 1-9) and these sentinel nodes were present in more than one nodal station in 8 patients (21%). In 6 patients in whom the sentinel node was negative for metastasis, non-sentinel nodes were positive for tumor cells (false-negative rate 6/39 = 15%). Micrometastases and isolated tumor cells were detected in 7/19 (37%) sentinel node-negative patients, but this finding did not affect the false-negative rate.

The term 'sentinel node' was introduced in 1960 by Gould *et al.* in their study to detect lymphatic metastases in parotid carcinoma.[16] The procedure was further refined in patients with melanoma[8] and in breast cancer.[9] Especially in breast cancer

it has become a widely accepted element in the routine surgical management.[17] In gastrointestinal tumors, the use of this procedure is still under investigation. Over the last 10 years a considerable number of clinical trials evaluating the feasibility and accuracy of the sentinel node procedure in mainly gastric and colorectal carcinoma have been published. With regards to gastric cancer, a complex lymphatic drainage is considered to result in high frequencies of skip metastases (15-20%).[18-19] These trials show high false-negative rates (ranging from 0 – 39%).[20-21] Hence, a tailored surgical approach with regard to lymphadenectomy on the basis of this procedure in gastric cancer patients is not justified. In colorectal cancer all regional lymph nodes are routinely removed with the resected bowel segment. Hence, minimizing surgical resection is not a major goal of the sentinel node procedure in colorectal cancer patients. Instead, improved prognostication is the main target here.[22]

The lymphatic drainage of the esophagus is complex with abundant lymph-capillary networks especially in the submucosa.[23,24] This results in a longitudinal lymphatic drainage which is presumed to be the reason for the phenomenon of skipping lymph node metastases in esophageal cancer.[25] Skip metastases are present when there is no tumor cell spread from the primary tumor into the adjacent peritumoral lymph nodes in the presence of positive distant nodes. For esophageal adenocarcinomas skip metastases have been reported in 0-35% of patients.[7,25,26] Since all patients from our study underwent transhiatal esophagectomy, a thorough analysis of the lymphatic spread in the upper part of the chest was not possible. In patients with a Siewert type I tumor the sentinel node was mainly located along the esophagus, whereas in most patients with a Siewert type II tumor the gastric nodal station contained the sentinel node. These results correlate with the patterns of lymphatic spread for these tumor entities as described in literature.[27] Furthermore, two sentinel nodes were found coincidentally in the cervical region (5%), representing the complex and unpredictable lymphatic spread of esophageal cancer cells.

We found a false-negative rate of 15%, which is higher in comparison with other malignancies. For example, false-negative rates between 0-5% for breast cancer have been reported and are worldwide considered to be acceptable.[17,28] Furthermore,

sentinel nodes were identified in more than one nodal station in 8 patients (21%), while ideally the sentinel node presents as a solitary lymph node, to enhance the usefulness of the sentinel node concept in clinical practice. Also, in our study a median of 4 sentinel nodes per patient were detected, indicating that half of the patients had 4 or more (up to 9) blue-stained nodes. These sentinel nodes were harvested during a transhiatal approach with a limited lymphadenectomy. One could hypothesize that, if a transthoracic esophagectomy with a two-field lymphadenectomy had been performed, the false-negative rate could have been lower (blue-stained lymph nodes may have been present in the upper mediastinum) as well as higher (if mediastinal non-sentinel nodes positive for tumor could have been detected). One study investigating the use of the sentinel node procedure in esophageal adenocarcinomas showed that when extended lymphadenectomy was performed, the lower paraesophageal and left gastric artery nodal stations were the most common sites of sentinel node identification.[29] In contrast, the upper mediastinal nodal basins accounted for only 3% (4/131) of the sentinel node stations. Therefore, we do not believe that the applied transhiatal approach with a limited lymphadenectomy is a major drawback in the current study.

Two other studies have been published thus far which investigated the sentinel node procedure in esophageal adenocarcinomas.[29,30] In the first study Lamb *et al.* identified at least one sentinel node by means of peritumoral injection (prior to the operation) of radioactive nanocolloid in all 57 patients with a type I or type II tumor (no blue dye was used).[29] A false-negative rate of 5% was reported. Hence, one could argue that in our study the use of blue dye only, is a technical shortcoming. However, this approach has been described before.[20,21] Nonetheless, in breast cancer blue dye and radiocolloid are considered to act as complementary techniques.[28]
The second study demonstrated that sentinel node detection was feasible in 17 out of 20 patients using a combination of blue dye and radiocolloid injection (N=10) and radiocolloid injection only (N=10).[30] No data were shown to clarify whether the use of blue dye and radiocolloid are complementary. Lymph node status was correctly predicted in all type I tumors, and in 75% of type II and type III tumors. Furthermore, advanced tumors had a higher false-negative rate and therefore it was concluded

that the sentinel node procedure was only applicable in patients with early (pT1-2) cancer. However, in our study both early and advanced tumors belonged to the group of false-negatives (one T1-, one T2- and four T3-tumors).
Overall, in our study the use of blue dye only without radiocolloid injection as a complementary technique, may be a potential drawback in this study. Nevertheless, in our opinion, the two above-mentioned studies together with our present findings do not justify the application of the sentinel node concept in clinical decision-making in patients with an adenocarcinoma of the distal esophagus or gastroesophageal junction in our institution at the moment.

In summary, we conclude that the sentinel node procedure is technically feasible during transhiatal esophagectomy for esophageal cancer. However, given the high false-negative rate and the high frequency of sentinel nodes in more than one nodal station, the clinical relevance of the sentinel node concept by application of the blue dye technique only in the current treatment of patients with an adenocarcinoma of the distal esophagus or gastroesophageal junction seems limited. More studies are needed that should focus on using the blue-dye technique in combination with radiocolloid injection as well as extended lymphadenectomy.

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7

Lymphatic micrometastases in patients with early esophageal adenocarcinoma

Brechtje A Grotenhuis
Mark van Heijl
Bas PL Wijnhoven
Mark I van Berge Henegouwen
Katharina Biermann
Fiebo JW ten Kate
Olivier RC Busch
Winand NM Dinjens
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

Introduction: Both endoscopic and surgical treatments are recommended for m3- or sm1-adenocarcinomas of the esophagus, depending on patients' lymph nodal status. Lymphatic dissemination is related to tumor infiltration depth, but varying incidences have been reported in m3- and sm1-adenocarcinomas. The study aim was to investigate whether the presence of occult tumor cells in lymph nodes could explain this variation.

Methods: Sixty-three node-negative (N0) patients with early esophageal adenocarcinoma (m2/m3/sm1-tumors) were included. Multilevel-sectioning of lymph nodes was performed; sections were stained by means of immunohistochemistry with cytokeratin-marker CAM5.2. Two pathologists searched for micrometastases (0.2-2.0mm) and isolated tumor cells (ITCs, <0.2mm).

Results: Positive CAM5.2 staining in lymph nodes was not seen in any of the 18 m2-patients. In 2/25 m3-tumors (8.0%) an ITC was found, but no micrometastases. Tumor cells were identified in 4/20 sm1-tumors (20.0%): 3 micrometastases and one ITC. Median follow-up was 121 months. Two m3-patients (3.2%) died due to disease recurrence, including one patient in whom an ITC was detected.

Conclusions: Lymphatic migration of tumor cells was found in node-negative m3- and sm1-adenocarcinomas of the esophagus (8.0% and 20.0%, respectively). However, the clinical relevance of these occult tumor cells should become apparent from large series of endoscopically treated patients.

Introduction

Lymph node status is the most important prognostic factor in esophageal cancer.[1-3] The presence of lymph node metastases has a strong adverse impact on patient survival, even after radical esophagectomy with extended lymphadenectomy. Some studies have shown that lymph node micrometastases and isolated tumor cells (ITCs) can be detected by immunohistochemistry (IHC) in up to 50% of N0-patients with esophageal cancer. However, contradictory results have been reported with regard to their prognostic importance.[4-16]

Current treatment options for early esophageal cancer (T1-tumors) vary from organ preserving endoscopic mucosal resection (EMR) to limited surgical resection (Merendino procedure or subtotal transhiatal esophagectomy) and radical esophagectomy with extended lymphadenectomy.[17-20] In patients with early esophageal cancer, otherwise fit for operation, the decision whether to perform EMR or an esophagectomy mainly depends on patients' lymph nodal status.[21] An overview of reports on the frequency of lymph node involvement in mucosal (m1-m2-m3) and submucosal (sm1-sm2-sm3) esophageal adenocarcinoma is given in Table 1.

Lymphatic dissemination is related to the depth of infiltration of the primary tumor.[22-25] Lymph node metastases have not been reported in m1 and m2 mucosal adenocarcinomas (thereby allowing endoscopic treatment), whereas positive lymph nodes can be found in 0-12% of m3 patients.[22-25] In submucosal adenocarcinomas, it appears that sm1 tumors are associated on average with a lower rate of lymph node metastases than sm2-sm3 tumors.[22-25] Because of the high incidence of lymph node metastases in patients with deep submucosal tumor infiltration, an esophagectomy is considered the standard therapy for sm2 and sm3 tumors.[19, 26, 27] Nevertheless, the management of carcinomas with invasion into the muscularis mucosae (m3) or the most superficial layer of the submucosa (sm1) is still under debate; on average 5-7% of m3- and sm1-patients are classified as N1 postoperatively (Table 1).

In a previous publication we reported that lymphatic dissemination was limited to tumors infiltrating the middle and deepest layer of the submucosa (sm2 and sm3).[23] However, subsequent reports from other institutes described lymphatic tumor spread already at an earlier stage (m3 and sm1). In an attempt to explain this discrepancy we re-evaluated the resection specimens from our previously published series and searched for micrometastases and ITCs. Also, we have attempted to address the question whether the presence of occult metastases in patients with m3 and sm1 adenocarcinomas will influence the decision to undertake an endoscopic or surgical resection.

Table 1. Overview of reports on the frequency of lymph node involvement in mucosal (m1-m2-m3) and submucosal (sm1-sm2-sm3) esophageal adenocarcinoma.

	m1		**m2**		**m3**		**sm1**		**sm2**		**sm3**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Liu[22]	36	0	--	--	17	12	12	8	--	--	25	36
Westerterp[23]	13	0	18	0	23	4	25	0	23	26	18	67
Bollschweiler[25, 47]	9	0	2	0	3	0	10	20	6	0	10	70
Ancona[24]	12 m1-m2-m3: 0%						12	0	7 sm2-sm3: 43%			
TOTAL	**58**	**0**	**20**	**0**	**43**	**7**	**59**	**5**	**29**	**21**	**53**	**53**

	T1a		**T1b**	
	n	%	n	%
TOTAL	**133**	**2**	**148**	**25**

n = number of included patients
% = percentage of patients with positive lymph nodes on routine examination

m1 = carcinoma in situ without invasion through the basement membrane
m2 = intramucosal carcinoma extending beyond the basement membrane into the lamina propria
m3 = carcinoma with invasion into the muscularis mucosae
sm1 = carcinoma infiltrating the submucosa but limited to the upper third of the submucosa
sm2 = carcinoma infiltrating the submucosa but limited to the middle third of the submucosa
sm3 = carcinoma infiltrating the submucosa but limited to the lower third of the submucosa

Patients and methods

Patients

The outcome of patients who underwent transhiatal esophagectomy with regional lymphadenectomy for early adenocarcinoma of the esophagus or gastro-esophageal junction (pT1-tumors) between 1980 and 2002 in two university hospitals in The Netherlands (Erasmus Medical Center, Rotterdam, and Academic Medical Center, Amsterdam) has been described previously.[23] Patients did not receive (neo)adjuvant chemo- and/or radiotherapy at that time. We have reported on the operation technique in previous studies.[28, 29] Patients in whom the tumor was classified as m2, m3 or sm1, and in whom the lymph nodes were scored as tumor-free (N0) on conventional histopathological examination (one N1-patient with a m3 tumor was excluded from the previous series), were selected for the present study (N=65). We included m2 tumors as a negative control, as we expected no lymphatic migration of tumor cells in these patients. Patients with sm2 or sm3 carcinomas were excluded in view of their high potential of lymphatic dissemination as already shown in the previous publication. Tissue blocks of resected lymph nodes of two patients were not available for this study. Therefore, a total number of 63 patients were included in the present series.

Histopathologic assessment

Tumors were assigned pathologic tumor-node-metastasis stages according to the Union Internationale Contre le Cancer (UICC) 2002 system.[30] The depth of tumor invasion was measured and subclassified based on the criteria proposed by the Japanese Society for Esophageal Disease.[31] Intramucosal carcinoma was defined as a tumor extending beyond the basement membrane into the lamina propria (m2). All carcinomas with invasion into the muscularis mucosae were classified as m3 tumors, irrespective of the presence of a double muscularis mucosae (*i.e.* a superficial and a deep layer as can be observed in Barrett's mucosa). Carcinomas infiltrating beyond the (deep) muscularis mucosae but limited to the upper third of the submucosa, were classified as sm1.

Immunohistochemistry

Sectioning at three different levels was performed in all lymph nodes. Sections of the 33 patients operated on in Rotterdam were processed in the Erasmus MC; sections of the remaining 30 patients were stained in the AMC, Amsterdam. IHC was performed using the Envision system (DAKO, Glostrup, Denmark). A mouse-monoclonal antibody against CAM5.2 (BD Biosciences, San Jose, CA, USA) was used for this experiment, which does not react with hematopoietic and lymphoid cells and which is specific for intracellular cytokeratin-7 and –8 (both cytokeratins are expressed in esophageal adenocarcinoma). Therefore, positive lesions indicate epithelial cell deposits in lymphoid tissue and it has previously been shown that micrometastases in esophageal adenocarcinoma can be detected with this marker.[14, 16, 32]

In brief, the sections were dewaxed in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was inhibited by incubating the sections in methanol with 3% hydrogen peroxide for 20 minutes. Microwave (700 W) pretreatment in Tris-EDTA (pH=9) was performed for 15 minutes. The antibody against CAM5.2 was incubated overnight at 4°C. This was followed by the secondary incubation step of the Envision system. Diaminobenzidene tetrachloride from the Envison kit, prepared according to kit instructions, was used for visualization. Tissues were counterstained with hematoxylin. Finally, the slides were dehydrated through a graded ethanol series, cleared in xylene and mounted in Malinol (Chroma-Gesellschaft, Köngen, Germany). As the sections were stained in two different centers with their own IHC protocol, a crosscheck was performed by staining sections of three patients in both centers, which showed similar results.

Definition of micrometastases and isolated tumor cells

All sections were examined independently by two GI pathologists (FJWtK, KB) to detect micrometastases (positive lesions larger than 0.2 mm in dimension but smaller than 2.0 mm) and ITCs (metastatic lesions no larger than 0.2 mm in dimension).[30, 33, 34] Only clusters of positive cells with malignant characteristics detected in the sinuses or lymphoid interstitium were designated as micrometastases. In contrast, tumor cells surrounding the lymph node were considered as contamination that most likely had occurred during the processing of the resection specimen.

Follow-up

Outcome data for all patients with esophageal cancer referred to both hospitals for treatment have been collected prospectively and stored in a database by data managers. Follow-up was recorded until January 2009 or until death if earlier, ensuring a follow-up of at least five years. There were no patients lost to follow-up. Disease recurrence was defined as locoregional or distant metastatic disease when radiologically and/or pathologically proven.

Statistics

Statistical analysis appropriate for non-parametric data was used. Grouped data were compared using the Chi-Square test. Overall survival was calculated from the date of operation until the date of last follow-up or death according to the Kaplan-Meier method. Disease-free survival was assessed from the date of operation until the date of disease recurrence in case of locoregional recurrence or distant metastases; patients were censored at the time of their last visit or when they died of non-disease related causes without a previous relapse. Data-analysis was carried out with SPSS version 15.0 (SPSS, Chicago, IL, USA). Two-sided p-values <0.05 were considered to be significant.

Results

Clinicopathological characteristics of the present study population are shown in Table 2. In 18 patients (28.6%) the tumor invaded into the lamina propria (m2), in 25 patients (39.7%) the tumor's deepest invasion was detected in the muscularis mucosae, and in 20 patients (31.7%) the tumor extended into the superficial layer of the submucosa (sm1). A median number of seven lymph nodes (range 1 – 30) was harvested per patient, all of which were processed for immunohistochemical analyses.

Table 2. Clinicopathological characteristics of 63 patients who underwent a transhiatal esophagectomy for early esophageal adenocarcinoma (m2, m3, sm1).

Age*	67 years (30-83)
Gender	53 (84.1%)
- *male*	10 (15.9%)
- *female*	
Tumor location	2 (3.2%)
- *mid esophagus*	38 (60.3%)
- *distal esophagus*	23 (36.5%)
- *gastroesophageal junction*	
Differentiation grade	14 (22.2%)
- *good*	44 (69.8%)
- *moderate*	5 (7.9%)
- *poor*	
Barrett's metaplasia	60 (95.2%)
- *yes*	3 (4.8%)
- *no*	
Tumor infiltration depth	18 (28.6%)
- *m2*	26 (41.3%)
- *m3*	19 (30.2%)
- *sm1*	

** Value presented as median (range in brackets)*

The results of the current IHC study are shown in Table 3. In six patients (9.5%) sections of lymph nodes stained positively for CAM5.2. Tumors of various differentiation grades were involved: one well-differentiated tumor, three tumors were of a moderate differentiation grade, and two tumors were poorly differentiated. In all six patients one single lymph node was affected in which tumor cell(s) had been detected. It concerned paraesophageal lymph nodes (N=4) or lymph nodes located at the lesser curvature (N=2). None of the 18 m2 tumors showed positive CAM5.2 staining in the lymph nodes. In two out of 25 m3 patients (8.0%) an ITC was found in the lymph nodes. The pathologists identified tumor cells in four out of 20 sm1 tumors (20.0%): three micrometastases (Figure 1) and one ITC (Figure 2). Hence, occult metastases as detected by positive CAM5.2 staining were seen more often in sm1 tumors than in m3 carcinomas: 20.0% versus 8.0%, respectively, although this difference did not reach statistical significance in this small series of patients (p=0.69). No macrometastases were found in any of the lymph nodes after multi-sectioning.

Table 3. Number of isolated tumor cells (ITCs) and micrometastases as detected by immunohistochemistry according to tumor infiltration depth.

Tumor infiltration depth	ITCs	Micrometastases	TOTAL
m2 (n=18)	0	0	0
m3 (n=25)	2 (8.0%)	0	2 (8.0%)
sm1 (n=20)	1 (5.0%)	3 (15.0%)	4 (20.0%)
TOTAL (n=63)	3 (4.8%)	3 (4.8%)	6 (9.5%)

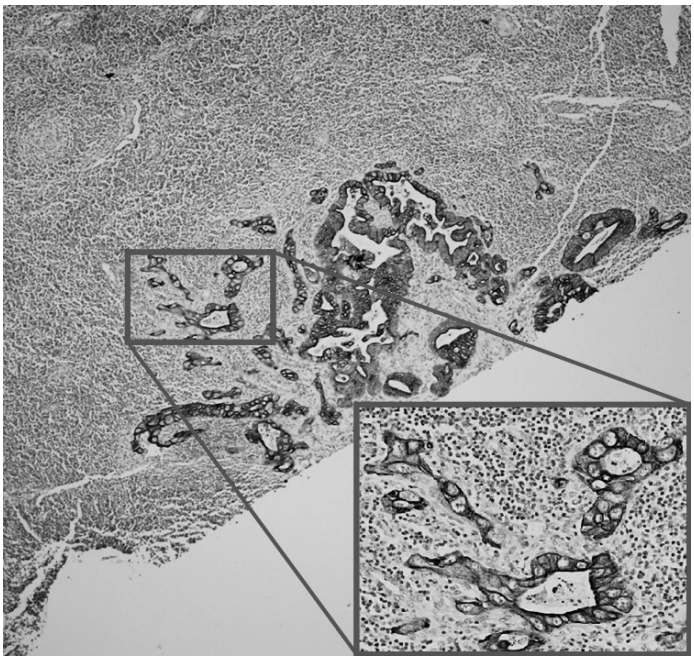

Figure 1. *An example of a micrometastasis (positive for CAM5.2) in a lymph node that was initially scored as tumor-free on conventional histopathological examination.*

Median follow-up was 121 months (range 10 – 187 months). Overall and disease-free five-year survival for this patient group (N=63) were 75.6% and 96.3%, respectively. Two out of 63 patients (3.2%) developed disease recurrence and died following locoregional tumor recurrence without evidence of distant metastastic disease. Both patients were diagnosed with an m3 tumor. In one of these patients who died due to tumor recurrence an ITC was identified on IHC. None of the other five patients with a detected micrometastasis or ITC developed disease recurrence.

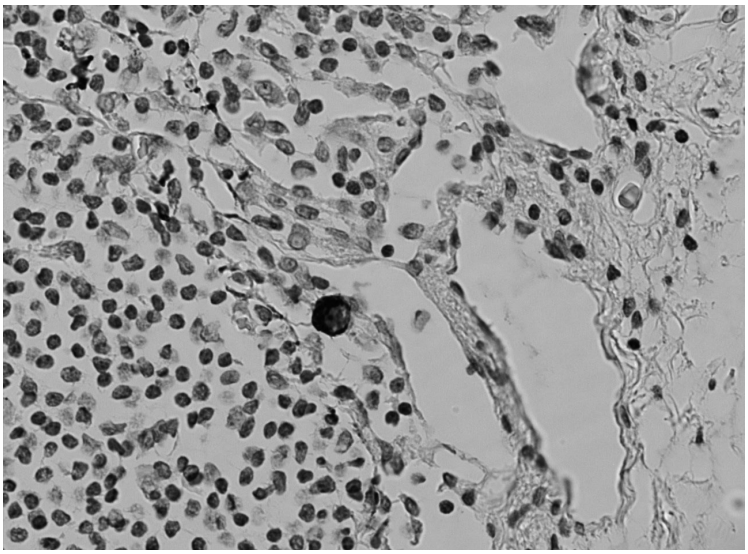

Figure 2. *An example of an isolated tumor cell (positive for CAM5.2) in a lymph node that was initially scored as tumor-free on conventional histopathological examination.*

Discussion

The aim of this study was to investigate whether the presence of occult tumor cells in early esophageal adenocarcinoma could (partly) explain the reported differences in the occurrence of lymphatic spread within the subgroup of m3 (0 – 12%) and sm1 adenocarcinomas (0 – 20%) as reported by our group and by others (Table 1).[22-25] Indeed, in the present series we have shown that lymphatic spread can be detected by means of additional IHC in conventionally node-negative patients in the event of tumor infiltration into the muscularis mucosae (m3 – 8.0%) or most superficial submucosal layer (sm1 – 20.0%). No micrometastases or ITCs were detected in the lymph nodes of patients with an m2-tumor, which supports the widespread use of endoscopic treatment in these patients.

Immunohistochemical techniques can identify micrometastases that are missed by standard H&E staining. Cytokeratin, a component of the cytoskeleton of epithelial cells,

is not found in normal lymph nodes, thus enabling monoclonal antibodies to specific cytokeratin markers (such as CAM5.2) to detect micrometastases. These techniques may identify single tumor cells or cell clusters in lymph nodes that have been staged as tumor free on routine H&E examination. The viability of micrometastatic tumor cells and their potential to form true metastases has been questioned, although evidence has been provided for the malignant potential of micrometastatic cells in esophageal cancer.[35, 36] However, searching for micrometastases by means of IHC remains a research tool and is not currently used in daily clinical practice. Furthermore, the prognostic outcome of the detection of micrometastases in esophageal cancer by IHC is controversial as some studies have found an association with increased risk of tumor recurrence and decreased survival[4, 6-9, 12, 15, 37], whereas others have not.[5, 10, 11, 13, 14] These findings were independent of histology type (squamous cell carcinoma versus adenocarcinoma). In T1-tumors in specific, the previously reported frequency of micrometastases or ITCs varied between 5 and 44%.[7, 10-13, 16] In the present series, sections stained positively for CAM5.2 in 9.5% of our patients who were initially staged as N0 with conventional techniques. It can be questioned whether stage migration could have played a role in the present study due to a limited number of harvested lymph nodes by performing a transhiatal esophagectomy (*e.g.* patients that have falsely been classified as N0 as positive mediastinal lymph nodes had not been removed). However, in that case it would have become apparent from a worse disease-free survival curve in the present series as N1-disease undoubtedly affects the risk of disease recurrence: disease-free five-year survival rate accounts 95% for T1N0 patients and not more than 35% for T1N1 patients after an esophagectomy with extended or limited lymphadenectomy.[23, 38] As the disease-free five-year survival was 96% in the current study, it is not likely that stage migration has played a substantial role.

The clinical relevance of these micrometastases and ITCs will not become clear from the results of surgical series such as the current one. Obviously, lymph nodes with occult tumor cells have already been resected and will not influence patient's long-term outcome. Hence, reports on endoscopically treated patients with early esophageal adenocarcinoma should provide evidence whether the presence of

occult tumor cells might be responsible for disease recurrence. Until now, several publications have shown that endoscopic resection of Barrett's intramucosal carcinoma is safe and effective in experienced hands.[39-43] Although local recurrences or metachronous lesions are known to be a major problem with endoscopic therapy, successful repeat endoscopic treatment has shown to be feasible in almost all of these patients.[44] Recently, long-term results of endoscopic resection in mucosal adenocarcinoma have been provided in two relatively large patient series presenting its successful outcome after five-year follow-up.[17, 45] Although none of the above-mentioned studies reported specifically on m3 adenocarcinomas, one may assume that this subpopulation is well-represented in the overall group of mucosal cancers. Furthermore, in a small series of patients who fulfilled the definition of low-risk submucosal esophageal adenocarcinoma (sm1 tumors, absence of lymphangio-invasion and no poor differentiation grade[37]), promising long-term results have been reported with regard to disease recurrence.[46] However, large series reporting on the endoscopic treatment of patients with sm1 adenocarcinomas are lacking.

In clinical practice, in patients with m3 or sm1 tumors without evidence of lymphatic dissemination it is still unclear whether a radical esophagectomy is indicated or if endoscopic treatment suffices. An esophagectomy with (extended) lymphadenectomy may prolong the recurrence-free period (5-7% of patients with m3 and sm1 adenocarcinomas are classified as N1 postoperatively, see Table 1), but major disadvantages are its invasiveness and accompanied morbidity. Moreover, even in specialized high-volume centers the mortality rate after esophagectomy is 2-5%.[2, 28, 29] Therefore, it is unlikely that the morbidity and mortality of a surgical resection is counter-balanced by a substantial gain in long-term survival for patients with m3 lesions. In our previous publication we stated that sm1 adenocarcinomas are potentially eligible for endoscopic therapy because of the very low risk of lymphatic dissemination in these tumors, provided that other groups could show similar results.[23] However, based on the presently available data and the results of the current study, we believe that in patients with sm1 tumors a surgical resection is most likely still indicated, as in 20% of these patients micrometastases or ITCs were detected on IHC. Again, final evidence with regard to the clinical relevance of occult

tumor cells should become apparent from large series of endoscopically treated patients with sufficiently long follow-up.

One drawback of the current study is its bicentric character. Sections of the selected patient group have been stained in two different hospitals, each with their own IHC protocols. However, a crosscheck was performed by staining sections of three patients in both centers, which showed similar results. Furthermore, one could criticize the antibody used in the present series (CAM5.2), as the antibodies AE1/AE3 and Ber-EP4 have been used more often in other studies. It has been shown previously that the antibody Ber-EP4 could well be used for the detection of clinically relevant micrometastatic disease in patients operated upon for esophageal adenocarcinoma because of its high sensitivity and specificity.[16] On the other hand, CAM5.2 has been incorporated in the day-to-day protocols of the two participating hospitals to detect occult tumor cells in resection specimens of several malignancies. Because of this wide experience with CAM5.2, we decided to employ CAM5.2 as the marker of choice in the current study.

In conclusion, lymphatic migration of tumor cells has been detected in conventionally node-negative lymph nodes of both m3 (8.0%) and sm1 (20.0%) adenocarcinomas of the esophagus, which can (partly) explain the reported variation in lymphatic dissemination in these tumors. However, the clinical relevance of these occult tumor cells should become apparent from large series of endoscopically treated patients with sufficiently long follow-up.

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8

Inter- and intraobserver variation in the diagnosis of early esophageal adenocarcinoma

Brechtje A Grotenhuis
Mark van Heijl
Fiebo JW ten Kate
Katharina Biermann
G Johan A Offerhaus
Bas PL Wijnhoven
Mark I van Berge Henegouwen
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

Introduction: According to the classification established by the Japanese Society for Oesophageal Disease, early oesophageal cancer can be subdivided into six successive layers of the mucosa or submucosa, which influences the treatment strategy and prognosis of the individual patient. However, the reproducibility of this classification in terms of inter- and intraobserver variability is unclear.

Methods: Histological slides from 105 surgical resection specimens of patients who had undergone oesophagectomy for early oesophageal adenocarcinoma were reviewed independently by three gastrointestinal pathologists, and were classified according to the Japanese criteria (m1/m2/m3/sm1/sm2/sm3 tumours). Inter- and intraobserver variation was determined by kappa-statistics.

Results: The interobserver reproducibility was good between pathologist 1 and 2 (κ=0.61, 95% confidence interval (CI) 0.55-0.67), and moderate between pathologist 1 and 3 (κ=0.51, 95% CI 0.45-0.57) and between pathologist 2 and 3 (κ=0.50, 95% CI 0.38-0.61). The intraobserver agreement as assessed by the expert pathologist was good (κ=0.76), with a 95% CI that was interpreted as good to very good (0.67-0.85). Most agreement was achieved at the lower (m1) and upper site (sm2, sm3) of the spectrum, whereas the m2 tumours reflected the most discrepant stage. The majority of the observed discrepancy included the variation in one substage only.

Conclusions: The reproducibility of the Japanese classification is good in terms of inter- and intraobserver variability when grading early oesophageal adenocarcinoma on surgical resection specimens. The present data confirm that dedicated gastrointestinal pathologists with broad experience are preferred when grading the resection specimens of patients with early oesophageal adenocarcinoma.

Introduction

Oesophageal adenocarcinoma is diagnosed with increasing frequency at an early stage, due to the increased awareness of the clinical importance of Barrett's oesophagus, more intensive surveillance programs, and improved imaging techniques.[1, 2] According to the UICC (Union Internationale Contre le Cancer) TNM classification, early carcinoma is defined as an invasive tumour limited to the mucosa (T1a) or submucosa (T1b), irrespective of the lymph nodal status.[3] This dichotomy is also incorporated in the revised Vienna classification that has been established to resolve the discrepancies in nomenclature between Western and Japanese pathologists with regard to the grading of gastrointestinal epithelial neoplasia (Table 1).[4, 5] In this classification intramucosal carcinoma (together with high-grade dysplasia and carcinoma in situ set in category 4) is separated from submucosal invasion (category 5).[4] Moreover, in 2001 the Japanese Society of Oesophageal Disease introduced its classification in which early cancers are subdivided in six successive layers of the mucosa (m1, m2, m3) or submucosa (sm1, sm2, sm3).[6] However, the usefulness and reproducibility of this more recently introduced, extended classification has not been studied yet.

Table 1. Revised Vienna classification that has been established to resolve the discrepancies in nomenclature between Western and Japanese pathologists with regard to the grading of gastrointestinal epithelial neoplasia.[4]

DIAGNOSIS	CATEGORY
Negative for neoplasia/dysplasia	1
Indefinite for neoplasia/dysplasia	2
Mucosal low-grade neoplasia/dysplasia	3
Mucosal high-grade neoplasia	
- high-grade adenoma/dysplasia	4.1
- non-invasive carcinoma (carcinoma in situ)	4.2
- suspicious for invasive carcinoma	4.3
- intramucosal carcinoma	4.4
Submucosal invasive carcinoma	5

In general, a classification should be simple, a reflection of available therapeutic options and reproducible. Simplicity means that a classification should have as few categories as possible, but as many as required clinically. Reflection of available therapeutic options should include the possibilities of careful observation, local endoscopic treatment or surgery. It appears that subdivision in six, rather than two categories is helpful in directing patients to the most optimal treatment strategy. Current treatment options for early oesophageal cancer vary from endoscopic mucosal resection (EMR) to surgical resection with extended or regional lymphadenectomy.[7-10] The decision whether to perform an endoscopic or a surgical resection is mainly dependent on patients' predicted lymph nodal status[11], which is related to the depth of infiltration of the primary tumour. Lymph node metastases have not been reported in m1 and m2 mucosal adenocarcinomas (thereby allowing endoscopic treatment), whereas positive lymph nodes can be found in 0-12% of m3 patients.[12-15] In submucosal adenocarcinomas, it appears that sm2-sm3 tumours are associated on average with a higher rate of lymph node metastases than sm1 tumours[12-15]; hence, an oesophagectomy is considered the standard therapy.[9, 16, 17] The optimal management of carcinomas with invasion into the muscularis mucosae (m3) or the most superficial layer of the submucosa (sm1) is still under debate. Finally, subdivision of early oesophageal cancer in six categories seems justified, as the treatment algorithm depends largely on the chance for lymphatic dissemination that is mainly related to the tumour infiltration depth.

Reproducibility of a classification includes the aspects of inter- and intraobserver variability. Thus far, these observer agreements have not been studied in the light of the Japanese classification of early oesophageal cancer. Therefore, the aim of the present study was to evaluate inter- and intraobserver variation among gastrointestinal pathologists in grading early oesophageal adenocarcinoma according to the criteria proposed by the Japanese Society for Oesophageal Disease, when analyzing surgical resection specimens.

Material and methods

Patient material

The outcome of 120 patients who underwent transhiatal oesophagectomy with regional lymphadenectomy for early adenocarcinoma of the distal oesophagus or gastro-oesophageal junction (pT1-tumours) between 1980 and 2002 in two university hospitals in The Netherlands (Erasmus Medical Centre, Rotterdam, and Academic Medical Centre, Amsterdam) has been described previously.[13] Patients did not receive neoadjuvant chemo(radio)therapy. Archival material of the resection specimens was used for the present study. Tissue blocks and slides of the resection specimens of thirteen patients were not available anymore, and in the resection specimens of another two patients the tumour was classified as a pT2 tumour (infiltration into the muscularis propria) by all three pathologists. Finally, resection specimens of 105 patients were used in the present study.

Histopathologic assessment

Tumours were assigned pathologic tumour-node-metastasis stages according to the UICC 2002 system.[3] The depth of tumour invasion was measured and subclassified based on the criteria proposed by the Japanese Society for Oesophageal Disease (Figure 1).[6] High-grade dysplasia or carcinoma in situ without invasion through the basement membrane was classified as m1. Intramucosal carcinoma was defined as a tumour extending beyond the basement membrane into the lamina propria (m2). All carcinomas with invasion into the muscularis mucosae were classified as m3 tumours, irrespective of the presence of a double muscularis mucosae (*i.e.* a superficial and a deep layer observed in Barrett's mucosa can be classified as a m3 and m4 lesion, respectively[18]). Carcinomas infiltrating beyond the (deeper) muscularis mucosae but limited to the upper third of the submucosa, were classified as sm1. Equally, carcinomas infiltrating the submucosa but limited to the middle or lower third of the submucosa were defined as sm2 or sm3, respectively.

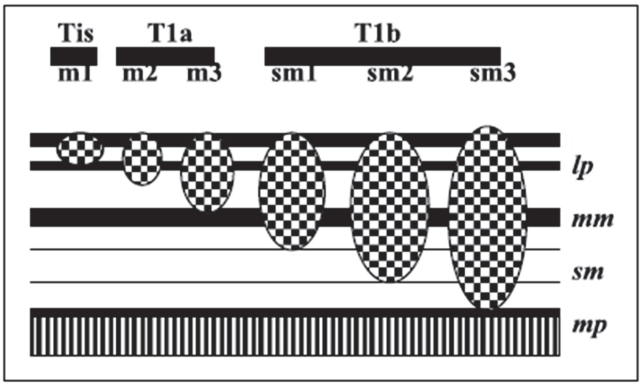

Figure 1. *The depth of tumour invasion based on the criteria proposed by the Japanese Society for Oesophageal Disease[6]; according to the Vienna classification m1, m2 and m3 lesions represent category 4, whereas sm1, sm2, sm3 tumours are classified as category 5.[4] Reprinted with permission, all rights reserved © 2006 The American Journal of Surgery.[32]*

lp = lamina propria
mm = muscularis mucosae
sm = submucosa
mp = muscularis propria

To assess interobserver agreement in analyzing early oesophageal cancer, histological slides of the primary tumour of 105 resection specimens were independently reviewed by three gastrointestinal pathologists. Pathologist 1 and pathologist 2 were highly experienced (more than 20 years experience) and had been closely working together in the same department. The third pathologist has a shorter work experience as a dedicated gastrointestinal pathologist (two years), but in daily practice evaluates all pathology specimens with regard to oesophageal cancer in a high-volume centre. The pathologists were blinded for the identity of the patient and the initial diagnosis. The resection specimen of each individual patient was categorized based on the deepest infiltration of the primary tumour according to the six classes of early oesophageal cancer (m1, m2, m3, sm1, sm2, sm3). To assess intraobserver agreement, the most experienced pathologist who is considered an expert on oesophageal cancer, re-reviewed all histological slides two weeks after this first assessment.

Statistics

Inter- and intra-observer agreement were determined by kappa statistics. Kappa statistics are widely used and accepted coefficients that provide a measure of observer agreement accounting for agreement other than that which occurs by chance alone.[19] Coefficients <0.21, 0.21-0.40, 0.41-0.60, 0.61-0.80, 0.81-1.00 represent a poor, fair, moderate, good and very good agreement, respectively.[20] Kappa-statistics with corresponding 95% confidence intervals were calculated with regard to interobserver agreement (pathologist 1 versus pathologists 2 and 3, respectively, and pathologist 2 versus pathologist 3) and intraobserver agreement (as assessed by pathologist 1). Data-analysis was carried out with SPSS version 15.0 (SPSS, Chicago, IL, USA).

Results

Clinicopathological characteristics of the present study population are shown in Table 2. The infiltration depth of the primary tumour was initially classified as carcinoma in situ in 10 patients (9.5%), and as invasive carcinoma in 95 patients (90.5%). One m3 patient, five sm2 patients and eleven sm3 patients were diagnosed with a N1 status.

Table 2. Clinicopathological characteristics of 105 patients who underwent oesophagectomy for carcinoma in situ (m1) or early oesophageal adenocarcinoma (m2, m3, sm1, sm2 or sm3).

Age*	66 years (30-82)
Gender	
- *male*	92 (87.6%)
- *female*	13 (12.4%)
Tumour location	
- *distal oesophagus*	93 (88.6%)
- *gastro-oesophageal junction*	12 (11.4%)
Differentiation grade	
- *Gx (undetermined)*	7 (6.7%)
- *G1 (good)*	20 (19.0%)
- *G2 (moderate)*	59 (56.2%)
- *G3 (poor)*	19 (18.1%)
Barrett's metaplasia	
- *yes*	95 (90.5%)
- *no*	10 (9.6%)
Lymph nodal involvement	
- pN0	88 (83.8%)
- pN1	17 (16.2%)
Radicality of resection (R0)	105 (100%)

** Value presented as median (range in brackets)*

The interobserver variation has been tested in three ways: pathologist 1 (expert on oesophageal cancer) versus pathologist 2, pathologist 1 versus pathologist 3, and pathologist 2 versus pathologist 3. The outcome with regard to this interobserver variation is shown in Table 3. The interobserver reproducibility between pathologists 1 and 2 was good (κ = 0.61), with a 95% confidence interval (CI) that can be interpreted as moderate to good (0.55-0.67). The interobserver agreement was graded as moderate between the pathologists 1 and 3 (κ = 0.51, 95% CI 0.45-0.57) and between the pathologists 2 and 3 (κ = 0.50, 95% CI 0.38-0.61). Furthermore, the most experienced pathologist re-reviewed all histological slides to assess the intraobserver variation of this classification. The intraobserver agreement was good with a kappa of 0.76, and a concomitant 95% confidence interval (CI) that was interpreted as good to very good (0.67-0.85).

Table 3. Inter- and intraobserver variation for the diagnosis of carcinoma in situ (m1) or early oesophageal adenocarcinoma (m2, m3, sm1, sm2 or sm3) as determined by the histopathological analysis of 105 resection specimens according to the Japanese classification.

	Kappa	95% CI	Interpretation*
Interobserver variation	0.61	0.55 – 0.67	good (moderate – good)
- pathologist 1 vs pathologist 2	0.51	0.45 – 0.57	moderate (moderate)
- pathologist 1 vs pathologist 3	0.50	0.38 – 0.61	moderate (fair – good)
- pathologist 2 vs pathologist 3			
Intraobserver variation	0.76	0.67 – 0.85	good (good – very good)
- pathologist 1 vs pathologist 1			

* Interpretation of kappa-statistics, with interpretation of 95% confidence interval in brackets.

CI = confidence interval

When analyzing the discrepancies between the observers with regard to the six different substages as shown in Table 4, it appeared that most agreement was achieved at the lower (m1) and upper site (sm2, sm3) of the spectrum. The m2 stage was the most discrepant stage (interobserver agreement 17% and 25% only), whereas in the m3 and sm1 stage pathologist 3 showed less agreement. However, when allowing variation in one substage (*i.e.* when the most experienced pathologist scored m3, both m2 and sm1 substages were not considered discrepant), it appeared

that there was only 4-5% discordance with regard to the interobserver variation, and 3% disagreement for the intraobserver variation of the most experienced observer (pathologist 1).

Table 4. Inter- and intraobserver agreement as assessed per tumour stage, defined by the depth of tumour infiltration according to the Japanese classification. Observations by pathologist 1 were considered the gold standard in this evaluation.

	Interobserver		Intraobserver
pathologist 1	**pathologist 2**	**pathologist 3**	**pathologist 1**
m1 (n=5)	4/5 (80%)	5/5 (100%)	5/5 (100%)
m2 (n=12)	2/12 (17%)	3/12 (25%)	11/12 (92%)
m3 (n=39)	29/39 (74%)	23/39 (59%)	28/39 (72%)
sm1 (n=18)	13/18 (72%)	8/18 (44%)	16/18 (89%)
sm2 (n=16)	12/16 (75%)	12/16 (75%)	13/16 (81%)
sm3 (n=15)	13/15 (87%)	13/15 (87%)	12/15 (80%)

Discussion

The reproducibility of the classifications that aim for the discrimination between Barrett's metaplasia, dysplasia and adenocarcinoma has been studied previously. Various groups have demonstrated that the use of such classifications (*e.g.* Vienna classification) is still accompanied by considerable interobserver variability.[21-23] Furthermore, studies have been undertaken to assess the observer variation in the diagnosis of high-grade dysplasia, intramucosal adenocarcinoma (T1a) or submucosal adenocarcinoma (T1b).[24, 25] In general, this discrimination in tumour infiltration depth will reflect the treatment options of endoscopic treatment (HGD, T1a) and surgery (T1b). When evaluating surgical resection specimens, the interobserver agreement was moderate when comparing HGD and intramucosal carcinoma, and good in case separation of intramucosal carcinoma from submucosal carcinoma was aimed for.[24] When preoperative biopsies were analyzed, these agreements appeared to be only fair and poor, respectively.[25] However, it has been suggested that interobserver agreement on EMR specimens is significantly higher compared to biopsy specimens.[26] Nevertheless, it may be a drawback of this study that interobserver variability has

been evaluated from surgical resection specimens rather than from EMR specimens, as the latter will largely influence clinical decision-making.

To our knowledge, this is the first study that reports on the reproducibility of the Japanese classification in grading early oesophageal adenocarcinoma into six categories. In the present series, the m2 stage was the most discrepant stage (interobserver agreement 17% and 25% only), although it can be questioned if the interpretation of a m2 tumour as a m1 or m3 lesion will affect largely the clinical decision making. In the m3 and sm1 stages, of which optimal treatment strategies are still under debate, pathologist 3 showed less agreement. Furthermore, there was no underdiagnosis of submucosal carcinoma that requires undoubtedly surgical resection as only little discordance in the sm2 and sm3 substages was noted, and in 16 out of 17 N1-patients a sm2 or sm3 tumour was observed. It can be questioned whether the differences in agreement between pathologist 1 and 2 versus the agreement between pathologist 3 and pathologists 1 and 2, respectively, reflect a difference in years of working experience, or whether the results are influenced by the fact that pathologists 1 and 2 had worked closely together in the same department for many years. Nevertheless, the present data confirm that dedicated gastrointestinal pathologists are preferred when grading the resection specimens of this subpopulation of oesophageal cancer patients.

The Japanese classification for grading early oesophageal cancer plays an important role in the phase prior to the onset of treatment as well as the time period after the patient has undergone treatment. In the post-treatment phase, the final tumour stage as determined by histology of the resection specimen will define patient's prognosis. In the pre-treatment phase its role is even more important, as the decision whether to perform an endoscopic or a surgical resection in patients with early oesophageal cancer is mainly dependent on patients' predicted lymph nodal status.[11] At present, all diagnostic modalities including endoscopic ultrasonography lack the ability to correctly stage the N-category with a very high accuracy (71-86% in literature).[27, 28] Nevertheless, on the basis of the T-stage as staged by the pathologist on an EMR-specimen, one can predict the chance for lymphatic dissemination because

of its relationship to the tumour infiltration depth as assessed by the Japanese classification.[12-15] EMR is advocated as a staging modality to determine the T-stage in patients with early oesophageal cancer[29, 30], although the assessment of the exact depth of submucosal invasion remains difficult as full thickness submucosa is virtually absent in these specimens.[11] Therefore, if the submucosal invasion exceeds 0.5 mm, one may consider the tumour infiltration beyond the sm1 layer. Other factors that may predict lymphatic involvement in early oesophageal cancer include a diameter greater than 3 cm, a poor differentiation grade and lymphangio-invasion.[27, 31, 32] Although indications for endoscopic (m1, m2 tumours) and surgical treatment (sm2, sm3 tumours) are clear, for each individual with a m3 or sm1 adenocarcinoma it should be carefully considered whether a radical oesophagectomy may be beneficial. Therefore, individual treatment plans and a close interdisciplinary cooperation between surgeon, gastroenterologist and pathologist are mandatory in this patient population.

In conclusion, the reproducibility of the Japanese classification system is good in terms of inter- and intraobserver variability when grading early oesophageal adenocarcinoma on surgical resection specimens. The present data confirm that dedicated gastrointestinal pathologists with broad experience are preferred when grading the resection specimens of patients with early oesophageal adenocarcinoma.

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Preoperative risk assessment and surgical treatment

9

Preoperative risk assessment and prevention of complications in patients with esophageal cancer

Brechtje A Grotenhuis
Bas PL Wijnhoven
Frank Grüne
Jasper van Bommel
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

In this review the preoperative risk assessment and prevention of complications in patients undergoing esophagectomy for cancer is discussed. Age, pulmonary and cardiovascular condition, nutritional status, and neoadjuvant chemo(radio)therapy are known predictive factors. None of these factors is a valid exclusion criterion for esophagectomy, but may help in careful patient selection. Both anesthetists and surgeons play an important role in intraoperative risk reduction by means of appropriate fluid management and application of optimal surgical techniques.

Introduction

Surgery is the primary curative therapy for patients with esophageal cancer. However, esophagectomy is associated with a high operative risk.[1,2] Although operative mortality is below 5% in high volume centers[1,3], esophageal resection is still accompanied by substantial morbidity. The impact of postoperative complications on quality of life plays an important role in decision making whether to proceed with an operation in a patient with esophageal cancer. Moreover, the limited overall 5-year survival that does not exceed 40% after esophagectomy[4,5], has raised concerns regarding the advisability of such an extensive procedure in elderly patients.

It is now widely recognized that certain high-risk surgical procedures have lower mortality and morbidity rates when performed in high-volume centers, which is also true for esophageal cancer surgery.[6,7] For continuous improvement of esophagectomy outcome, an optimal treatment strategy should be based on proper patient selection by means of accurate staging and preoperative risk assessment. In this review we will discuss patients' preoperative risk assessment and the potential risk factors that can be taken care of both preoperatively and intraoperatively in order to prevent postoperative complications in patients with esophageal cancer.

Methods

A review of the recent English-language literature (January 1990 – June 2009) concerning esophageal cancer was performed. We focused on the one hand on studies that discussed patients' preoperative risk assessment and scoring systems that facilitate individualized preoperative risk stratification, and on the other hand on studies discussing the possibility of reducing these risks. With regard to the first part of this review (preoperative setting), we confined our search for potential risk factors to those that have been revealed in studies applying multivariate analysis to identify risk factors predicting morbidity and mortality after esophagectomy for cancer. This chapter will include an in-depth discussion of these potential risk factors. In the

second part we summarize the most recent advances in both anesthetic and surgical techniques that are thought to have an impact on patients' postoperative course.

1. Preoperative risk assessment and risk reduction

Several studies have focused on predisposing factors for complications after esophageal surgery for cancer. Patient characteristics have been combined in multivariate prognostic models for the prediction of short-term morbidity and mortality after esophagectomy. In general, these multivariate analyses revealed that advanced age, pulmonary dysfunction, a poor preoperative general performance status and application of neoadjuvant chemo- and/or radiotherapy are pronounced risk factors. [8-12] To summarize the most relevant studies published on these topics that can be evaluated in daily practice by both anesthetist and surgeon, we will discuss the potential risk factors age, pulmonary condition, cardiovascular status, nutritional status and application of neoadjuvant chemo- and/or radiotherapy.
However, we have to keep in mind that these studies have often been based on selected patient groups in specialized centers, are retrospective in nature, and the prognostic models have not been tested in independent patient cohorts, thus limiting the generalizability of the conclusions.

1.1 Risk factors

Age

In the past, advanced age was considered a relative contraindication for esophagectomy because of high operative mortality rates. Multivariate analyses of patient cohorts have identified advanced age as an independent risk factor for patients eligible for esophagectomy.[8-10] However, more recent studies showed acceptable results in patients 70 years and older undergoing esophagectomy, with morbidity (25-49%) and mortality rates (2-8%) that are comparable to those in younger counterparts.[13, 14] This is true even in the presence of significantly more preoperative risk factors (pulmonary dysfunction, cardiac disease, hypertension, diabetes mellitus) in elderly patients.[15] Moreover, disease-specific 5-year survival

did not differ between older and younger patients.[13-15] The acceptable outcome for elderly patients can most likely be attributed to an overall improvement in surgical technique and perioperative patient care. For instance, avoidance of a thoracotomy in elderly patients with esophageal cancer may lead to a lower cardiopulmonary-related mortality.

A few papers on esophagectomy in octogenarians have been published.[16-18] The percentage of patients above 80 among esophageal cancer patients is approximately 5%, and 20-30% of these patients is eligible for surgery.[18] In this highly-selected patient group morbidity (33-45%) and mortality (0-11%) rates were acceptable.[16, 18] However, in one study a 19% mortality rate was observed in the octogenarian patients, and it was calculated that age above 80 years is associated with a significantly increased perioperative mortality.[17]

From these studies, it can be concluded that advanced age is not a valid exclusion criterion for esophagectomy. There is substantial evidence that well selected elderly patients (based on a proper preoperative assessment of all potential risk factors) can survive surgical treatment with acceptable postoperative morbidity and mortality rates. A plausible explanation for these findings is that coexisting disease has more impact on perioperative morbidity and mortality than age alone.[19] For octogenarians, more evidence is needed to draw firm conclusions.

Pulmonary condition

Pulmonary complications such as pneumonia, atelectasis and respiratory insufficiency occur at high incidence rates in patients undergoing esophagectomy. In fact, pulmonary complications are the most common cause of postoperative mortality.[2, 20, 21] Surgical exploration in two or even three separate body compartments may be an explanation for the high pulmonary risk after esophagectomy. The extent of surgical trauma is related to the extent of postoperative immune depression, resulting in a failing host immunity against postoperative infections.[22] Furthermore, disruption of bronchial innervation, postoperative dysfunction of respiratory muscles and poor airway protection in case of recurrent laryngeal nerve injury are also likely

to play a role.[23] Patient-bound factors that contribute to postoperative pulmonary complications are advanced age, abnormal lung function test, and poor performance status.[11, 20, 21]

Preoperative pulmonary dysfunction is significantly associated with the development of postoperative pulmonary complications.[11, 21, 24] Poor pulmonary status (forced vital capacity (FVC) of less than 80% and forced expiratory volume in one second (FEV1) less than 70% of the predicted value) is related with both pulmonary and non-pulmonary postoperative morbidity.[24] Furthermore, impaired pulmonary function (in particular FEV1 <65% of predicted) is associated with prolonged mechanical ventilation and prolonged hospital stay.[11]

In general, preoperative assessment of patient's pulmonary function is a valuable risk indicator of postoperative pulmonary complications. Following the guidelines of the American College of Physicians[25], at least patients older than 60 years, with a history of smoking and/or signs of pulmonary obstruction should perform a lung function test. Respiratory medicine consultation should be considered in patients with FEV1 <70% of the predicted value. Whether the risk of postoperative pulmonary complications can be reduced in patients undergoing esophagectomy by means of improving respiratory muscle strength and performance status is not clear yet. One study showed that preoperative respiratory muscle training in patients undergoing thoracic surgery in general (including esophagectomy) may prevent postoperative pulmonary complications by increasing both inspiratory and expiratory muscle strength.[26] Other promising results have been reported in a randomized clinical trial (RCT) that included patients undergoing coronary artery bypass grafting: pre-operative intensive inspiratory muscle training reduced significantly the incidence of postoperative pulmonary complications.[27]

Cardiovascular status

Perioperative cardiac arrhythmias occur with an incidence of approximately 20%.[2, 20, 28] Atrial fibrillation (AF) in particular might reflect underlying pulmonary problems or surgical sepsis[28], but the exact mechanisms responsible for AF after

esophagectomy remain speculative. The reported incidence of myocardial infarction after esophagectomy is low (1-2%).[2, 9, 20] Nevertheless, because of the extensiveness of the surgical procedure, a thorough preoperative cardiac evaluation is indicated based on the level of exercise tolerance and appearance of cardiac risk predictors according to the Revised Cardiac Risk Index (RCRI), see Figure 1.[29]

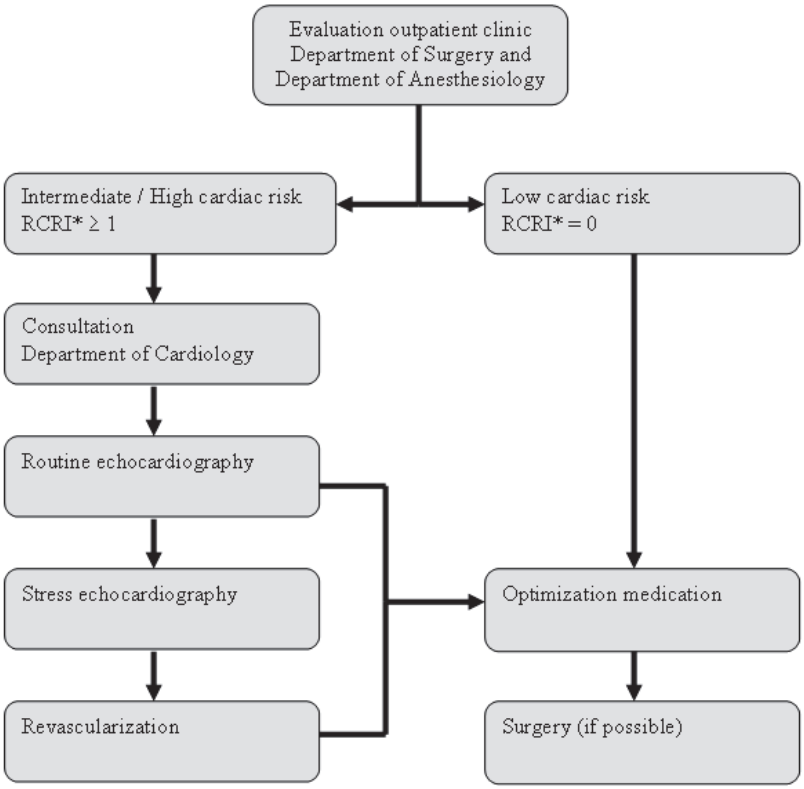


Figure 1. *Preoperative cardiac evaluation of esophageal cancer patients scheduled for high-risk surgery (esophagectomy) at the outpatient clinic of the Department of Surgery and Department of Anesthesiology.*

Little is known about the value of routine preoperative cardiac stress testing in patients undergoing esophagectomy in predicting outcome. Dobutamine stress echocardiography is able to provide prognostic information on myocardial ischemia

and contractile reserve in patients with ischemic left ventricular function.[30] One study investigated the usefulness of cardiopulmonary exercise testing, in which oxygen uptake at increasing levels of exercise determined the cardiopulmonary performance under conditions of stress, thereby imitating the operative situation.[31] However, the maximum oxygen uptake ($V\dot{O}_2$-max) appeared to be a poor predictor of cardiopulmonary morbidity in these patients. In general, grading and rating literature on cardiac stress testing is a difficult process due to great variability in endpoints and results. More research is needed to evaluate the most optimal way of cardiac stress testing in patients undergoing esophagectomy.

Although it is probably beneficial for high-risk patients in general to receive perioperative beta-blocker therapy[32], its role in patients undergoing esophagectomy has not been investigated thoroughly yet. A recent meta-analysis of RCTs in patients having non-cardiac surgery showed no clear benefit of perioperative beta-blockers for the prevention of cardiovascular outcomes.[33] In fact, beta-blockers seemed to increase the risk of stroke, bradycardia and hypotension. Therefore, it is advised that beta-blockers should not be routinely used for perioperative treatment of patients undergoing non-cardiovascular surgery unless patients are already taking them for clinically indicated reasons following the guidelines of the European Society of Cardiologists (*i.e.* heart failure, coronary artery disease, previous myocardial infarction).[34]

Statin use has been associated with a decreased incidence of atrial fibrillation after non-cardiac thoracic surgery[35] as well as with decreased mortality after non-cardiac surgery.[36] This is probably due to its anti-inflammatory and plaque-stabilizing effects. Moreover, as shown in patients undergoing major vascular surgery, discontinuation of statin therapy is associated with an increased postoperative cardiac risk.[37] It may be valid to extrapolate this to esophagectomy patients by restarting this medication as soon as possible after the operation.
In many patients anti-platelet drugs like aspirin will complement this combination therapy; such medications are generally ceased 7-10 days prior to surgery. A large meta-analysis on the risk of increased bleeding in surgical patients using low-dose aspirin has demonstrated that aspirin increases the rate of bleeding complications by

a factor 1.5.[38] However, this does not implicate an increase in morbidity or mortality in the postoperative phase. Moreover, acute withdrawal of these drugs is associated with an increased risk of development of thrombotic events.[39] It has been reviewed that the risk of coronary thrombosis on anti-platelet drugs withdrawal is higher than the risk of surgical bleeding when maintaining them.[40] Aspirin should be considered a lifelong therapy and should not be stopped before surgery or before insertion of an epidural catheter when it has been prescribed as a secondary prevention after stroke, myocardial infarction or revascularization.[40] As a primary prevention it can be withdrawn one week before surgery, and should be restarted as soon as the epidural catheter has been removed. There is no literature to support the use of therapeutic heparin as an alternative.[41]

More recently, the role of B-type natriuretic peptide (BNP) and its N-terminal fragment (NT-pro-BNP) as a biomarker in predicting cardiac complications during the postoperative course has been evaluated. BNP and NT-pro-BNP are neurohormones that are released in response to ventricular wall stress and have shown to be sensitive and specific predictors of left ventricular systolic dysfunction.[42] In patients undergoing esophagectomy, it appeared that elevation of the perioperative plasma BNP level is a strong and independent predictor of postoperative AF, suggesting that patients at higher risk of postoperative AF can be identified and should be considered for preventive antiarrhythmic therapy.[43] However, it is unlikely that this single parameter will change the decision on patient's operability.

In conclusion, until now there is no evidence for routine stress-testing or routine prophylactic combination therapy (beta-blocker, statin, aspirin). It remains unclear whether such combination therapy is beneficial to esophagectomy patients, or whether it is at the cost of potential adverse effects (*e.g.* strokes and conduit necrosis). If there is an indication for secondary prevention after stroke, myocardial infarction or revascularization, the combination therapy should be continued during the perioperative process. In general, the guidelines on perioperative cardiovascular evaluation for noncardiac surgery formulated by the American College of Cardiology/ American Heart Association can be followed.[44]

Nutritional status: obesity and malnutrition

The increased incidence of adenocarcinoma of the esophagus and gastroesophageal junction in recent decades parallels the increasing prevalence of obesity. Surgery and anesthesia are more hazardous in overweighted patients, not least because of the increased incidence of cardiorespiratory comorbidity.[45] In general, obese patients are at higher risk for hypoxia from atelectasis, because pulmonary function in obese patients is characterized by reductions in functional residual capacity and expiratory reserve volume.[45] One retrospective study showed that obese patients (Body Mass Index = BMI >30) were more likely to develop respiratory complications than non-obese patients.[46] Furthermore, the presence of excessive subcutaneous fat, with its relatively low blood perfusion and oxygen tension, may predispose obese patients to impaired wound healing and wound infections. However, obesity is not associated with an increased operative risk nor with worse long-term outcomes after esophagectomy.[46-48] Thus, obesity cannot be considered a contra-indication for surgery.

In general, malnutrition is associated with an increased risk for postoperative infectious complications.[49] Pretreatment nutritional support could potentially decrease postoperative complications, but preoperative nutritional condition has received only minor attention in risk analyses for esophagectomy. One study showed that preoperative nutritional status determined by prognostic nutritional index (PNI) and nutritional risk index (NRI) has only limited value in predicting postoperative infectious complications.[50] However, hypoalbuminemia as a marker of malnutrition is associated with greater risk of adverse surgical outcome in terms of morbidity and mortality.[51, 52] Decreased serum albumin level may indicate protein-energy malnutrition, which results from increased protein and energy requirements associated with the stress of illness, injury or infection.[51] In patients undergoing esophagectomy for cancer, serum albumin concentration on the first postoperative day was independently associated with postoperative complications.[53]

Neoadjuvant chemo- and/or radiotherapy

The average 5-year survival for esophageal cancer patients does not exceed 40% after esophagectomy.[4, 5] As further improvement in survival from a single modality

approach such as surgery is less likely, interest in multimodal approaches has considerably grown. Strategies combining neoadjuvant chemo(radio)therapy and surgery have been investigated as a means of enhancing locoregional control and improving survival among patients with localized esophageal cancer.[54] However, multivariate analyses focusing on risk factors for complications after esophagectomy revealed that neoadjuvant therapy was a predictor for early postoperative mortality and development of pulmonary complications.[11, 12] Furthermore, concerns have been raised for a suppressed immune system[55] and delayed wound healing.[14] In meta-analyses of RCTs comparing neoadjuvant therapy and surgery to surgery alone for resectable esophageal cancer, a significant effect of neoadjuvant chemoradiation on postoperative mortality was shown in one study[56], whereas a nonsignificant trend toward increased mortality for the neoadjuvant therapy group was seen in other reports.[57, 58] It is clear that more studies are required to investigate this potential negative effect of multimodality treatment on postoperative mortality rates.

1.2 Scoring systems

Accurate individualized preoperative risk stratification can help in informing patients, choosing the optimal extent of surgery, and guiding patients to high-volume centers when necessary. First of all, the O-POSSUM score (adapted from the Physiological and Operative Severity Score for the enUmeration of Mortality and morbidity-POSSUM[59]) has been developed for the individualized prediction of postoperative mortality and morbidity in esophageal and upper gastro-intestinal surgery.[60] In-hospital mortality can be predicted based on a combination of patients' age, a physiological score (based on 12 variables) and three intra-operative parameters. However, external validation of the O-POSSUM score revealed its inability to predict accurately postoperative mortality and morbidity in patients undergoing esophagectomy.[61, 62]

Various other risk-adjusted models have been developed by means of multivariable prognostic models to predict in-hospital mortality for individual patients undergoing esophagectomy.[9, 63, 64] However, these models have often been based on selected patient groups in specialized centers and on retrospective data, thereby limiting the generalizability of the results. Moreover, independent validation on new patients or other patient cohorts was often not accomplished or was performed in relatively small external series. One study managed to analyze predictive variables in a large

patient cohort, to develop a simple risk score and to validate this score in three other cohorts (Figure 2).[65] The risk score showed good agreement of predicted risks with observed mortality rates (calibration), but low discrimination. Therefore, this risk score is probably insufficient for risk assessment in the individual patient. Recently, three existing prognostic models for mortality after esophagectomy[63-65] were validated in two independent patient cohorts in Australia and Switzerland, but it was concluded that none of the scores was suitable for application in daily practice.[66]

Score Chart to Estimate 30-Day Mortality After Cancer-Directed Surgery for Esophageal Cancer

Characteristic	Score
Age, years	
50	−1
65	0
80	1
Comorbidity	
Pulmonary	1
Cardiovascular	1
Diabetes	1
Hepatic	1
Renal	1
Neoadjuvant therapy	
Radiotherapy	1.5
Chemoradiotherapy	1
Hospital volume; No. of esophagectomy/year	
Low (≤ 1)	0
Intermediate (1.1-2.5)	−0.5
High (≥ 2.6)	−1.5
Very high (± 50)	−2

NOTE. Sum score is obtained by adding scores. Intermediate scores for age can be approximated by linear interpolation. For example, age 72 corresponds to a score of + 0.5. The formula to calculate the predicted probability of surgical mortality is P(mortality) = 1/[1+ exp(2.41 − 0.32 × score)].

Figure 2. *Score chart to estimate 30-day mortality after surgery for esophageal cancer. Reprinted with permission, all rights reserved © 2006 American Society of Clinical Oncology.*

Predicting the severity of complications after esophagectomy may supply important information for both patient and surgeon. Although not independently validated, a risk score has been developed in order to predict pulmonary complications based on three preoperative variables (age, FEV1 and performance status).[21] Furthermore, a nomogram (giving a graphical representation of the predictive strength of specific predictors and enabling the calculation of an overall risk score for the individual patient) has been developed to predict the severity of complications in esophagectomy patients.[67] Factors including high age, myocardial infarction,

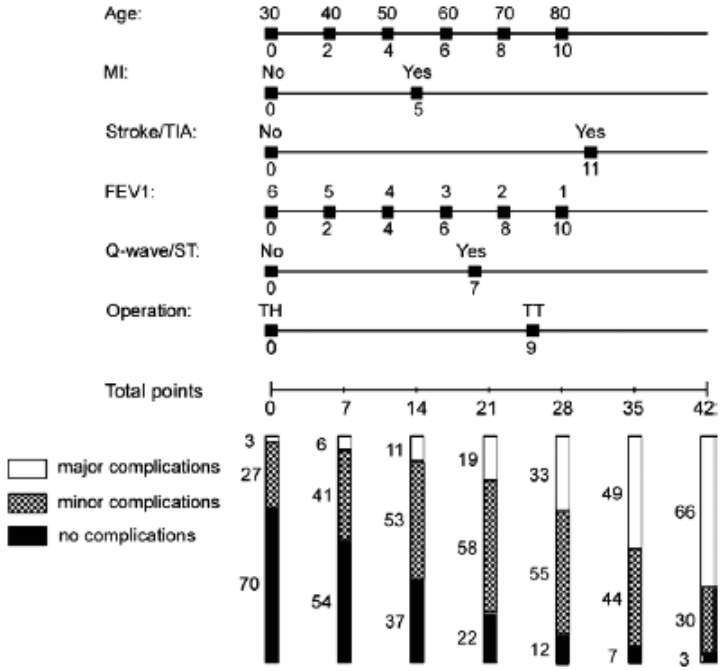
stroke or transient ischemic attack in the medical history, low FEV1, presence of electrocardiographic changes and more extensive surgery (*i.e.* transthoracic esophagectomy) were taken into account (Figure 3). This model was validated in a second group of patients within the same institution, which showed a good overall agreement, but a substantial overlap in the risk scores between patient groups resulted in a moderate discrimination. Overall, this nomogram may play a role in preoperative risk assessment of morbidity after esophagectomy, provided that external validation will show good calibration and discrimination.[67]

In conclusion, there is no scoring system available yet that can be used preoperatively in order to predict reliably patient's morbidity and mortality after esophagectomy.

2. Intraoperative risk reduction

2.1 Optimal intraoperative management: role of the anesthetist

It is evident that perioperative anesthetic management contributes directly to the postoperative course after esophagectomy. Intraoperative cardiorespiratory instability (measured by intraoperative hypoxemia, hypotension, need for inotropic support) has shown to be associated with the occurrence of acute lung injury in the postoperative course.[68] During transthoracic esophagectomy one-lung ventilation (leading to a total collapse of the other lung) is used to create surgical exposure to the esophagus. Hypoxia may occur in this stage due to shunting of blood via the non-ventilated lung or surgical compression of the mediastinum.[69] Furthermore, patients undergoing esophagectomy are at risk of aspiration. Prophylactic pharmacological management of gastroesophageal reflux, application of continuous low-grade nasogastric suction, adequate tracheal cuff inflation, use of a gel lubricant on the tracheal cuff and avoiding vocal cord injury at intubation will be beneficial in reducing the risk of tracheal aspiration.[70, 71]



Nomogram for prediction of severity of complications with use of preoperative risk-factors. Complication categories are graded according to severity; no complications (category 0), minor to moderate complications (category 1), and major complications (category 2). Instruction: Locate the Age on the axis. Determine how many points the patient receives. Repeat this for each axis. Sum the points for all predictors and locate the sum on the Total points axis. Draw a line straight down to the bar graphs. Bar graphs represent the chance for an individual patient after esophagectomy for cancer to develop major, minor-to-moderate, or no complications. (FEV_1 = forced expiratory volume in the first second; MI = myocardial infarction; TIA = transient ischemic attack.)

Figure 3. *Nomogram for prediction of severity of complications with use of preoperative risk factors. Reprinted with permission, all rights reserved © 2008 Annals of Thoracic Surgery.*

More important for patient's postoperative course is an appropriate fluid management in the intraoperative setting. A strict balance is required between the maintenance of cardiac output and perfusion pressure for oxygen-delivery of vital organs and the prevention of pulmonary and peripheral edema. Performing esophagectomy induces a systemic stress response with subsequent release of inflammatory mediators causing vasodilatation, severe capillary leakage and a fluid shift from the intravascular space into the interstitium.[72] Together with the vasodilatory effects of general anesthetics and thoracic epidural analgesia, this can easily lead to excessive fluid administration and, subsequently, pulmonary edema. In patients undergoing esophagectomy it was found in retrospective analyses that limited intraoperative fluid administration is associated with a low morbidity rate in general, reduced postoperative pulmonary complications and shortened length of hospital stay.[73, 74] However, it remains unclear whether restriction of fluid administration or use of diuretics after esophagectomy can reduce postoperative morbidity and mortality; prospective studies are needed in order to address these issues. Until then, the amount of fluid should be titrated for the individual patient according to the perioperative hemodynamic changes as detected by intraoperative monitoring.

In order to reduce the systemic inflammatory response to surgical trauma, it has been suggested that intraoperative steroid therapy could reduce the inflammatory cytokine release from macrophages. However, it is believed that steroids may also suppress wound healing and the patient's immune defense after operation. One RCT showed that preoperative administration of steroid therapy significantly decreased postoperative morbidity following esophagectomy.[75] No adverse clinical effects were observed, especially no increase in infectious complications or delayed wound healing.[75] Careful investigation to what extent the release of proinflammatory cytokines should be suppressed is required.

Another factor that influences the postoperative course is the application of thoracic epidural analgesia. Potential benefits after major surgery include proper pain relief and reduction in respiratory complications. Pain relief provided by epidural analgesia has been shown superior in achieving effective cough and early mobilization in the

postoperative period.[76, 77] A reduced incidence of respiratory complications with epidural analgesia after esophagectomy has been demonstrated, which can lead to a reduced length of stay at the intensive care unit.[78] Moreover, the absence of effective epidural analgesia is an independent risk factor for pneumonia.[79]

1.2 Prevention of complications – surgical technique

The optimal surgical approach for patients with esophageal cancer is still debatable. Three operation techniques are widely performed nowadays: transhiatal esophagectomy without formal intrathoracic lymphadenectomy, extended transthoracic esophagectomy with *en bloc* lymphadenectomy, and esophagectomy with extension of the lymphadenectomy to the cervical region ('three-field dissection'). One RCT in which the transhiatal and transthoracic approach in patients with adenocarcinoma of the esophagus were compared, it was shown that an ongoing trend towards better overall 5-year survival could be achieved with the extended transthoracic esophagectomy, especially in patients with a Siewert type I tumor.[80] However, the additive thoracotomy may be at the cost of increased postoperative morbidity; in particular a higher incidence of pulmonary complications was seen.[81] Hence, it is believed that an extended transthoracic esophagectomy is the procedure of first choice in patients with a Siewert type I tumor, and a limited transhiatal esophagectomy in type II carcinoma. A transhiatal approach may also be considered the optimal surgical strategy in patients with significant co-morbidities or in elderly patients, in order to minimize the risk of postoperative complications. It is believed that the high prevalence of cervical lymph node involvement (approximately 30% in certain series) is the rationale for three-field lymphadenectomy.[82, 83] Depending on the level of the primary tumor, cervical lymph nodes are involved in 60, 20 and 12% of upper, middle and lower third tumors, respectively.[82, 83] However, a convincing survival benefit for three-field lymphadenectomy has not been proven[84] and the high incidence of recurrent laryngeal nerve palsy, related to extensive dissection of the recurrent laryngeal nerve chain, remains a major concern.[85]

As surgical blood loss correlates with postoperative mortality, excessive bleeding should be prevented.[2] Recurrent laryngeal nerve function should be carefully

preserved because it is required for effective tracheal clearance.[23] The integrity of the thoracic duct or the adequacy of its distal ligation should always be checked with care in order to prevent the development of a chylothorax.

Anastomotic leakage and conduit necrosis are amongst the most serious postoperative complications. Measurement of gastric tissue blood flow reflecting the quality of arterial and venous blood supply, can predict anastomotic failure.[86, 87] Other factors that influence the risk of anastomotic leakage include tension on the anastomosis, location of the anastomosis and, most likely, the experience of the operating surgeon (reviewed in reference[88]). No clear difference in outcome could be demonstrated between circular stapled and hand-sewn anastomoses.[89, 90] More recently, the modified Collard anastomotic technique has been described, which encompasses a semi-mechanical (*i.e.* partially stapled, partially hand-sewn) technique for a side-to-side cervical anastomosis.[91] Although not randomized, this trial suggests a reduced anastomosis-related morbidity risk.
In cases of a major anastomotic leakage, the leak can be accompanied by (partial) conduit necrosis, which may induce a severe sepsis. Etiologic factors are comparable to those indicated for anastomotic leaks, but include also conduit torsion and lesions caused during gastric pull-up.[92] It has been hypothesized that the use of pharmacological agents enhancing conduit's blood flow may decrease anastomotic failure. Prostaglandin E1 has been studied extensively, but this powerful vasodilator did not show beneficial effects on conduit viability or anastomotic leak rate.[93] Furthermore, a RCT was performed to evaluate the role of nitroglycerin, which can lead to an enhanced microvascular blood flow of the gastric tube.[94] Administration of intravenous nitroglycerin during gastric tube reconstruction could not prevent deterioration in gastric microvascular perfusion and no significant difference in anastomotic failure could be detected between the nitroglycerin and control group.[94]

To improve the anatomic vascular supply of the gastric conduit, several experimental studies have concentrated on preoperative gastric ischemic conditioning ('delay phenomenon').[95] The procedure consists of surgical or radiological partial gastric devascularization by occlusion of the left gastric vessels, thereby inducing an ischemic

insult leading to the development of collateral submucosal blood flow. Ideally, this procedure should result in an improved gastric tissue perfusion prior to pull-up and anastomosis to the esophagus. Recently, gastric ischemic conditioning was reported in patients by laparoscopic mobilization of the stomach including partial gastric cardia stapling with division of the left gastric and gastroepiploic arteries.[96] After a mean delay of 4 days, a conventional transthoracic esophagectomy was performed, after which anastomotic leakage was reported in 6% of patients.[96] Overall, laparoscopic ischemic conditioning of the gastric conduit is feasible, but its effectiveness in reducing the incidence of anastomotic leakage remains to be determined.

Conclusion

Esophagectomy is still associated with high operative risks. Age, pulmonary and cardiovascular condition, nutritional status, and application of neoadjuvant chemo- and/or radiotherapy have shown to be important predictive factors for postoperative morbidity and mortality that can be evaluated preoperatively. None of these factors is in itself a valid exclusion criterion for esophagectomy, but may contribute to careful patient selection. Both the surgeon and the anesthetist play an important role in intraoperative risk reduction. Adequate fluid management and application of appropriate surgical techniques are of great importance in terms of risk reduction in patients with esophageal cancer. In order to improve the short-term outcome of esophagectomy, an optimal perioperative treatment strategy can be tailored for the individual patient by a multidisciplinary team consisting of surgeons, physicians, anesthetists and intensive care specialists.

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10

Prognostic value of body mass index on short-term and long-term outcome after resection of esophageal cancer

Brechtje A Grotenhuis
Bas PL Wijnhoven
Gijsbert J Hötte
Eric P van der Stok
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

Introduction: Cachexia and obesity have been suggested to be risk factors for postoperative complications. However, high BMI might result in a higher R0-resection rate because of the presence of more fatty tissue surrounding the tumor. The aim of this study was to investigate whether BMI is of prognostic value with regard to short-term and long-term outcome in patients undergoing esophagectomy for cancer.

Methods: In 556 patients who underwent esophagectomy (1991-2007), clinical and pathological outcome were compared between different BMI classes (underweight, normal weight, overweight, obesity).

Results: Overall morbidity, mortality, and reoperation rate did not differ in underweight and obese patients. However, severe complications seemed to occur more often in obese patients ($p=0.06$), and the risk for anastomotic leakage increased with higher BMI (12.5% in underweight patients to 27.6% in obese patients, $p=0.04$). Histopathological assessment showed comparable pTNM stages, although an advanced pT-stage was seen more often in patients with low/normal BMI ($p=0.02$). A linear association between BMI and R0-resection rate was detected ($p=0.02$): 60.0% in underweight patients to 81.0% in obese patients. However, unlike pT-stage ($p<0.001$), BMI was not an independent predictor for R0-resection ($p=0.12$). There was no significant difference in overall or disease-free five-year survival between the BMI classes ($p=0.25$ and $p=0.60$, respectively).

Conclusion: BMI is not of prognostic value with regard to short-term and long-term outcome in patients undergoing esophagectomy for cancer, and is not an independent predictor for radical R0-resection. Patients oncologically eligible for esophagectomy should not be denied surgery on the basis of their BMI class.

Introduction

Among patients with esophageal cancer weight loss is common, which is caused by malnutrition due to reduced food intake (mostly related to dysphagia) and increased demands because of systemic inflammation (cancer cachexia).[1] Malnutrition has long been recognized as a condition associated with an increased risk of postoperative complications[2], in particular infectious complications.[3] It is unclear which mechanism is responsible for the relationship between malnutrition and an adverse short-term clinical outcome, but a combination of immune, inflammatory and metabolic processes is thought to play a role.[3]

The prevalence of obesity (defined as body mass index (BMI) > 30.00) among adults has increased over the past decades in the Western world.[4, 5] Obesity is associated with several medical comorbidities, including diabetes, hypertension and coronary artery disease.[6] In addition, increased BMI is associated with a higher risk of several types of cancers[7], including esophageal adenocarcinoma.[8] Surgery and anesthesia are more hazardous in overweight patients, not least because of the increased incidence of cardiorespiratory comorbidity.[9] Obese patients are at higher risk for developing respiratory complications, because their pulmonary function is characterized by reductions in functional residual capacity and expiratory reserve volume.[9, 10 11] The presence of excessive subcutaneous fat may also predispose obese patients to impaired wound healing and wound infections.[12] On the other hand, it might be hypothesized that in patients with a high BMI (and increased visceral fatty tissue) a higher percentage of tumor-free circumferential resection margins might be achieved because of the presence of more fatty tissue surrounding the tumor as compared to patients with a low BMI when performing esophagectomy for cancer.

To improve outcome after esophagectomy, an optimal treatment strategy should be based on appropriate patient selection, which is strongly influenced by preoperative risk assessment. However, preoperative nutritional condition has received minor attention in risk analyses for esophagectomy thus far. The aim of the present study was to investigate whether BMI is of prognostic value with regard to postoperative

short-term and long-term outcome in patients undergoing esophagectomy for cancer. We hypothesized on the one hand that patients with either underweight or obesity are at higher risk for perioperative morbidity and mortality after esophagectomy, and that on the other hand increased BMI with excessive peritumoral fatty tissue might facilitate a radical (R0-) resection.

Patients and methods

Patients

Between January 1991 and December 2007, 791 patients underwent an esophagectomy for cancer of the esophagus or gastroesophageal junction in the Erasmus Medical Center in Rotterdam, The Netherlands. Only patients in whom no chemo- and/or radiotherapy was applied and who underwent a transthoracic or transhiatal esophagectomy were included in the present study. In the Erasmus MC, patients received neoadjuvant chemo(radio)therapy in the context of clinical trials.[13] Induction chemo- and/or radiotherapy was given to patients with either a cT4-tumor without distant metastases or to patients with gross involvement of celiac trunk lymph nodes (M1a), who were not considered eligible for primary surgical therapy. There were 214 patients who were excluded because of chemo- and/or radiotherapy prior to surgery, and 21 patients because of unknown preoperative BMI. The remaining 556 patients were included in this study.

Surgery

The majority of patients underwent a transhiatal esophagectomy (N=541). The primary tumor and its adjacent lymph nodes were dissected under direct vision through the widened hiatus of the diaphragm up to the level of the inferior pulmonary vein. In addition, all adjacent fatty tissue surrounding the tumor was removed simultaneously, until the lateral resection margins were reached (diaphragm, pleura, pericardium, aorta). Subsequently, a gastric tube was created. The left gastric artery was transected at its origin, with resection of celiac trunk lymph nodes. After mobilization and transection of the cervical esophagus, the normal intrathoracic

esophagus proximal to the primary tumor was mobilized bluntly from the neck to the abdomen with a vein stripper. Esophagogastrostomy (hand-sewn or by using a circular stapler) was performed in the neck. Posterolateral thoracotomy was the first step in transthoracic resection with extended lymphadenectomy (N=15). The thoracic duct, azygos vein, ipsilateral pleura, and all periesophageal tissue in the posterior mediastinum were dissected *en bloc*. The resection specimen included the lower and middle mediastinal, subcarinal, and right-sided paratracheal lymph nodes. The aortapulmonary-window nodes were dissected separately. Through a midline laparotomy, the paracardial, lesser curvature, left-gastric-artery, celiac trunk, common-hepatic-artery, and splenic-artery nodes were dissected, and a gastric tube was constructed. The cervical phase of the transthoracic procedure was identical to that of the transhiatal procedure. Tumors were assigned pathologic tumor-node-metastasis stages according to the Union Internationale Contre le Cancer (UICC) 2002 system.[14]

Data collection

Patients' weight and length were measured at their first visit to the outpatient clinic. Patients' BMI was calculated and classified according to the World Health Organization criteria (underweight: BMI < 18.50 kg/m^2, normal weight: BMI 18.50 – 24.99 kg/m^2, overweight: BMI 25.00 – 29.99 kg/m^2, obesity: BMI ≥ 30.00 kg/m^2).[15] Outcome data (including half-yearly follow-up) for all patients with esophageal cancer referred to our hospital for further analysis and treatment had been collected prospectively and stored in a database by a specialized data manager. Follow-up was recorded until December 2008 or until death if earlier, and was complete for all patients.

Statistics

To address the question whether patients with either underweight or obesity are at higher risk for perioperative morbidity and mortality after esophagectomy, short-term outcome of underweight and obese patients was compared with outcome of the control group (patients with normal weight or overweight). The potential impact of BMI on histopathological outcome was considered to be on a linear scale (*i.e.* increased BMI might result in a higher percentage R0-resections), and was analyzed by comparing patients' outcome of the four BMI classes.

Statistical analysis appropriate for non-parametric data was used. Grouped data as presented in Table 2 and 3 were compared using the Chi-Square-, Mann-Whitney U- or Kruskall-Wallis H-test. In case these tests were significant (thereby indicating that there were differences but not indicating the where the differences are), groups were compared pair-wise with adding the Bonferroni correction. Trend-analysis and logistic regression were performed to reveal linear associations with regard to BMI.
Overall survival was calculated from the date of operation until the date of last follow-up or death according to the Kaplan-Meier method. Patients who died due to complications following esophagectomy (in-hospital mortality) were not excluded from survival-analysis. Univariate analyses were performed to identify prognostic variables associated with overall survival after esophagectomy.
Data-analysis was carried out with SPSS version 15.0 (SPSS,Chicago,IL,USA). Two-sided p-values <0.05 were considered to be significant.

Results

Patients' characteristics

Patients' characteristics are described in Table 1. Forty patients (7.2%) were classified as underweight (BMI < 18.50 kg/m^2), 244 patients (43.9%) had a normal weight (BMI 18.50 – 24.99 kg/m^2), 214 patients (38.5%) were overweight (BMI 25.00 – 29.99 kg/m^2), whereas obesity (BMI > 30.00) was diagnosed in 58 patients (10.4%).

Short-term outcome

Median operative time for all patients was 280 minutes (range 120-572 minutes). Operative time increased among the different BMI classes: median operative time was 266 minutes in underweight patients, 274 minutes in normal weight, 285 minutes in overweight and 307 minutes in obese patients (p=0.04). There was no significant difference in length of ICU-MCU- (median 4 days) or hospital-stay (median 14 days) between the different groups.

Table 1. Clinicopathological characteristics of 556 patients who underwent surgical resection for esophageal cancer.

Age (in years)*		65 (range 28 – 89)
Gender		
- *Male*		450 (80.9%)
- *Female*		106 (19.1%)
ASA classification		82 (14.7%)
- *I*		364 (65.5%)
- *II*		106 (19.1%)
- *III*		4 (0.7%)
- *IV*		
BMI class		40 (7.2%)
- *Underweight*	*(BMI < 18.50)*	244 (43.9%)
- *Normal weight*	*(BMI 18.50 – 24.99)*	214 (38.5%)
- *Overweight*	*(BMI 25.00 – 29.99)*	58 (10.4%)
- *Obesity*	*(BMI ≥ 30.00)*	
Tumor location		9 (1.6%)
- *Proximal esophagus*		32 (5.8%)
- *Mid esophagus*		232 (41.7%)
- *Distal esophagus*		283 (50.9%)
- *Gastroesophageal junction*		
Histology		83 (14.9%)
- *Squamous cell carcinoma*		473 (85.1%)
- *Adenocarcinoma*		

***Age is given as median*
ASA classification = American Society of Anesthesiologists classification
BMI = body mass index

When analyzing the effect of either underweight or obesity on patients' short-term outcome, overall morbidity, reoperation rate and in-hospital mortality were similar to those of the patients in the control group (normal weight or overweight; Table 2). However, when the grade of complications was taken into account, severe complications seemed to occur more often in obese patients than in the control group (36.4% versus 21.8%, p=0.06). Furthermore, the incidence of anastomotic leakage (radiological as well as clinical) did not seem comparable between the three groups (p=0.09). On trend-analysis, a linear association between BMI and anastomotic leakage was detected (p=0.04): the risk for anastomotic leakage increased with higher BMI (from 12.5% in underweight patients to 27.6% in obese patients). Also, a trend for a linear association between BMI and wound infection was seen (p=0.07): from 2.5% in underweight patients to 12.1% in obese patients.

Table 2. Impact of underweight and obesity on postoperative complications and in-hospital mortality in patients undergoing esophagectomy for cancer, as compared to the control group (normal weight and overweight patients).

	Underweight *BMI <18.50 N=40*	***Control group*** *BMI 18.50 – 29.99 N=458*	***Obesity*** *BMI ≥ 30.00 N=58*	***p-value***
Postoperative complications	20 (50.0%)	294 (64.2%)	34 (58.6%)	0.17
Surgical complications	1 (2.5%)	13 (2.8%)	0 (0.0%)	0.43
-Bleeding	1 (2.5%)	12 (12.6%)	1 (1.7%)	0.92
-Chyle leakage	5 (12.5%)	77 (16.8%)	16 (27.6%)	0.09
-Anastomotic leakage	0 (0.0%)	11 (2.4%)	1 (1.7%)	0.59
-Conduit necrosis	4 (10.0%)	67 (14.6%)	4 (6.9%)	0.16
-Vocal cord paresis	1 (2.5%)	43 (9.4%)	7 (12.1%)	0.25
-Wound infection				
Medical complications	1 (2.5%)	29 (6.3%)	3 (5.2%)	0.60
-Sepsis	11 (27.5%)	149 (32.5%)	12 (20.7%)	0.16
-Pneumonia	5 (12.5%)	40 (8.7%)	6 (10.3%)	0.69
*-Respiratory insufficiency**	3 (7.5%)	39 (8.5%)	3 (5.2%)	0.67
-Atrial fibrillation	0 (0.0%)	5 (1.1%)	2 (3.4%)	0.24
-Myocardial infarction	0 (0.0%)	10 (2.2%)	2 (3.4%)	0.51
-Thromboembolism				
Reoperation	3 (7.5%)	54 (11.8%)	5 (8.6%)	0.58
In-hospital mortality	1 (2.5%)	27 (5.9%)	5 (8.6%)	0.45

***** *Respiratory insufficiency was defined as pulmonary dysfunction requiring prolonged ventilation (>10 days) or reintubation.*

BMI = body mass index

Histopathological assessment for the four different BMI classes is shown in Table 3. Advanced pT-stage was seen significantly more often in patients with a low or normal BMI (p<0.01). Patients with overweight or obesity appeared to participate more often in a surveillance program than patients with a low or normal BMI (28.8% versus 17.1%, p=0.21). Underweight patients mostly suffered from squamous cell carcinoma (55%), whereas the majority of patients with normal weight, overweight or obesity were diagnosed with adenocarcinoma (89%; p<0.001). A trend towards a higher rate of radical resection (R0) across the BMI classes was noted (p=0.06). On trend-analysis, a linear association between BMI and R0-resection rate was detected (p=0.02): 60.0% in underweight patients versus 81.0% in obese patients. However, unlike pT-stage (p<0.001), BMI was not an independent predictor for R0-resection on logistic regression (p=0.12). Stratification for tumor location (esophagus versus gastroesophageal junction) did not influence the R0-resection rate.

Table 3. Histopathological assessment of the resection specimens in relation to four BMI classes in 556 patients who underwent surgical resection for esophageal cancer.

	Underweight *BMI <18.50 N=40*	***Normal weight*** *BMI 18.50 – 24.99 N=244*	***Overweight*** *BMI 25.00 – 29.99 N=214*	***Obesity*** *BMI ≥ 30.00 N=58*	***p-value***
Histology	22 (55.0%)	29 (11.9%)	26 (12.1%)	6 (10.3%)	***<0.001***
- *SCC*	18 (45.0%)	215 (88.1%)	188 (87.9%)	52 (89.7%)	
- *AC*					
pT-status	10 (25.0%)	65 (26.6%)	83 (38.8%)	23 (39.7%)	***0.02***
- *T1 – T2*	30 (75.0%)	179 (73.4%)	131 (61.2%)	35 (60.3%)	
- *T3 – T4*					
pN-status	16 (40.0%)	84 (34.4%)	81 (37.9%)	24 (41.4%)	0.94
- *N0*	24 (60.0%)	160 (65.6%)	133 (62.2%)	34 (58.6%)	
- *N1*					
pM-status	32 (80.0%)	194 (79.5%)	164 (76.6%)	47 (81.0%)	0.84
- *M0*	8 (20.0%)	50 (20.5%)	50 (23.4%)	11 (19.0%)	
- *M1a – M1b*					
Differentiation grade	4 (10.0%)	14 (5.7%)	17 (7.9%)	4 (6.9%)	0.17
- *G1 (good)*	26 (65.0%)	111 (45.5%)	95 (44.4%)	22 (37.9%)	
- *G2 (moderate)*	10 (25.0%)	119 (48.8%)	102 (47.7%)	32 (55.2%)	
- *G3 (poor)*					
Type of resection	24 (60.0%)	167 (68.4%)	154 (72.0%)	47 (81.0%)	***0.06***
- *R0*	16 (40.0%)	77 (31.6%)	60 (28.0%)	11 (19.0%)	
- *R1 – R2*					
Number of positive lymph nodes*	1 (1 – 19)	2 (0 – 23)	2 (0 – 43)	1 (0 – 11)	0.14
Total number of harvested lymph nodes*	10 (3 – 45)	12 (1 – 56)	10 (1 – 43)	9 (2 – 31)	0.10
Lymph node ratio*	0.10	0.17	0.16	0.10	0.14

Values presented as median (range in brackets)

BMI = *body mass index*
SCC = squamous cell carcinoma
AC = *adenocarcinoma*
Lymph node ratio = *number of positive lymph nodes / total number of harvested lymph nodes*

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Long-term outcome

Median survival time was 20 months (range 0-193 months). Overall five-year survival was 27.9%, whereas disease-specific five-year survival was 33.4%. Disease recurrence was noted in 315 patients (56.7%): locoregional recurrence in 27.2% and distant metastases in 47.1% of patients. There was no significant difference in overall five-year survival between the different BMI classes (Figure 1: underweight 26.8%, normal weight 25.2%, overweight 28.5% and obesity 34.4%; p=0.25) or in disease-free five-year survival (28.8%, 32.5%, 33.2%, and 35.1%, respectively; p=0.60).

Parameters found to be associated with overall survival in univariate analyses are shown in Table 4. Age younger than 65 years, early pT-stage (pT1 or pT2), no lymph node involvement (pN0), absence of distant metastatic disease (pM0), lymph node ratio smaller than 0.17, good differentiation grade of the tumor, and R0-resection were favorable for improved survival.

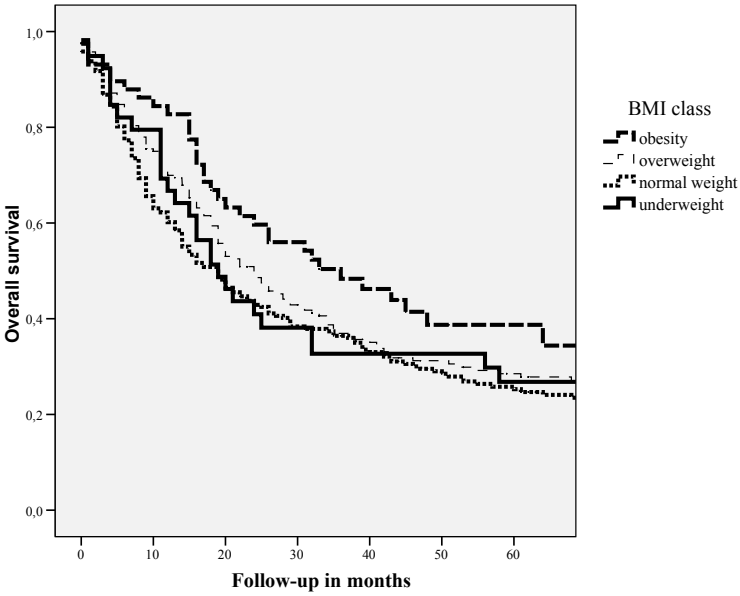

Figure 1. Overall five-year survival in relation to BMI classes in 556 patients who underwent surgical resection for esophageal cancer.

No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
Underweight	40	26	15	11	11	9
Normal weight	244	147	97	75	56	46
Overweight	214	138	89	59	47	40
Obesity	58	47	33	23	14	10

Table 4. Univariate analyses of variables associated with overall survival in esophageal cancer patients who underwent surgical resection.

VARIABLE	5-YEAR SURVIVAL	p-value
BMI class	26.8%	0.25
Underweight	25.2%	
Normal weight	28.5%	
Overweight	34.4%	
Obesity		
Gender	26.6%	0.50
Male	33.4%	
Female		
Age	33.7%	***<0.001***
< 65 years	22.3%	
≥ 65 years		
ASA classification	28.5%	0.11
I / II	25.9%	
III / IV		
Histology	26.0%	0.53
Squamous cell carcinoma	28.3%	
Adenocarcinoma		
pT-stage	55.5%	***<0.001***
pT1-2	14.5%	
pT3-4		
pN-stage	52.7%	***<0.001***
pN0	12.9%	
pN1		
pM-stage	32.3%	***<0.001***
pM0	8.1%	
pM1a / M1b		
Lymph node ratio	44.9%	***<0.001***
< 0.17	11.5%	
≥ 0.17		
Differentiation grade of tumor	70.2%	***<0.001***
Good	30.3%	
Moderate	19.1%	
Poor		
Radicality of resection	37.9%	***<0.001***
R0	4.8%	
R1 / R2		

BMI = *body mass index*
ASA classification = *American Society of Anesthesiologists classification*

Discussion

In this large cohort of patients who underwent esophagectomy for cancer, no differences in overall morbidity, in-hospital mortality, or reoperation rate were detected among the different BMI classes. However, severe complications seemed to occur more often in obese patients than in the control group. Furthermore, a linear association between BMI and anastomotic leakage was detected: the risk for anastomotic leakage increased with higher BMI.

Cancer cachexia has been recognized as a condition associated with an increased risk of postoperative complications[2], in particular infectious complications.[3] However, in the present series it appeared that underweight did not influence patient's short-term outcome after esophagectomy. When analyzing infectious complications in particular (esp. wound infection and pneumonia), incidences in underweight patients were similar to those in the control group.

Obese patients are also thought to have a higher risk for developing wound infections.[6, 12, 16, 17] This might be related to the presence of excessive fatty tissue (with a low regional oxygen tension), which may predispose to impaired wound healing. Furthermore, it has been shown that obese patients have an underlying immune impairment, which may further contribute to higher rates of wound infection.[18] Although other reports could not confirm this hypothesis[11, 19, 20], the risk for wound infection seemed to increase with higher BMI on trend-analysis in the present series. Furthermore, we found a linear association between BMI and anastomotic leakage: a higher incidence of anastomotic leakage was found with increasing BMI. In one study in which a significantly increased incidence of anastomotic leakage in obese patients was demonstrated, it was speculated that this might be due to a compromised vascularity of the conduit (because of an increased tension on the conduit in the thoracic compartment or because of medical comorbidities in general such as diabetes or cardiovascular disease).
In contrast to previous studies, we did not find differences in respiratory complications nor in laryngeal nerve injury between the BMI classes.[11, 20] This discrepancy with

the literature might be explained by a difference in the applied surgical technique. While the majority of our patients underwent a transhiatal esophagectomy, in the previously reported patient group a transthoracic technique was applied.[11] It is known that transthoracic esophagectomy is associated with more pulmonary complications compared with the transhiatal approach.[21]

In this study advanced pT-stage was seen significantly more often in patients with a low or normal BMI when compared to patients with a high BMI (p=0.02). This might be explained by the fact that overweight people experience more reflux symptoms, and are offered upper gastro-intestinal endoscopy more frequently, thereby facilitating an early detection of the esophageal tumor. Furthermore, in the underweight group patients with a squamous cell carcinoma were overrepresented, who would not have entered a Barrett's esophagus surveillance program. Indeed, patients with overweight or obesity were participating more often in a surveillance program than patients with a low or normal BMI (28.8% versus 17.1%), although this difference did not reach statistical significance (p=0.21). It is well-established that stage of disease and survival are more favorable for patients who have undergone endoscopic surveillance than for patients who have not participated in a surveillance program.[22, 23]

We hypothesized that a higher percentage of tumor-free circumferential resection margins might be achieved when performing esophagectomy for cancer in overweight or obese patients because of the presence of more substantial fatty tissue surrounding the tumor. Indeed, on trend-analysis, a significant linear association between BMI and R0-resection rate was found (p=0.02): 60.0% in underweight patients versus 81.0% in obese patients. However, pT-stage acted as a confounder for this relationship, as BMI was not an independent predictor for R0-resection on logistic regression. Therefore, our hypothesis that a higher percentage of tumor-free circumferential resection margins might be achieved in obese patients because of the presence of more fatty tissue surrounding the tumor could not be confirmed. Furthermore, higher BMI did not result in an improved long-term survival following resection and BMI class was not a predictor of survival after esophagectomy in univariate analysis.

Selection-bias can be a limitation of the present study. Only patients who have been considered fit enough for operation have entered this study. Therefore, severely malnourished patients or patients with severe obesity-associated medical comorbidites (including diabetes, hypertension and coronary artery disease) are more likely to have been excluded from surgery. This may have lead to a properly selected patient group, sufficiently fit to undergo esophagectomy, in whom no impact of either underweight or obesity on patient's short-term and long-term outcome could thus be demonstrated.

In summary, BMI is not of prognostic value with regard to short-term complications or long-term survival in patients undergoing esophagectomy for cancer. A radical R0-resection was achieved more often in patients with high BMI. However, this was not due to the presence of more substantial fatty tissue surrounding the tumor (potentially facilitating a radical resection). This observation should rather be explained by a confounding effect of pT-stage, as early pT-stage was seen more often in patients with high BMI. We conclude that patients who are oncologically eligible for esophagectomy should not be denied surgery on the basis of their BMI class only.

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11

Delay in diagnostic work-up and treatment of esophageal cancer

Brechtje A Grotenhuis
Pieter van Hagen
Bas PL Wijnhoven
Manon CW Spaander
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

Introduction: Esophageal cancer should preferably be detected and treated at an early stage, but this may be prohibited by late onset of symptoms and delays in referral, diagnostic work-up and treatment. The aim of this study was to investigate the impact of these delays on outcome in patients with esophageal cancer.

Methods: For 491 patients undergoing esophagectomy for cancer between 1991 and 2007, patients' short-term and long-term outcome were analyzed according to different time intervals between onset of symptoms, diagnosis and surgical treatment.

Results: Length of prehospital-delay (from onset of symptoms until endoscopic diagnosis) did not affect patient's short-term or long-term outcome. A shorter hospital-delay between establishing the diagnosis of esophageal cancer on endoscopy and surgery was associated with lower overall morbidity and in-hospital mortality. Patients of ASA-class I and II experienced a shorter hospital-delay than patients of ASA-class III and IV. Length of hospital-delay between endoscopic diagnosis and surgery did not affect pTNM-stage or R0-resection rate. Longer hospital-delay did not result in worse survival: overall survival after esophagectomy for cancer was not significantly different between patients with hospital-delay <5 weeks, 5-8 weeks or >8 weeks (24.7%, 21.7% and 32.3%, respectively; p=0.12).

Conclusion: A longer hospital-delay (between endoscopic diagnosis and surgery) resulted in worse patient's short-term outcome (higher overall morbidity and mortality rates), but not in a worse long-term outcome (overall survival). This may be explained by a more time-consuming diagnostic work-up in patients with a poorer physical status and not by tumor progression.

Introduction

The five-year survival rate for esophageal cancer patients after esophagectomy with curative intent has improved up to 40%.[1-3] As further improvement in survival from a single modality approach such as surgery is unlikely, considerable interest has grown in other strategies that may improve patients' survival (neoadjuvant chemo- and/or radiotherapy in particular). In many types of cancer, the prognosis of patients with small, localized tumors is better than with locally advanced or metastatic disease. Similar to other malignancies such as colorectal and breast cancer, the outcome of esophageal cancer is related to the pTNM-stage of the disease.[2, 4, 5] Therefore, detection and treatment of esophageal cancer at an early stage could also improve long-term survival.

Early detection of esophageal cancer may be prohibited not only by the late onset of symptoms, but also by delays in referral to an appropriate specialist, establishment of the diagnosis, further diagnostic work-up, and start of treatment. However, the impact of these delays on both short-term and long-term outcome for patients undergoing esophagectomy for cancer is unclear.

In patients with breast cancer, delays of 3-6 months between the onset of symptoms and start of treatment are associated with lower survival, caused by a more advanced tumor stage.[6] In two systematic reviews, no association was found between diagnostic and therapeutic delay and survival in colorectal cancer patients[7], nor between these delays and disease stage.[8] A few studies have investigated the impact of delays in diagnosis and treatment of esophageal cancer. Drawbacks of these studies are small numbers of patients included[9], analyses that do not cover the complete track between onset of symptoms and surgical treatment[10, 11], combined patient groups with gastric and esophageal carcinoma[12, 13], and studies lacking survival analyses.[9, 11-13]

We hypothesized that longer delays between onset of symptoms, endoscopic diagnosis and surgical treatment are associated with a worse short-term outcome (morbidity, reoperation rate and in-hospital mortality), worse tumor-stage and,

hence, worse long-term outcome (overall survival) following potentially curative esophagectomy in patients with esophageal cancer.

Patients and methods

The Erasmus Medical Center in Rotterdam is a tertiary referral center for patients with esophageal cancer in The Netherlands. Most patients are referred to the Erasmus MC outpatient clinic for (surgical) treatment after the diagnosis of esophageal cancer has been established in a referring hospital (group A). The minority of patients is directly referred by the general practitioner (GP) to the Erasmus MC for clinical investigations of symptoms suggestive of cancer (group B). In all patients (group A and B) upper gastro-intestinal endoscopy with biopsy is (re)done in the Erasmus MC to confirm the diagnosis of esophageal cancer and to determine the exact location of the tumor. Staging is performed routinely with endoscopic ultrasonography (EUS), CT scanning of thorax and abdomen and external ultrasound of the neck. Every patient is discussed in a weekly multidisciplinary oncology meeting in which a definitive treatment plan is designed. If eligible for surgery, patients are put on the waiting list for surgery. On the same day, the patient is referred to the Department of Anesthesiology for preoperative counseling. If needed, additional cardiac and/or pulmonary function tests are scheduled.

Between January 1991 and December 2007, 791 patients underwent esophagectomy for cancer of the esophagus or gastroesophageal junction in the Erasmus MC. To obtain a homogeneous cohort of patients in terms of treatment and to circumvent possible stage migration following chemo- and/or radiotherapy, patients receiving (neo) adjuvant therapy were excluded from this analysis. In our hospital, patients received neoadjuvant chemo(radio)therapy in the context of randomized controlled trials.[14, 15] Induction chemo- and/or radiotherapy was given in patients with either a cT4-tumor without distant metastases or in patients with gross involvement of celiac trunk lymph nodes (M1a), who were not considered eligible for primary surgical therapy. There were 214 patients who were excluded because of chemo- and/or radiotherapy prior to surgery. In 44 patients the hospital-delay from endoscopic diagnosis to surgery

could not be calculated, as the date of their first upper gastro-intestinal endoscopy performed in the referring hospital was unknown. Another 42 patients were excluded as they participated in a Barrett's esophagus surveillance program. Over recent years, multiple attempts for endoscopic treatment of early lesions delayed referral to the Department of Surgery in such way that this group was not representative for patients treated for (more advanced) esophageal cancer. Finally, data of 491 patients were analyzed in the present study. The vast majority of these patients underwent a transhiatal esophagectomy with locoregional lymphadenectomy only (N=477). In 14 patients a transthoracic resection with extended lymphadenectomy was performed. The applied surgical techniques have been described previously.[3, 16] Tumors were assigned pathologic tumor-node-metastasis stages according to the Union Internationale Contre le Cancer (UICC) 2002 system.[17]

Data on patients' demographics, diagnostic tests, surgery, postoperative morbidity, in-hospital mortality and survival have been collected prospectively and stored in a database by a data manager. From this database the following time points were defined:

- Date of upper gastro-intestinal endoscopy in the referring hospital, on which the diagnosis of esophageal cancer had been established by histology from biopsies (only applicable for group A);
- Date of first visit at the Erasmus MC outpatient clinic: Department of Surgery, Gastroenterology or Medical Oncology;
- Date of upper gastro-intestinal endoscopy in the Erasmus MC, on which the diagnosis of esophageal cancer had been established by histology from biopsies;
- Date of the multidisciplinary oncology meeting, after which the patient had been put on the operative waiting list if eligible for surgery;
- Date of surgery.

To summarize all different time points that have been marked in the process between onset of symptoms and surgery, we divided this time-span into two major time-intervals that have been analyzed separately: pre-hospital and hospital-delay (see

Figure 1). Subsequently, data were analyzed in three different ways:

- Impact of prehospital-delay: time from onset of symptoms until diagnosis on first endoscopy (either in the referring hospital for group A or in Erasmus MC for group B);
- Impact of hospital-delay: time from diagnosis on patient's first endoscopy undertaken until surgery;
- Impact of specific time intervals between diagnosis on first endoscopy and surgery. In order to examine the hospital-delay in more detail, the effect of specific time intervals between diagnosis in the referring hospital, first visit at the outpatient clinic in Erasmus MC, diagnosis on endoscopy in Erasmus MC, multidisciplinary oncology meeting and surgery on short-term and long-term outcome were analyzed. For this purpose, only data from patients in group A were used.

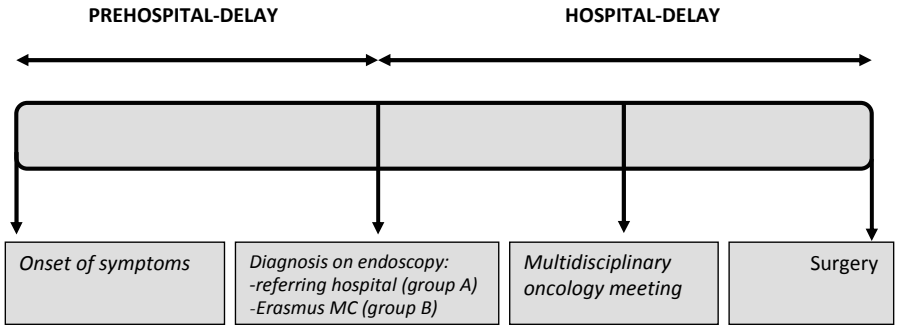


Figure 1. *Analysis of prehospital-delay and hospital-delay encountered by patients who underwent surgical resection for esophageal cancer in Erasmus MC.*

Statistics

Follow-up was recorded until December 2008 or until death if earlier, and was complete for all patients. Statistical analysis for non-parametric data was used. Grouped data were compared using the Chi-Square-, Mann-Whitney U- or Kruskall-Wallis H-test. Patients who died due to complications following esophagectomy (in-hospital mortality) were not excluded from survival-analysis. Overall survival was calculated from the date of operation until the date of last follow-up or death according to the

Kaplan-Meier method. Disease-free survival was assessed from the date of operation until the date of disease recurrence in case of locoregional recurrence or distant metastases. Univariate analyses were performed with the log-rank test to identify prognostic variables associated with overall survival after esophagectomy. Data-analyses were carried out with SPSS version 15.0 (SPSS, Chicago, IL, USA).

Results

Patients' characteristics are shown in Table 1. Three hundred and sixty-five patients (74.3%), in whom the diagnosis esophageal cancer was established in an other hospital, were referred to the Erasmus MC for further staging and treatment (group A). Hundred and twenty-six patients (25.7%) were referred directly to the Erasmus MC by the general practitioner for investigation of symptoms suggestive of esophageal cancer (group B). Patients' first visit to the Erasmus MC was at the Department of Surgery (N=338, 68.8%), Department of Gastroenterology (N=147, 29.9%) or Department of Medical Oncology (N=6, 1.3%).

Table 1. Clinicopathological characteristics of 491 patients who underwent surgical resection for esophageal cancer and who were included in the present study.

Age (in years)*	65 (28 – 89)
Gender	
- *Male*	399 (81.3%)
- *Female*	92 (18.7%)
ASA classification	
- *I*	77 (15.7%)
- *II*	316 (64.4%)
- *III*	96 (19.6%)
- *IV*	2 (0.4%)
Tumor location	
- *Proximal esophagus*	8 (1.6%)
- *Mid esophagus*	27 (5.5%)
- *Distal esophagus*	196 (39.9%)
- *Gastroesophageal junction*	260 (53.0%)
Histology	
- *Squamous cell carcinoma*	73 (14.9%)
- *Adenocarcinoma*	418 (85.1%)

Age is given as median (range)
ASA classification = American Society of Anesthesiologists classification

Impact of prehospital-delay: time from onset of symptoms until first endoscopy

The majority of patients underwent endoscopy for investigation of obstructive symptoms suggestive of cancer like dysphagia, odynophagia and weight loss (N=462, 94.1%). Other indications for endoscopy encompassed investigation of hematemesis (N=12, 2.4%), anemia (N=9, 1.8%), or melena (N=8, 1.6%). Prehospital-delay (from onset of symptoms until first endoscopy) lasted a median time period of 3.0 months (range 0 – 36 months). Patient's short-term (morbidity, reoperation rate and in-hospital mortality) and long-term outcome (overall five-year survival) after esophagectomy were comparable for patients who experienced symptoms for a period of 3 months or less versus more than 3 months until endoscopy was performed (Table 2).

Table 2. Impact of prehospital-delay from onset of symptoms to first endoscopy on short-term and long-term outcome after esophagectomy; comparison of prehospital-delay ≤ 3 months (N=308) versus > 3 months (N=183).

	Prehospital-delay ≤3 months N=308	Prehospital-delay >3 months N=183	p-value
Morbidity	199 (64.6%)	104 (56.8%)	0.09
Reoperation	34 (11.0%)	16 (8.7%)	0.42
In-hospital mortality	18 (5.8%)	9 (4.9%)	0.66
Overall five-year survival	24.0%	29.3%	0.10

Impact of hospital-delay: time from endoscopic diagnosis until surgery

The hospital-delay from establishing the diagnosis of esophageal cancer on endoscopy (either in the referring hospital for group A or in Erasmus MC for group B) until surgery was 49 days (given as median, range 5 – 175 days). This delay encompassed a median time period of 28 days (range 0 – 147 days) from diagnosis on patient's first endoscopy until the multidisciplinary oncology meeting (staging-delay), and a median time period of 15 days from this meeting until surgery (operative waiting list, range 1 – 67 days). Median hospital-delay between diagnosis and surgery increased during the study period (1991-2007): 3.9 weeks in 1991 towards 10.9 weeks in 2007 (Figure 2). This increase in hospital-delay should rather be ascribed to the 3.4 times increase in length of the operative waiting list (1.6 weeks in 1991 towards 5.6 weeks

in 2007) than to the 1.5 times increase in staging-delay (3.3 weeks in 1991 towards 4.9 weeks in 2007).

A shorter hospital-delay between establishing the diagnosis of esophageal cancer on patient's first endoscopy and surgery was associated with significantly lower overall morbidity and mortality (Table 3). These associations appeared to be linear: morbidity (p=0.001) and in-hospital mortality (p=0.01) increased with longer hospital-delay. Patients of ASA-class I and II experienced a shorter hospital-delay than patients of ASA-class III and IV (hospital-delay <5 weeks 28.8%, 5-8 weeks 36.9% and >8 weeks 34.4% versus < 5 weeks 15.3%, 5-8 weeks 41.8% and > 8 weeks 42.9%, respectively; p=0.02). Length of hospital-delay did not affect pTNM-stage or R0-resection rate (Table 3).

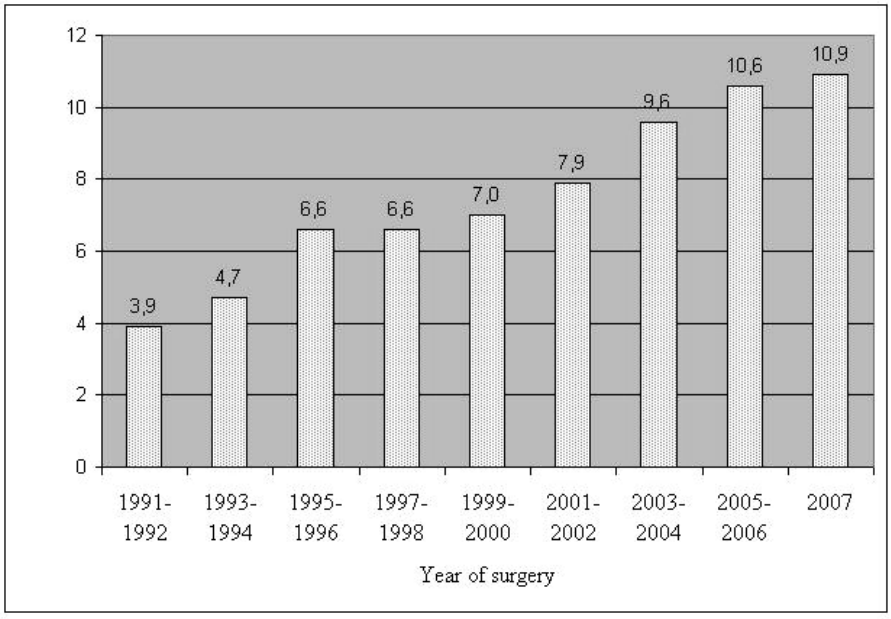
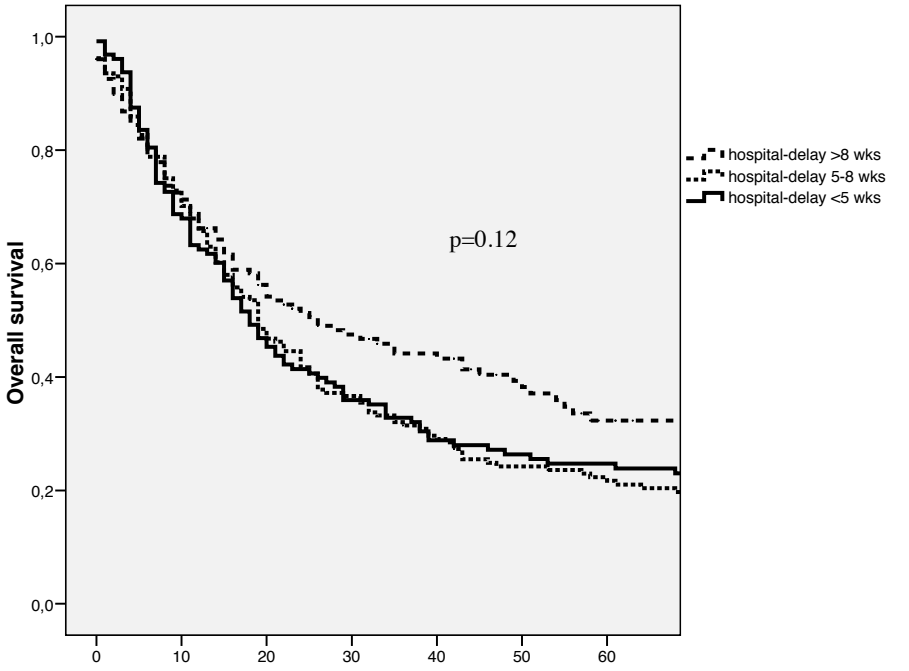


Figure 2. *Median hospital-delay (in weeks) between endoscopic diagnosis and surgery increased during the study period (1991-2007): 3.9 weeks in 1991 towards 10.9 weeks in 2007.*

Table 3. Impact of the hospital-delay from diagnosis on patient's first endoscopy until surgery: hospital-delay < 5 weeks (N=128), 5-8 weeks (N=186) and > 8 weeks (N=177).

	Delay <5 weeks N=128	Delay 5-8 weeks N=186	Delay >8 weeks N=177	p-value
Morbidity	62 (48.4%)	122 (65.6%)	119 (67.2%)	***<0.01***
In-hospital mortality	2 (1.6%)	10 (5.4%)	15 (8.5%)	***0.03***
Reoperation	7 (5.5%)	20 (10.8%)	23 (13.0%)	0.10
pT-stage	30 (23.4%)	57 (30.6%)	54 (30.5%)	0.31
- pT1-pT2	98 (76.6%)	129 (69.4%)	123 (69.5%)	
- pT3-pT4				
pN-stage	42 (32.8%)	66 (35.5%)	62 (35.0%)	0.88
- pN0	86 (67.2%)	120 (64.5%)	115 (65.0%)	
- pN1				
pM-stage	103 (80.5%)	150 (80.6%)	131 (74.0%)	0.24
- pM0	25 (19.5%)	36 (19.4%)	46 (26.0%)	
- pM1a-M1b				
Radicality of resection	86 (67.2%)	124 (66.7%)	130 (73.4%)	0.32
- R0	42 (32.8%)	62 (33.3%)	47 (26.6%)	
- R1-R2				

Longer hospital-delay did not result in worse survival (Figure 3): overall five-year survival was 24.7% in patients with a hospital-delay less than 5 weeks, 21.7% in patients with a hospital-delay between 5 and 8 weeks, and 32.3% in patients in whom the hospital-delay was more than 8 weeks. Although overall survival appeared to be longer in patients with a longer hospital-delay, this difference was not statistically significant (p=0.12). Parameters found to be associated with overall survival in univariate analyses are shown in Table 4: age younger than 65 years, early pT-stage (pT1 or pT2), no lymph node involvement (pN0), absence of distant metastatic disease (pM0), good differentiation grade of the tumor, R0-resection and lymph node ratio smaller than 0.24 were favorable of improved overall survival. Survival analysis with regard to five-year disease-free survival paralleled the overall five-year survival curves (27.0%, 27.7% and 38.3%, respectively; p=0.09).



No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
> 8 weeks	177	107	68	48	38	24
5 – 8 weeks	186	122	78	54	39	34
< 5 weeks	128	80	53	42	32	30

Figure 3. *Overall five-year survival for esophageal cancer patients appeared longer for patients with a hospital-delay between diagnosis on first endoscopy and surgery > 8 weeks (N=177) versus patients with a hospital-delay < 5 weeks (N=128) or 5 – 8 weeks (N=186), although this difference did not reach statistical significance (p=0.12).*

Table 4. Univariate analyses of potential prognostic variables associated with overall survival after esophagectomy for cancer (N=491).

VARIABLE	FIVE-YEAR SURVIAL	P-VALUE
Age		
≤ 65 years	30.2%	***0.001***
> 65 years	21.4%	
Sex		
Male	25.4%	0.84
Female	28.5%	
ASA classification		
I – II	27.0%	0.12
III – IV	22.2%	
pT-stage		
pT1 – T2	53.3%	***<0.001***
pT3 – T4	15.0%	
pN-stage		
pN0	50.3%	***<0.001***
pN1	12.2%	
pM-stage		
pM0	39.8%	***<0.001***
pM1a – M1b	9.5%	
Histology		
Squamous cell carcinoma	27.1%	0.98
Adenocarcinoma	25.8%	
Differentiation grade of tumor		
Good	69.1%	***<0.001***
Moderate	29.5%	
Poor	16.0%	
Radicality of resection		
R0	35.5%	***<0.001***
R1 – R2	5.5%	
Lymph node ratio		
≤ 0.24	36.0%	***<0.001***
> 0.24	12.0%	
Referral		
By another hospital (group A)	25.9%	0.65
By GP (group B)	26.2%	
Prehospital-delay		
≤ 3 months	24.0%	0.10
> 3 months	29.3%	
Hospital-delay		
<5 weeks	24.7%	0.12
5 – 8 weeks	21.7%	
>8 weeks	32.3%	

ASA classification = American Society of Anesthesiologists classification
GP = general practitioner

Impact of specific time intervals between endoscopic diagnosis and surgery (group A)
The median hospital-delay was 53 days (range 5 – 175 days) for patients in group A in whom the diagnosis esophageal cancer had been established in an other hospital and who were referred to the Erasmus MC for surgical treatment (N=365). The breakdown of this delay is shown in Table 5, according to the different time intervals between diagnosis in the referring hospital, first visit to the outpatient clinic in Erasmus MC, diagnosis on endoscopy in Erasmus MC, multidisciplinary oncology meeting and surgery.

Table 5. Delays encountered by esophageal cancer patients who have been referred from an other hospital to the Erasmus MC for surgical treatment (group A, N=365). Lengths of delays are given as a median values with the corresponding range in brackets.

Diagnosis on endoscopy elsewhere → First visit outpatient clinic Erasmus MC	17 days (1 – 138)
First visit outpatient clinic Erasmus MC → Diagnosis on endoscopy Erasmus MC	6 days (0 – 36)
Diagnosis on endoscopy Erasmus MC → Multidisciplinary oncology meeting	7 days (0 – 95)
Multidisciplinary oncology meeting → Surgery	15 days (1 – 67)
HOSPITAL-DELAY Diagnosis on endoscopy elsewhere → Surgery	53 days (5 – 175)

When analyzing the impact of the separate time intervals, it appeared that the delay between the multidisciplinary oncology meeting and surgery (median 15 days, reflecting the length of the operative waiting list) was the only time interval that influenced short-term outcome post-esophagectomy. Although in-hospital mortality was comparable between patients who had been on the waiting list for 15 days or shorter versus patients who were waiting for more than 15 days (p=0.14), length of the operative waiting list did influence morbidity (55.7% versus 67.1%, p=0.03) and a trend towards an increased reoperation rate could be noted (7.8% versus 13.9%, p=0.06). However, in contrast with the hospital-delay between endoscopic diagnosis and surgery, none of the separate time intervals affected long-term survival.

Discussion

When initiating the current study, we hypothesized that longer delays between onset of symptoms, diagnosis and surgical treatment are associated with worse short-term outcome (in terms of morbidity, reoperation rate and mortality) and worse long-term outcome (overall survival) following esophagectomy for cancer. In the present series, it appeared that length of prehospital-delay (from onset of symptoms until endoscopic diagnosis) did not influence patient's short-term outcome or overall five-year survival. Onset of symptoms is a subjective measurement, and it may be that patients are not able to recall the exact moment that they first experienced discomfort. Furthermore, although little information is known about the tumor doubling time of esophageal cancer, the period of time in which a patient is symptomatic may be relatively short when compared to the total period between the first presence of malignant cells in the esophagus and the diagnosis of esophageal cancer. Unfortunately we did not have information on delays caused by the GP (*i.e.* time between onset of symptoms and referral for endoscopy). Nevertheless, we do want to emphasize the importance of both patient and primary care education that will result in earlier notification of alarming symptoms such as dysphagia and weight loss.

A longer hospital-delay from endoscopic diagnosis until surgery was associated with higher overall morbidity and mortality. This could be explained by a more thorough and time-consuming diagnostic work-up in patients with a poorer physical status. Indeed, in the present study patients of ASA-class I and II experienced a shorter hospital-delay than patients of ASA-class III and IV. Alternatively, a longer delay prior to surgery may also have caused a worse physical status in esophageal cancer patients by means of malnutrition. However, this remains speculative, as our database did not provide detailed information with regard to patients' preoperative nutritional status (e.g. nutritional risk indices). When analyzing the impact of the separate time intervals between patient's first endoscopy and surgery, it appeared that the length of the operative waiting list was the time interval that influenced short-term outcome following esophagectomy the most. From the literature, it is also known that the quality of life in newly diagnosed esophageal cancer patients

who are waiting for surgery is seriously impaired.[18] Hence, it should be aimed for to keep this time-interval to a minimum.

Our second hypothesis was that patients with longer delays would generally present with more advanced disease and that this relation between delay and stage would result in a poorer survival. However, pTNM-stages were comparable in patients with a hospital-delay <5 weeks, 5–8 weeks or >8 weeks between endoscopy and surgery. Surprisingly, it appeared that overall survival was improved in patients with a longer hospital-delay, although this difference was not statistically significant. This is in line with the results of Kötz et al. who showed that a longer delay between diagnosis and surgical resection was associated with improved survival in esophageal cancer patients.[10] However, the delay between diagnosis and surgery was not an independent prognostic variable on multivariate analysis in their study. Kötz et al. noted that patients with a longer delay had a higher rate of complete tumor resection suggesting that they were more appropriately selected for surgical treatment.[10] In our series we could not find evidence that patients were selected more appropriately, as both pTNM-stage and R0-resection rate did not differ between patients with a shorter or longer hospital-delay. However, hospital-delay substantially increased especially over the last few years in our hospital (Figure 2). This can probably explain the counter-intuitive correlation between longer hospital-delay and improved long-term survival, which is rather reflecting state-of-the-art staging modalities, refined surgical techniques and improved intensive care that have been introduced over the past years. Theoretically, it could also be possible that in our hospital patients did not undergo surgery anymore after a longer hospital-delay in case the tumor progressed to a stage that was considered irresectable. However, in our patient group the increased hospital-delay can rather be ascribed to an increase in length of the operative waiting list than to an increased staging-delay. As the decision on whether to operate or not has been made during the multidisciplinary oncology meeting, it is unlikely that a longer hospital-delay led to a dropout of patients with irresectable tumors and, hence, a more selected patient group that underwent esophagectomy.

It is evident that efforts are taken to minimize delays experienced by patients with esophageal cancer between onset of symptoms, diagnosis and surgical treatment. The National Health Service cancer plan was implemented in 2000 in the UK, indicating that all patients with relevant symptoms and suspected cancer should be able to see a specialist within two weeks of their GP referral. The introduction of these guidelines was associated with reductions in times to first outpatient visit, endoscopy and diagnosis in patients with upper gastrointestinal cancer (esophageal or gastric).[19, 20] However, the effectiveness of the NHS cancer plan is uncertain, as it can be questioned whether the slightly improved survival rates after 2000 can be ascribed to this plan.[21]

In our hospital, we recently introduced a new schedule of diagnostic services for patients with suspected esophageal cancer. It is attempted to see patients at the outpatient clinic of the Department of Surgery or Department of Gastroenterology within one week after referral. Furthermore, patients are offered all imaging modalities in one week, including upper gastro-intestinal endoscopy, endoscopic ultrasonography, CT-scanning of thorax and abdomen and external ultrasound of the neck. Aim of this schedule is to minimize the delay between referral to our hospital and establishment of a definitive treatment plan for each individual patient.

In conclusion, length of prehospital-delay (from onset of symptoms until diagnosis) did not affect patient's short-term or long-term outcome. A longer hospital-delay (between endoscopic diagnosis and surgery) resulted in worse patient's short-term outcome (higher overall morbidity and mortality rates), but not in worse long-term outcome (overall survival). This may be explained by a more time-consuming diagnostic work-up in patients with a poorer physical status and not by tumor progression.

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12

Validation of a nomogram predicting the occurrence and severity of complications after esophagectomy for cancer

Brechtje A Grotenhuis
Pieter van Hagen
Johannes B Reitsma
Sjoerd M Lagarde
Bas PL Wijnhoven
Mark I van Berge Henegouwen
Hugo W Tilanus
J Jan B van Lanschot

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Abstract

Introduction: A nomogram has been developed recently in order to predict the occurrence and severity of postoperative complications after esophagectomy for cancer. In the present study, we externally validated this nomogram in a new cohort of patients who underwent esophagectomy for cancer in a different high-volume center.

Methods: An independent dataset of 777 patients who underwent esophagectomy for cancer was used for validation. The discriminatory capability of the nomogram was determined by using the concordance index (*c*-statistic). Calibration was evaluated by comparing the observed with the expected number of patients with complications, as predicted by the original nomogram across patients with different risk profiles. We also examined whether adjusting the value of the original coefficients of the predictors or adding new predictors would improve the fit of the nomogram.

Results: Discrimination of the original nomogram was similar in the validation cohort: the *c*-statistic hardly decreased from 0.65 in the original cohort to 0.64 in the validation cohort. Observed and expected number of patients with complications were in close agreement, reflecting a good calibration (p=0.84). Re-estimation of the coefficients in the validation cohort did not lead to any significant changes of the original nomogram values.

Conclusion: External validation of a nomogram predicting the occurrence and severity of complications after esophagectomy showed that the model is applicable in other high-volume hospitals. Nevertheless, preoperative prediction of complications in individual patients remains difficult, most likely due to the complexity of mechanisms causing these complications.

Introduction

Surgery is the primary curative therapy for patients with esophageal cancer. However, esophagectomy is associated with a high operative risk.[1, 2] Although operative mortality is below 5% in high volume centers[1, 3], esophageal resection is still accompanied by substantial morbidity. Early postoperative complication rates vary between 40% and 80%, depending on the applied criteria and on the extent of resection.[1, 4, 5] Complications can range from minor complications (*e.g.* urinary tract infection) to major complications (*e.g.* respiratory failure). Several previous studies focused on predisposing factors for complications after esophagectomy for cancer, but this did not result in reliable predictive models[6-10], except for the prediction of pulmonary complications.[11]

Predicting the severity of complications after esophagectomy may supply important information for both patient and surgeon. The impact of postoperative complications on quality of life plays an important role in decision making whether to proceed with an operation in an individual patient with esophageal cancer. Furthermore, the prediction of severity of complications in the preoperative phase may help in choosing the extent of the operation and in informing patients. Recently, a nomogram has been developed to predict the occurrence and severity of complications in esophagectomy patients.[12] A nomogram gives a graphical representation of the predictive strength of specific predictors and enables clinicians to calculate an overall risk score for individual patients reflecting their personal risk. In this nomogram the severity of complications was predicted at three levels: no complications, minor complications or severe complications. Specific patients' characteristics (*i.e.* more advanced age, myocardial infarction, stroke or transient ischemic attack in the medical history, lower forced expiratory volume in one second (FEV_1) and presence of electrocardiographic changes) and the application of more extensive surgery (*i.e.* transthoracic esophagectomy) were associated with a higher risk of (more severe) postoperative complications.

This new nomogram has been validated in a second group of patients within the same institution (temporal validation), which did not reveal any statistically significant changes in the predictive strength of the included prognostic factors. However, the power of that validation study was only moderate given the limited sample size of 100 patients[13], and validation within the same institute may not reveal inherent problems of a prognostic model that can become apparent when differences in patient populations, surgeons, applied surgical techniques and postoperative care between institutions are taken into account.[14] Therefore, the aim of the present study was to externally validate this nomogram in a new and large cohort of patients who underwent esophagectomy for cancer in a different high-volume center.

Patients and methods

Patients

Between January 1991 and September 2008, a consecutive series of 777 patients underwent a potentially curative esophagectomy for adenocarcinoma or squamous cell carcinoma of the esophagus or gastroesophageal junction (GEJ) in the Erasmus Medical Center, a tertiary referral center with wide experience in esophageal surgery. Patients who received neoadjuvant chemo(radio)therapy or induction chemotherapy were not excluded from the present study. In this hospital, patients mainly received neoadjuvant chemo(radio)therapy in the context of randomized controlled trials.[15, 16] Induction chemotherapy was given in patients with either a cT4-tumor without distant metastases or in patients with gross involvement of celiac trunk lymph nodes (M1a), who were not considered eligible for primary surgical therapy. Transhiatal esophagectomy (THE) was the preferred technique in this series of patients (N=744). A minority of patients (N=33) underwent transthoracic esophagectomy (TTE), mainly in the context of a randomized controlled trial.[1, 17] Surgical techniques have been described before.[1, 17] Clinicopathological data of all patients had been collected routinely in an ongoing registry.

The Medical Ethics Committees of the participating hospitals that provided the derivation cohort (Academic Medical Center, Amsterdam) and the validation cohort

(Erasmus Medical Center, Rotterdam) have approved the ongoing registry of data of esophageal cancer patients in prospective databases that are managed by dedicated data managers.

Definitions of complications

The severity of postoperative complications was graded according to the morbidity scale proposed by Dindo and colleagues.[18] This classification system is based on the therapeutic consequences of complications and consists of five grades and two subgrades. Grade I complications do not need any medical or surgical intervention (*e.g.* atelectasis, vocal cord paralysis, radiological anastomotic leakage). Grade II complications need pharmacologic treatment (*e.g.* pneumonia, chyle leakage, pulmonary embolus). Grade III complications need an intervention (grade IIIa: *e.g.* anastomotic leakage requiring drainage of the neck, pneumothorax or pleural empyema requiring drainage of the chest; grade IIIb: any reoperation). Grade IV complications are life threatening and represent single organ failure (grade IVa: *e.g.* pulmonary dysfunction requiring artificial ventilation, heart failure requiring intravenous inotropic agents, renal insufficiency requiring dialysis) or multiorgan failure (grade IVb). Finally, grade V complications are complications leading to death. Grading of complications was performed according to the most severe complication in each patient. Similar to the original study, complications were categorized into three groups: no complications, minor to moderate complications (grades I to IIIb) and severe complications (grades IVa, IVb, and V).

Statistical analysis

Clinicopathological characteristics of the derivation cohort (Academic Medical Center, Amsterdam) and the validation cohort (Erasmus Medical Center, Rotterdam) were analyzed in a descriptive way. Data on daily alcohol intake and presence of preoperative dyspnea were not available for the Rotterdam patients. In Rotterdam, weight loss was classified as a categorical value rather than a continuous variable (Amsterdam).

Ordinal logistic regression was used to examine the association between predictors and the occurrence of complications classified in three categories of severity (no complications, minor complications, severe complications). The ordinal logistic regression (proportional odds model) is an extension of the binary logistic regression model that is used in case of three or more outcome states that are naturally ordered.[19] Univariate analyses of potential predictors for the severity of complications were performed with the data set of the Rotterdam cohort. Because missing data result in loss of statistical power and can lead to possible bias, multiple imputation techniques were applied.[20] All predictors as well as the observed outcome were used to impute missing values based on multivariate normal distributions using the Markov chain Monte Carlo method. The coefficients of ten rounds of imputations were combined to obtain the final estimates of odds ratios and their 95% confidence intervals.

The nomogram predicting the occurrence and severity of complications previously developed in Amsterdam was validated in several ways on the Rotterdam patient cohort. First, discrimination was evaluated by calculating a risk score for each patient in the validation cohort based on the original coefficients from the derivation cohort. The discriminatory properties of the model were examined by visualizing the distribution and overlap in risk scores of individual patients within and between the three outcome categories. Furthermore, the discriminative capability was quantified by using the concordance *(c*) statistic. The *c*-statistic is a measure that can be interpreted as the probability among all possible pairs between patients from different outcome categories that the patient with the more severe complication also has the higher risk score. Values can range from 0.5 (due to chance, no discrimination) to 1.0 (perfect discrimination). We calculated *c*-statistics for the three categories (no complications versus minor complications, minor complications versus severe complications, no complications versus severe complications) as well as the overall *c*-statistic in the validation cohort. These data were compared with the original *c*-values in the derivation cohort.

Second, calibration was evaluated by comparing the expected and observed number of patients in each of the three outcome categories across seven-quantiles of

expected risk (*i.e.* seven groups of each 111 patients with an increasing mean risk score). Calibration was tested for significance by using an extension of the Hosmer-Lemeshow goodness-of-fit statistic.[21]

The third analysis examined whether the importance of the individual predictors or intercepts within the original nomogram was different in the validation cohort. For each predictor a proportional odds model was fitted with only that predictor using the risk score based on the original coefficients as an offset variable. Ideally, if the weight (importance) of the predictor is comparable between the validation cohort and the derivation cohort, the coefficient of each predictor would be zero as its weight is already incorporated in the risk score. The likelihood ratio test was used to indicate whether reweighing of the predictor will significantly improve the model. Similarly, potential predictors that did not significantly improve the prognostic performance of the original nomogram were re-evaluated in the derivation cohort whether they improved the prognostic performance of the model in the new cohort.

All analyses were performed using SAS software version 9.1 (SAS Institute Inc, Cary, NC, USA).

Results

Clinicopathological characteristics of the two study populations are shown in Table 1. The groups were comparable with regard to general patient characteristics such as age, sex, ASA-classification, BMI and comorbidities. More patients with a tumor located in the proximal or mid-esophagus underwent an esophagectomy in the Amsterdam group, which is also reflected in the higher proportion of patients who underwent an extended lymphadenectomy by means of a transthoracic esophagectomy (TTE). The median preoperative risk score as calculated by the original nomogram was significantly lower in Rotterdam than in Amsterdam (0.82 versus 1.07, $p<0.001$), indicating that the case mix with respect to the presence of predictors included in the nomogram was more favorable in Rotterdam.

Table 1. Clinicopathological characteristics and risk score of patients who underwent esophagectomy for cancer in Rotterdam (validation cohort) and Amsterdam (derivation cohort).

	ROTTERDAM validation cohort N=777	**AMSTERDAM derivation cohort N=663**
age*	64 (55–70)	64 (56-71)
sex	150 (19%)	154 (23%)
-female	627 (81%)	509 (77%)
-male		
ASA	117 (15%)	112 (17%)
-1	508 (65%)	404 (62%)
-2	147 (19%)	137 (21%)
-3	5 (1%)	3 (0%)
-4		
BMI*	24.7 (22.5-27.4)	24.5 (21.9-26.9)
smoking	278 (36%)	275 (42%)
history pulmonary disease	99 (13%)	52 (8%)
preoperative FVC*	4.11 (3.37-4.74)	4.19 (3.43-4.95)
preoperative FEV1*	2.89 (2.29-3.50)	3.07 (2.41-3.60)
serum creatinin*	79 (69-90)	75 (64-84)
history MI	98 (13%)	44 (7%)
history stroke/TIA	34 (4%)	22 (3%)
history hypertension	209 (27%)	140 (21%)
ECG changes	58 (8%)	54 (8%)
history DM	54 (7%)	39 (6%)
neoadjuvant therapy	221 (28%)	140 (21%)
location tumor	72 (9%)	150 (23%)
-proximal or mid-esophagus	705 (91%)	513 (77%)
-distal esophagus or GEJ		
type operation	33 (4%)	239 (36%)
-transthoracic esophagectomy	744 (96%)	424 (64%)
-transhiatal esophagectomy		
risk score* **	0.82 (0.51-1.16)	1.07 (0.71-1.58)

* Values presented as median, with 25th and 75th percentiles within brackets
** Preoperative risk score based on the original nomogram

BMI = body mass index
ASA = American Society of Anesthesiologists
FVC = forced vital capacity (lung function)
FEV1 = forced expiratory volume in one second (lung function)
MI = myocardial infarction
TIA = transient ischemic attack
ECG changes = q-waves and/or ST-T changes on electrocardiogram
DM = diabetes mellitus
GEJ = gastroesophageal junction

The frequency and severity of complications in the validation cohort (Rotterdam) and the derivation cohort (Amsterdam) are shown in Table 2. More patients in Rotterdam did not develop any complications postoperatively (40% Rotterdam versus 30% Amsterdam). In-hospital mortality was comparable (5% Rotterdam versus 4% Amsterdam).

Table 2. Frequency and severity of complications in the Rotterdam study population (validation cohort) versus the Amsterdam patients (derivation cohort).

	ROTTERDAM validation cohort N=777	**AMSTERDAM derivation cohort N=663**
complication category	N (%)	N (%)
no complications	307 (40%)	197 (30%)
minor complications	367 (47%)	354 (53%)
- grade I	*90 (11%)*	*133 (20%)*
- grade II	*211 (27%)*	*113 (17%)*
- grade IIIa	*26 (3%)*	*36 (5%)*
- grade IIIb	*42 (5%)*	*72 (11%)*
severe complications	103 (13%)	112 (17%)
- grade IVa	*47 (6%)*	*71 (11%)*
- grade IVb	*15 (2%)*	*17 (3%)*
- grade V	*41 (5%)*	*24 (4%)*

In the first validation step, we examined the discriminatory capability of the original risk score in the validation cohort. The distribution and overlap in risk scores of individual patients within and between the three outcome categories are shown in Figure 1. The mean risk scores (± SD) in the three complication categories were significantly different: 0.74 (±0.48) in patients without complications, 0.94 (±0.59) in patients with minor complications and 1.30 (±0.70) in patients with severe complications ($p<0.001$). However, there was a substantial overlap in scores between the three categories (Figure 1). This was also reflected in the pairwise *c*-statistics (overall measure of discriminatory capability): 0.61 for the discrimination between the group without complications versus patients with minor complications, 0.68

for the group with minor complications versus severe complications, and 0.77 for patients without complications versus severe complications. The overall *c*-statistic of the model in the Rotterdam validation cohort was 0.64, which was only marginally lower than the *c*-statistic (0.65) in the original Amsterdam derivation cohort[12], reflecting a moderate individual discriminatory capability of the model.

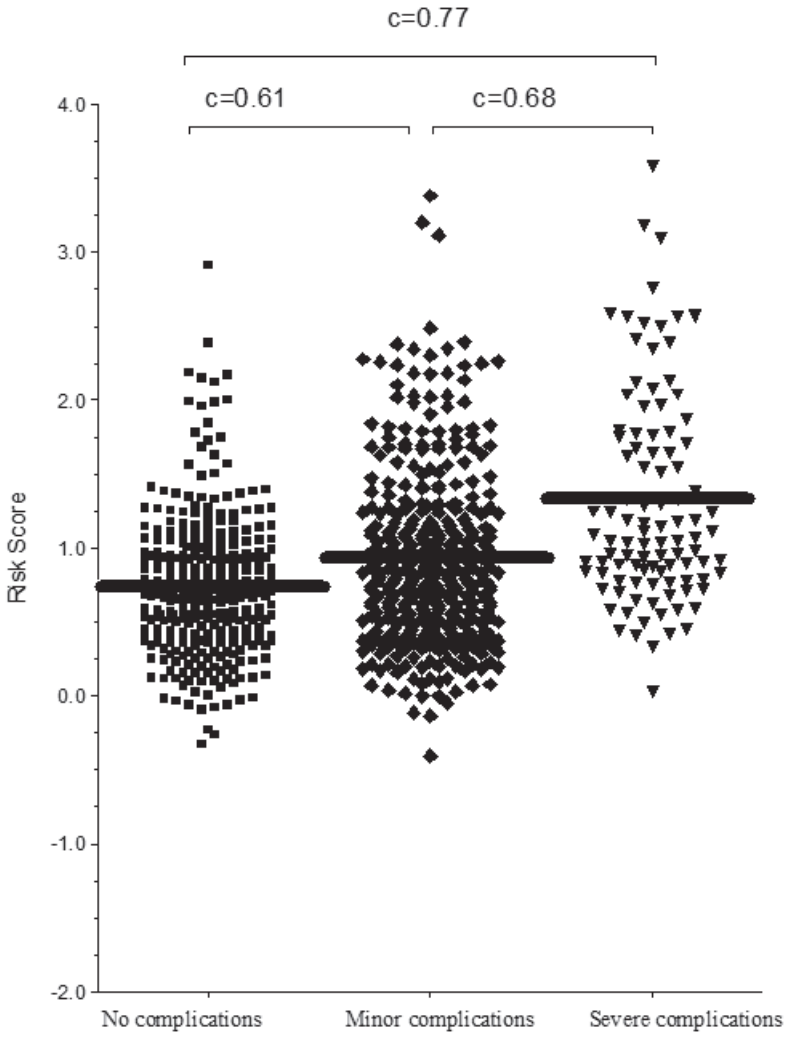

Figure 1. Distribution of risk score (as calculated with the original nomogram) in the patients of the validation cohort who experienced no complications, minor complications or severe complications.

Concordance *(c*) statistic = a measure that can be interpreted as the probability among all possible between patients from different outcome categories that the patient with the more severe complication also has the higher risk score.

Secondly, we evaluated the calibration of the nomogram score in the validation cohort *(i.e.* the closeness of predicted and observed frequency of outcomes). In Figure 2 the expected number of patients in each outcome category is depicted next to the observed number of patients in each category, across seven equally sized groups of 111 patients in the validation cohort ordered according to their risk score. In general, there was a tendency that complications in the validation cohort occurred less frequently or were less severe than expected (except for group 4 in which more severe complications were observed than expected). These discrepancies became smaller when the intercepts (*i.e.* background risk for an individual hospital) were re-estimated in the validation cohort while still using the original risk score (data not shown). The fit of the nomogram was evaluated by means of the goodness-of-fit test (p=0.84), which indicated that the differences between the probabilities predicted by the model and the actual probabilities were small and non-significant.
In the third validation step, we examined whether adjusting the original coefficients (weight) of the predictors would improve the fit of the nomogram model in the validation cohort. The outcome is shown in Table 3: only for the predictor age there was an indication (p=0.07) that a change in coefficient would improve the model. Furthermore, the optimal coefficients in the validation cohort did not significantly differ from the coefficients of the original nomogram model. Finally, predictors which were not incorporated in the original nomogram were re-evaluated in the derivation cohort in order to determine whether they might improve the prognostic performance of the nomogram in the new cohort. The addition of ASA classification (p=0.14), BMI (p=0.62), smoking (p=0.47), or application of neoadjuvant chemo(radio) therapy (p=0.17) to the existing model did not result in a significant improvement in predicting the occurrence and severity of postoperative complications in the validation cohort.

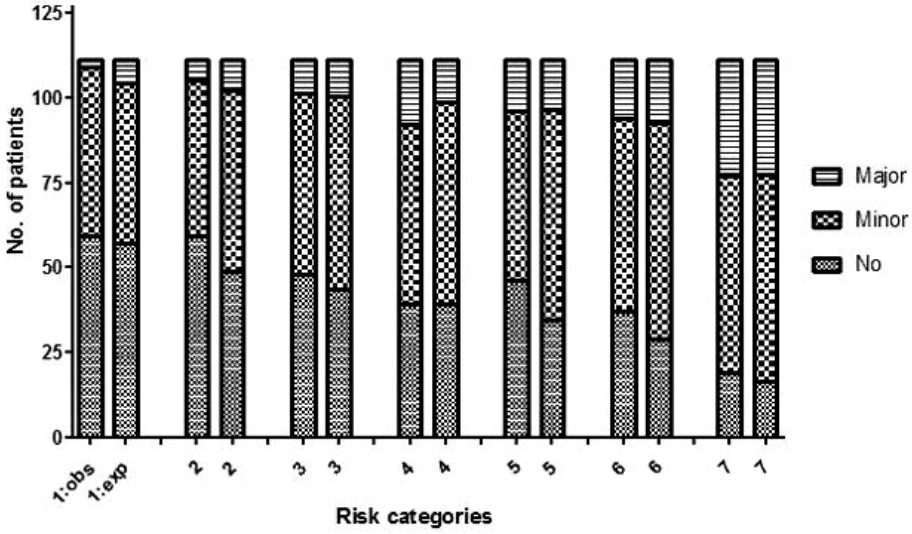

Figure 2. Expected (exp) versus observed (obs) number of patients in each of the three outcome categories (no complications, minor complications or severe complications) across seven-quantiles of increasing expected risk, with a p-value for goodness-of-fit of 0.84 (*i.e.* differences between observed and expected number of patients are non-significant).

Table 3. Comparison of the importance of each predictor in the validation cohort with that within the derivation cohort (original nomogram). Coefficients are presented as odds ratios with their 95% confidence intervals in brackets.

Predictor	validation coefficient	derivation coefficient	difference	p-value for difference
age (one year increment)	1.04 (1.02-1.05)	1.02 (1.00-1.04)	+0.02	0.07
history of MI	1.45 (0.95-2.22)	1.79 (0.96-3.32)	-0.34	0.33
history of stroke/TIA	2.59 (1.32-5.12)	3.06 (1.33-7.05)	-0.47	0.62
preoperative FEV1 (per l/s)	0.92 (0.76-1.11)	0.81 (0.67-0.98)	+0.11	0.20
ECG changes	1.63 (0.96-2.78)	2.16 (1.23-3.81)	-0.53	0.32
type of operation (TTE versus THE)	3.50 (1.78-6.90)	2.64 (1.91-3.66)	+0.86	0.42

MI = myocardial infarction
TIA = transient ischemic attack
FEV1 = forced expiratory volume in one second (lung function)
ECG changes = q-waves and/or ST-T changes on electrocardiogram
TTE = transthoracic esophagectomy
THE = transhiatal esophagectomy

Discussion

In the present study we externally validated a previously published nomogram predicting the occurrence and severity of complications in an independent cohort of patients who underwent esophagectomy for cancer in a different high-volume center.[12] This nomogram had been developed to assist surgeons in assessing preoperatively the risk of complications after esophagectomy. In the original nomogram, there was a considerable overlap in nomogram scores between patients with no, minor or severe complications (*i.e.* moderate discriminative capability), despite the identification of several factors associated with postoperative complications.[12] It was concluded that the complication nomogram could be of value if an adequate performance of the model would be examined in other settings (hospitals) with possible differences in case mix, surgical procedures and perioperative care.

The clinicopathological characteristics of patients undergoing esophagectomy for cancer in the validation cohort in Rotterdam and the derivarion cohort in Amsterdam were comparable with regard to potential prognostic factors such as age, ASA classification, BMI and comorbidities (Table 1). However, the median preoperative risk score as calculated by the original nomogram was significantly lower in Rotterdam than in Amsterdam. Apparently, the preoperatively determined risk factors were more prevalent in Amsterdam. An other explanation could be the fact that a TTE was performed more often in the original cohort (36% Amsterdam versus 4% Rotterdam). The weight or importance of the predictor operation technique in the validation cohort did not significantly differ from that in the original nomogram, but it can be questioned whether the power of this parameter in the validation cohort was sufficient to evaluate this issue adequately. Overall, no large differences in grades of complications impeded an adequate evaluation of the two patient cohorts in this study (Table 2), although the comparison of occurrence and severity of complications between hospitals is subject to bias because of variable definitions of complications and different scoring systems.

When analyzing the discriminatory capability of the original risk score in the validation cohort, it appeared that the mean risk scores in the three outcome categories were significantly different, but a substantial overlap in scores between the three categories was noted (Figure 1). This was also reflected in the pairwise *c*-statistics between the three complication categories, and the overall moderate discriminative power of the model (0.64 in the validation cohort, 0.65 in the derivation cohort). Apparently, patient related factors are not the only determinators responsible for developing complications after esophageal cancer surgery. The intraoperative course (*e.g.* fluid management by the anesthetist[22, 23] and surgical complications resulting in a longer operative time or more blood loss[2]) as well as the early postoperative phase (*e.g.* application and effectiveness of epidural analgesia[24-26]) will also influence the final outcome of patients who undergo esophagectomy for cancer. However, these factors cannot be predicted in a preoperative risk assessment.

Nevertheless, the overall fit of the model and the calibration of the nomogram score in the validation cohort *(i.e.* the closeness of predicted and observed frequency of outcomes) were good: the predictions for groups of patients with a similar risk profiles matched the observed probabilities (Figure 2). The expected frequency of patients with no or minor complications was higher than expected on basis of the nomogram, while these differences improved after adjusting the intercept (*i.e.* background risk for the new cohort) suggesting that the general level of complications was lower in the validation cohort. One could hypothesize that this may reflect differences in postoperative care between the two hospitals (*e.g.* more specialized ICU-care and faster detection of complications, preventing an increase in complication grade). On the other hand it can be questioned whether the complication registration in the validation cohort was as efficient and complete as in the derivation cohort.

Despite the complexity of mechanisms that can lead to the development of complications (preoperative, intraoperative and early postoperative factors as well as intrinsic (patient-related) and extrinsic (hospital-related) elements) and the subjectivity in the registration of complications, the optimal coefficients of the prognostic factors in the validation cohort did not significantly differ from

the coefficients of the original nomogram model. This indicates that the current nomogram is applicable in other high-volume hospitals performing esophagectomies for cancer. The model can give a preoperatively estimated risk of the occurrence and severity of postoperative complications. Furthermore, as we have shown that adequate model performance can be achieved, this model can be used to adjust for case-mix when comparing hospital performances: the nomogram can play a role in the risk-adjusted audit of morbidity after esophagectomy for cancer.

In conclusion, a recently developed nomogram predicting the occurrence and severity of complications was externally validated in a new cohort of patients who underwent esophagectomy for cancer in a different high-volume center. The model showed good overall calibration when applied in the validation cohort. Re-estimating the coefficients of the prognostic factors within the nomogram in the validation cohort did not reveal significant improvement compared to the original values. Nevertheless, preoperative prediction of complications in individual patients remains difficult, most likely due to the complexity of mechanisms causing these complications.

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Surgical management of submucosal esophageal cancer: extended or regional lymphadenectomy?

Brechtje A Grotenhuis
Mark van Heijl
Joerg Zehetner
Johnny Moons
Bas PL Wijnhoven
Mark I van Berge Henegouwen
Hugo W Tilanus
Tom R DeMeester
Toni Lerut
J Jan B van Lanschot

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Abstract

Introduction: Radical esophagectomy is considered the standard therapy for tumors that infiltrate the submucosa of the esophagus (T1b) as the prevalence of lymph node metastases has been reported in up to 40% of these patients. It remains unclear whether a radical esophagectomy with extended lymphadenectomy is needed or whether a surgical procedure with only regional lymphadenectomy suffices. The aim of this study was to compare outcome of patients who underwent esophagectomy for T1b cancer through a transthoracic approach with extended lymphadenectomy (TTE), with that of patients in whom a transhiatal esophagectomy (THE) was performed with a regional lymph node dissection.

Methods: Patients who underwent esophagectomy for T1b cancer between 1990 and 2004 and who did not receive (neo) adjuvant therapy were included. Data were collected from prospective databases of four centers. In Leuven, Belgium (n=101) and Los Angeles, USA (n=31), patients with T1b-tumors had been operated via TTE with extended lymphadenectomy while in Amsterdam (n=43) and Rotterdam (n=47), The Netherlands, THE with regional lymphadenectomy had been performed.

Results: The two patient groups (TTE, n=132; THE, n=90) were comparable with regard to age, BMI and ASA classification. Operative time was longer in patients who underwent TTE (390 minutes) versus THE (250 minutes; $p<0.001$). The yield of lymph nodes resected was higher in the TTE group (median 32) versus THE (median 10); $p<0.001$. Overall morbidity, in-hospital mortality and length of hospital stay were comparable between both groups. In the TTE-group 27.3% of complications were classified as major versus 14.4% in the THE-group ($p<0.001$) however the reoperation rate was higher after THE (12.2%) versus TTE (3.8%, $p=0.01$). There was no difference in pathological outcome (infiltration depth, pN-stage, pM-stage, positive lymph node ratio) between both groups. Overall five-year survival (63.4% TTE versus 69.4% THE, $p=0.55$) and disease-free five-year survival (76.9% TTE versus 78.3% THE, $p=0.65$) were comparable between both groups. In patients with N1 disease, disease-free five-year survival was 49.8% in the TTE-group versus 40.0% in the THE-group ($p=0.57$).

Conclusions: In patients with submucosal esophageal cancer (T1b), TTE with extended lymphadenectomy and THE with regional lymphadenectomy had similar short-term outcome and long-term survival. In the selected group of T1bN1 patients TTE may be the preferred operative technique due to a potential disease-free survival benefit; in patients with T1bN0 disease THE with en bloc dissection of the esophagus and regional lymph nodes offers an oncologically safe and less invasive treatment.

Introduction

The management of early esophageal cancer is controversial. Current treatment options range from endoscopic organ preserving mucosal resection or limited surgical resection with or without regional lymphadenectomy to radical esophagectomy with extended lymphadenectomy.[1-3] Complete macroscopic and microscopic tumor resection with an adequate tumor free margin and removal of all potentially involved lymph nodes are known to be key factors for cure. It would therefore be helpful to have greater insight into the relationships between the stage of disease, the extent of the surgical procedure and outcome.

Lymph node metastases are very rare in patients with esophogeal carcinoma limited to the mucosa.[4-7] In contrast, the prevalence of lymph node metastases in patients with submucosal tumor infiltration (T1b), is 20-25% in adenocarcinomas and can be up to 40% in squamous cell carcinomas.[2, 4-6, 8-10] Therefore, endoscopic treatment options are reserved for patients with strictly mucosal disease. A surgical esophagectomy is still considered the standard therapy for tumors that extend into the submucosa.[3, 11, 12]

At present, it is unclear whether an esophagectomy with extended lymphadenectomy is needed or if a surgical procedure with regional lymphadenectomy may suffice in order to obtain optimal outcome for patients with T1b esophageal cancer.[13] Extended lymphadenectomy is believed to prolong the recurrence-free period, but it has the major disadvantages of being more invasive with concomitant higher morbidity.[14, 15] On the other hand, the five-year recurrence-free survival of patients with early tumors and lymph nodal invasion after a transhiatal resection with limited regional lymphadenectomy has been reported to be as low as 35%.[4, 9] Thus a more extended lymphadenectomy by means of a transthoracic approach might be justified, if it leads to a higher chance for cure or a prolonged disease free survival notwithstanding a potentially higher surgical complication rate.

The aim of the present study was to compare short-term and long-term outcomes of patients who underwent esophagectomy for cancer with submucosal tumor infiltration (T1b) through a transthoracic approach with extended lymphadenectomy, to those of patients in whom a transhiatal esophagectomy was performed with only regional lymph node dissection. To address this issue, data were collected from four large expert centers, where patients with submucosal esophageal cancer had been operated predominantly either through the transthoracic route (Leuven, Los Angeles) or through the transhiatal route (Amsterdam, Rotterdam).

Patients and methods

Patients

Patients with squamous cell carcinoma or adenocarcinoma of the esophagus or gastroesophageal junction with infiltration into the submucosa were included. Only patients who did not receive (neo) adjuvant chemo- and/or radiotherapy and of whom complete five-year follow-up data were available were included. Patients with submucosal cancer of the cervical or proximal thoracic esophagus were excluded, as a transhiatal resection is generally considered inappropriate under these circumstances. Staging routinely included CT scanning of thorax and abdomen and external ultrasound of the neck. Endoscopic ultrasonography (EUS) was incorporated in staging as it became available in the four centers.

In total, 248 patients met the inclusion criteria in four centers (Leuven – Belgium, Los Angeles – USA, Amsterdam and Rotterdam – The Netherlands). Over a 14-year period (May 1990 – June 2004), these patients underwent transthoracic esophagectomy (TTE) or transhiatal esophagectomy (THE) for cancer limited to the submucosa (T1b) and located below the carina. TTE was the preferred operation technique in Leuven (TTE, n=101) and Los Angeles (TTE, n=31; THE, n=15), whereas THE was preferred in Amsterdam (THE, n=43; TTE, n=9) and Rotterdam (THE, n=47; TTE, n=2). To avoid selection bias (especially in patients who underwent THE in Los Angeles because of severe co-morbidities that precluded a thoracotomy), only patients who underwent TTE in Leuven (n=101) or Los Angeles (n=31) were included. In Amsterdam and

Rotterdam a few patients underwent TTE in the context of a randomized controlled trial (n=11), in which the outcome of patients with adenocarcinoma of the esophagus or gastroesophageal junction who underwent extended transthoracic esophagectomy was being compared to the outcome of those in whom limited transhiatal esophagectomy was performed.[15, 16] To minimize the number of hospitals per treatment arm (TTE versus THE), only patients who underwent THE in Amsterdam (n=43) and Rotterdam (n=47) were included in the present study. Thus, it was possible to compare short- and long-term outcome for patients with T1b esophageal cancer who had been operated upon by either TTE (n=132) or THE (n=90).

Surgery

A thoracotomy was the first step in a transthoracic esophagectomy with extended lymphadenectomy (n=132). The surgical techniques used in Leuven (left thoraco-abdominal approach in 87 patients and right posterolateral thoracotomy in 14 patients) and in Los Angeles (right posterolateral thoracotomy) have been described previously.[17-20] The thoracic duct, ipsilateral pleura, and all periesophageal tissue in the posterior mediastinum were dissected *en bloc*. The resection specimen included the lower and middle mediastinal, subcarinal, and paratracheal lymph nodes. The aortopulmonary-window nodes were dissected separately. Through a midline laparotomy or left-sided thoracotomy with peripheral phrenotomy, the paracardial, lesser curvature, left-gastric-artery, celiac trunk, common-hepatic-artery, and splenic-artery nodes were dissected, and a gastric tube was constructed. The cervical phase of the transthoracic procedure was identical to the transhiatal procedure, unless a formal cervical lymphadenectomy was performed (three-field lymphadenectomy).[17] The surgical technique for THE (N=90) performed in Amsterdam as well as in Rotterdam has been described previously.[15, 16] In short, the primary tumor and its adjacent lymph nodes were dissected *en bloc* under direct vision through the widened hiatus of the diaphragm up to the level of the inferior pulmonary vein. Subsequently, a gastric tube was created. The left gastric artery was transected at its origin, with resection of celiac trunk lymph nodes. After mobilization and transection of the cervical esophagus, the intrathoracic esophagus (up to the level of the inferior pulmonary vein) was mobilized with a vein stripper. Esophagogastrostomy was performed in the neck, without a formal cervical lymphadenectomy.

Tumors were assigned pathologic tumor-node-metastasis stages according to the tumor–node–metastasis (TNM) cancer staging system of the American Joint Committee on Cancer and the International Union for Cancer Control.[21] Based on the depth of invasion, submucosal lesions were classified by expert pathologists as sm1 for tumors invading the more superficial layer of the submucosa (corresponding to one-third of its thickness), sm2 for those invading the middle-third, and sm3 for those invading the deeper submucosal layer.[22]

Data collection

Data in all four centers were collected prospectively.. Severity of complications was scored according to Dindo et al.[23] Retrospectively, complications were classified as minor (grade 1, 2, 3a) or major (grade 3b, 4a, 4b, 5). Patient outcome was recorded until April 2009 or until death if earlier, ensuring a follow-up of at least five years. Disease recurrence was primarily suspected on clinical grounds, which lead to further investigations. Recurrence was defined as locoregional or distant metastatic disease when radiologically or pathologically proven.

Statistics

Data-analysis was carried out with SPSS version 15.0 (SPSS, Chicago, IL, USA). Statistical analysis appropriate for non-parametric data was used. Grouped data were compared using the Chi-Square, Mann-Whitney U or Kruskall-Wallis H test when appropriate. Overall survival was calculated from the date of operation until the date of last follow-up or death according to the Kaplan-Meier method. Patients who died due to complications following esophagectomy (in-hospital mortality) as well as deaths from all causes during follow-up were not excluded from survival-analysis. The primary endpoint in the present study was disease-free survival, which was assessed from the date of operation until the date of disease recurrence in case of locoregional recurrence or distant metastases; patients were censored at the time of their last visit or when they died of non-disease related causes without a previous relapse. Univariate analyses were performed to identify prognostic variables associated with disease-free survival after esophagectomy. Variables achieving a probability of less than 0.10 in univariate analysis were introduced in a multivariate

proportional-hazard analysis (Cox model) to identify those variables independently associated with disease-free survival. Results are given as hazard ratios (HR) or relative risks with their 95% confidence interval (CI).

Results

Patients' characteristics are shown in Table 1. The two groups were comparable with regard to age, BMI, ASA-classification, but there were significantly more females in the TTE-group. Early cancer was predicted correctly more often in the TTE-group (cT1-stage 45.5% versus 33.3% in the THE-group, p=0.01); preoperative lymph node status was comparable between both groups. In patients operated through the transthoracic route, more squamous cell carcinomas were seen (p<0.001) and more tumors were located in the mid-esophagus than in patients who underwent THE.

Operative time was significantly longer in the TTE-group (median 390 minutes, range 240-780) when compared to that in the THE-group (median 250 minutes, range 141-468; p<0.001). Intraoperative complications occurred in eleven patients (7.9%) in the TTE-group (myocardial infarction or splenectomy), and in four patients (4.4%) in the THE-group (ventricular fibrillation or splenectomy; p=0.25). Median length of hospital stay did not differ between both groups (16 days in both groups, p=0.26).

Postoperative short-term outcome is shown in Table 2. Overall morbidity was comparable for both groups (TTE 45.5% versus THE 55.6%; p=0.14). However, when the grade of complications was taken into account, it appeared that more complications were classified as major in patients operated through the transthoracic route (27.3% versus THE 14.4%; p<0.001) (Table 2) Vocal cord paralysis occured more often in the THE-group, whereas more patients in the TTE-group required prolonged ventilation or re-intubation due to respiratory insufficiency. Reoperation rate was higher for patients in whom a limited lymphadenectomy was performed (TTE 3.8% versus THE 12.2%; p=0.01). In-hospital mortality was comparable for both groups (TTE 4.5% versus THE 4.4%, p=0.97).

Table 1. Clinical characteristics of 222 patients who underwent primary surgical resection by TTE or THE for esophageal cancer limited to the submucosa.

	TTE (N=132)	THE (N=90)	p-value
Age*	63 years (37-85)	66 years (40-83)	0.29
Gender	100 (75.8%)	79 (87.8%)	***0.03***
- *Male*	32 (24.2%)	11 (12.2%)	
- *Female*			
Body Mass Index*	25.4 kg/m^2 (16.7-45.4)	25.4 kg/m^2(17.2-37.3)	0.55
ASA classification	86 (65.2%)	66 (73.3%)	0.49
- *I/II*	37 (28.0%)	19 (21.1%)	
- *III/IV*	9 (6.8%)	5 (5.6%)	
- *Unknown*			
Preoperative T-stage**	12 (9.1%)	15 (16.7%)	***0.01***
- *cTx*	60 (45.5%)	30 (33.3%)	
- *cT1*	60 (45.4%)	45 (50.0%)	
- *cT2-3*			
Preoperative N-stage**	95 (72.0%)	73 (81.1%)	0.12
- *cN0*	37 (28.0%)	17 (18.9%)	
- *cN1*			
Histology	46 (34.8%)	12 (13.3%)	***<0.001***
-*Squamous cell carcinoma*	86 (65.2%)	78 (86.7%)	
-*Adenocarcinoma*			
Tumor location	33 (25.0%)		***<0.001***
- *Mid-esophagus*	75 (56.8%)	8 (8.9%)	
- *Distal esophagus*	24 (18.2%)	47 (52.2%)	
- *GE-junction*		35 (38.9%)	
Barrett's metaplasia	74 (56.1%)	60 (66.7%)	0.11
- *Yes*	58 (43.9%)	30 (33.3%)	
- *No*			

* *Values presented as median (range in brackets)*

** *Preoperative T- and N-stage as determined by endoscopic ultrasound. In case the endoscopic ultrasound had not been incorporated in the staging procedure, the tumor was diagnosed as cTx and its N-stage was determined by CT-scanning.*

ASA classification = American Society of Anesthesiologists classification
GE-junction = gastroesophageal junction

Table 2. Postoperative complications and in-hospital mortality in relation to operation technique (TTE versus THE) in 222 patients who underwent primary surgical resection for submucosal esophageal cancer.

	TTE (N=132)	THE (N=90)	p-value
Overall morbidity	60 (45.5%)	50 (55.6%)	0.14
Severity of complications*		37 (41.1%)	
- *Minor*	24 (18.2%)	13 (14.4%)	***<0.001***
- *Major*	36 (27.3%)		
Surgical complications	2 (1.5%)	2 (2.2%)	0.70
- *Bleeding*	1 (0.8%)	2 (2.2%)	0.35
- *Chyle leakage*	11 (8.3%)	12 (13.3%)	0.23
- *Anastomotic leakage*	2 (1.5%)	9 (10.0%)	***<0.01***
- *Vocal cord paralysis*	8 (6.1%)	5 (5.6%)	0.88
- *Wound infection*			
Non-surgical complications	4 (3.0%)	2 (2.2%)	0.72
- *Sepsis*	37 (28.0%)	21 (23.3%)	0.43
- *Pneumonia*	23 (17.4%)	5 (5.6%)	***0.01***
- *Respiratory insufficiency***	11 (8.3%)	4 (4.4%)	0.26
- *Pleural effusion requiring drainage*	2 (1.5%)	3 (3.3%)	0.37
- *Myocardial infarction*	10 (7.6%)	2 (2.2%)	0.08
- *Thromboembolism*			
Re-operation	5 (3.8%)	11 (12.2%)	***0.01***
In-hospital mortality	6 (4.5%)	4 (4.4%)	0.97
Median length of hospital stay (range)	16 days (9 – 132)	16 days (1 – 171)	0.26

Severity of complications as determined by Dindo[23]:
Minor complications (grade 1, 2, 3a, 3b) versus major complications (grade 4a, 4b, 5)

*** Respiratory insufficiency was defined as pulmonary dysfunction requiring prolonged ventilation (>10 days) or re-intubation.**

Histopathological assessment (Table 3) showed no differences in pT-stage (tumor infiltration depth) or pN-stage (lymph node involvement) between both groups, despite a higher number of harvested lymph nodes in the TTE-group (mean 32 lymph nodes versus 10 lymph nodes in the THE-group, p<0.001). Lymph nodal status was related to infiltration depth of the tumor: in 9.9% of sm1-patients positive lymph nodes were found, while 28.8% of sm2 patients and 45.2% of sm3 patients were diagnosed with N1-status. Histology type did not influence pN-stage: 24.1% of patients with squamous cell carcinoma were lymph node positive versus 25.6% of patients with adenocarcinoma.

Table 3. Histopathological assessment of the resection specimens in relation to operation technique (TTE versus THE) in 222 patients who underwent primary surgical resection for submucosal esophageal cancer.

	TTE (N=132)	THE (N=90)	p-value
pT-status			
- *sm1*	64 (48.5%)	37 (41.1%)	
- *sm2*	25 (18.9%)	27 (30.0%)	0.19
- *sm3*	36 (27.3%)	26 (28.9%)	
- *unspecified*	7 (5.3%)	0 (0.0%)	
pN-status			
- *N0*	97 (73.5%)	69 (76.7%)	0.59
- *N1*	35 (26.5%)	21 (23.3%)	
pM-status			
- *M0*	122 (92.4%)	86 (95.6%)	0.35
- *M1*	10 (7.6%)	4 (4.4%)	
Differentiation grade			
- *G1 (good)*	13 (19.8%)	13 (14.4%)	0.10
- *G2 (moderate)*	60 (45.4%)	49 (54.4%)	
- *G3 (poor)*	59 (44.7%)	28 (31.1%)	
Type resection			
- *R0*	132 (100.0%)	88 (97.8%)	0.09
- *R1*	0 (0.0%)	2 (2.2%)	
Number positive lymph nodes*	1 (0 – 31)	1 (0 – 8)	0.40
Total number harvested lymph nodes*	32 (1 – 104)	10 (1 – 28)	***<0.001***
Lymph node ratio*	0.04 (0 – 1.00)	0.06 (0 – 0.60)	0.92

*****Values presented as mean (range in brackets)

Lymph node ratio = number of positive nodes / total number of harvested nodes

Ten patients who underwent an extended lymphadenectomy were classified postoperatively as M1 because of positive lymph nodes in the cervical (N=9) and/ or lower abdominal lymph node stations below the superior mesenteric artery (N=4). Location of positive lymph nodes in relation to histology type and operation technique is shown in Table 4. No patients with squamous cell carcinoma who underwent THE showed positive lymph nodes. In 12 of 35 (34%) patients with N1 disease who underwent TTE with extended lymphadenectomy, positive lymph nodes were located high in the mediastinum (6 adenocarcinomas and 6 squamous cell carcinomas). Five of these 12 patients were initially staged as cN0 and seven as cN1.

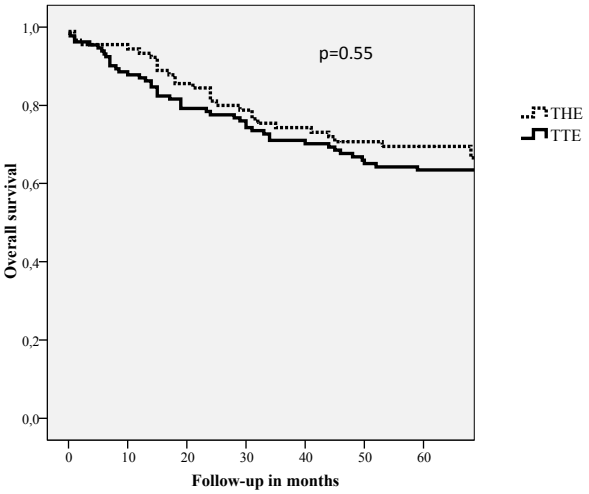
Table 4. Location of positive lymph nodes in 56 patients diagnosed with pN1-stage, in relation to histology and operation technique (patients can have positive nodes in more than one location).

		Cervical	Thoracic	Abdominal-regional	Abdominal-distant
Squamous cell carcinoma					
	TTE (N=14)	6	7	8	2
	THE (N=0)	0	0	0	0
Adenocarcinoma					
	TTE (N=21)	3	9	15	2
	THE (N=21)	0	0	21	0

Median survival time was 124 months (range 0-209 months). For both groups combined, overall and disease-free five-year survival rates were 65.8% and 77.5%, respectively. Disease-free five-year survival was influenced by depth of tumor infiltration (sm1 89.2%, sm2 76.1%, sm3 58.1%, $p<0.001$), but did not depend on histology (squamous cell carcinoma 79.5% versus adenocarcinoma 76.7%, $p=0.47$). Disease-recurrence was noted in 46 patients (20.7%): TTE 21.2% versus THE 20.0% ($p=0.83$). Type of recurrence was comparable: 9.8% locoregional recurrence and 14.4% distant metastases in the TTE-group versus 12.2% and 13.3%, respectively, in the THE-group. Overall five-year survival (TTE 63.4% versus THE 69.4%, $p=0.55$; Fig 1A) or disease free survival (TTE 76.9% versus THE 78.3%, $p=0.65$; Fig 1B) did not differ between the two groups. In the subgroup of patients with adenocarcinoma (Fig 2), disease-free five-year survival was also similar for both groups (TTE 77.3% versus THE 76.2%, $p=0.81$). For the subset of patients with N1 disease disease-free five-year survival was 49.8% in the TTE-group versus 40.0% in the THE-group; $p=0.57$ (Fig 3).

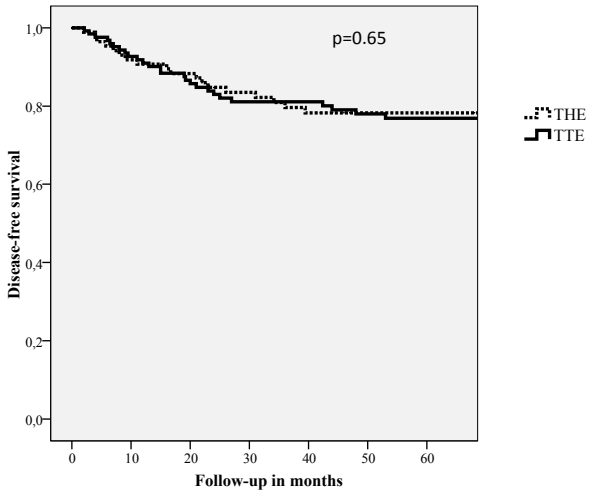
Figure 1A-B. *Overall survival (1A) and disease-free survival (1B) in relation to operation technique (TTE versus THE) in 222 patients who underwent primary surgical resection for submucosal esophageal cancer.*

Figure 1A.



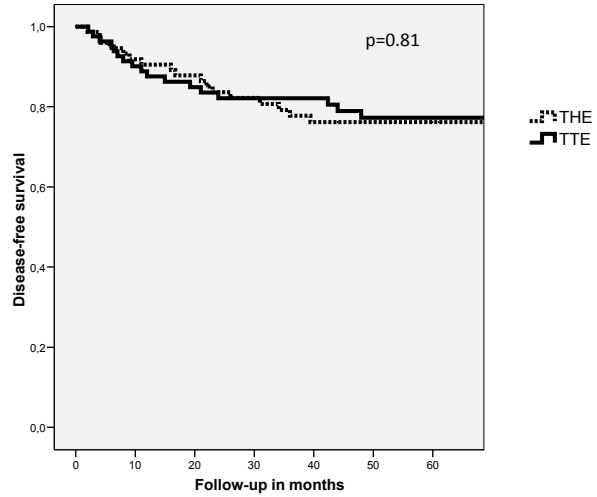
No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
TTE	132	113	97	86	79	73
THE	90	84	74	64	55	51

Figure 1B.



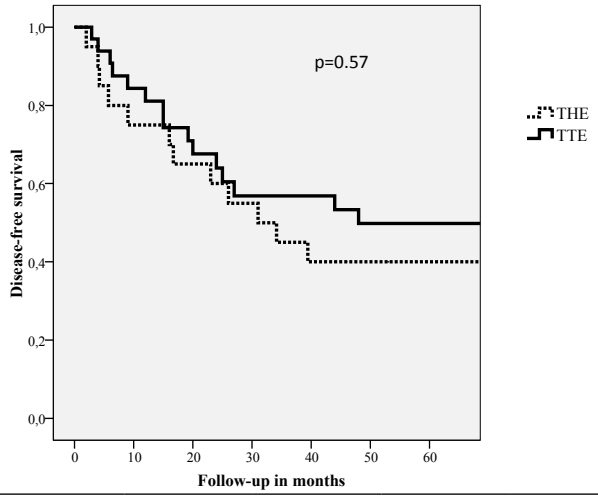
No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
TTE	132	108	90	86	73	68
THE	90	78	69	61	53	49

Figure 2. *Disease-free survival in relation to operation technique (TTE versus THE) in 164 patients who underwent primary surgical resection for submucosal esophageal adenocarcinoma.*



No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
TTE	86	69	59	52	46	44
THE	78	66	59	52	44	40

Figure 3. *Disease-free survival in relation to operation technique (TTE versus THE) in 56 patients who underwent primary surgical resection for submucosal esophageal cancer in whom positive lymph nodes were found (pN1).*



No. at risk	0 months	12 months	24 months	36 months	48 months	60 months
TTE	35	25	18	16	14	12
THE	21	15	12	9	6	5

Parameters found to be associated with disease-free survival in univariate analyses are shown in Table 5. Infiltration depth of the tumor, lymph node involvement (pN-stage), distant metastatic disease (pM-stage), and lymph node ratio were related to survival. On multivariate analysis, pN-stage was the only independent prognostic variable for disease-free survival (HR 3.9, 95% CI 1.6 – 9.7; p=0.004). Infiltration depth of the tumor (entered as a categorical variable, with sm1 as the reference category) appeared to be a borderline independent prognostic factor (sm2 HR 1.2, 95% CI 0.5 – 3.1; sm3 HR 2.3, 95% 1.1 – 5.1; p=0.07).

Table 5. Univariate analyses of variables associated with disease-free survival in esophageal cancer patients who underwent surgical resection.

VARIABLE	FIVE-YEAR SURVIVAL	p-value
Type of surgery		
-TTE	76.9%	0.65
-THE	78.3%	
Gender		
- Male	76.0%	0.24
- Female	83.7%	
Age		
≤ 64 years	75.2%	0.59
> 64 years	80.4%	
ASA classification		
- I / II	77.7%	0.93
- III / IV	76.5%	
Location of the tumor		
- Mid-esophagus	82.1%	0.59
- Distal esophagus / GEJ / gastric cardia	76.5%	
Histology		
- Squamous cell carcinoma	79.5%	0.47
- Adenocarcinoma	76.7%	
Tumor infiltration depth		
- sm1	89.2%	***<0.001***
- sm2	76.1%	
- sm3	58.1%	
pN-stage		
- N0	88.4%	***<0.001***
- N1	45.7%	
pM-stage		
- M0	78.3%	***0.07***
- M1	64.2%	
Lymph node ratio		
≤ 0.05	86.4%	***<0.001***
>0.05	46.0%	
Differentiation grade tumor		
- G1 (good)	83.0%	0.29
- G2 (moderate)	79.1%	
- G3 (poor)	72.3%	

ASA Classification = American Society of Anesthesiologists Classification
GEJ = Gastroesophageal junction

Discussion

Long-term outcome in terms of overall and disease-free five-year survival (68.6% and 77.5%, respectively) in the present series are at the upper limits of reported survival rates for submucosal esophageal cancer (33-79%).[13] Lymphatic dissemination was related to infiltration depth of the tumor, which is known to decrease patients' disease-free survival.[4, 5, 7, 24] Some previous reports have indicated considerably higher rates of lymph node involvement and worse disease-free survival inpatients with early squamous cell carcinomas.[5, 6, 24] However, in the present study the results of both submucosal adenocarcinomas and squamous cell carcinomas were combined as histology type did not influence pN-stage, M-stage or patients' disease-free survival.

The aim of the present study was to compare short-term and long-term outcomes of patients who underwent esophagectomy for cancer with submucosal tumor infiltration (T1b) through a transthoracic approach with extended lymphadenectomy, to those of patients in whom a transhiatal esophagectomy was performed with regional lymph node dissection. With regard to the short-term outcome, a well-known disadvantage of an extended lymphadenectomy is its surgical invasiveness at the cost of increased postoperative morbidity particularly pulmonary complications[14, 15] In the present study, overall morbidity was comparable for both groups, but more complications were classified as major in patients operated through the transthoracic route. This was mainly due to a higher rate of post operative respiratory insufficiency, which is in line with the corresponding literature.[14, 15] On the other hand, a higher incidence of reoperations was noted in the THE group.

A transthoracic approach may be justified if it leads to improved survival. To date only one study has shown a favorable long-term outcome in patients with T1b carcinoma operated through extended radical esophagectomy (including three-field lymphadenectomy) compared to a less radical esophagectomy (described as 'lesser extent of esophagectomy and lesser extent of lymphadenectomy').[9] However, the number of included patients in that particular study was small (n=78) and 30% received adjuvant chemo- or radiotherapy. In the present larger study (n=222), disease-free survival and overall five-year survival did not differ between the two groups.

The observation that TTE leads to an increased number of severe complications but not to improved survival in the current study population suggests that the transhiatal approach with regional lymphadenectomy is the preferred operation technique in patients with T1b esophageal cancer. Nevertheless, one should take into account that the survival curves in the present study include the total population of patients, of whom the majority (75%) did not have lymph nodal involvement. pN0-patients are unlikely to benefit from an extended lymphadenectomy. However, in the subgroup of pN1-patients, a disease-free five-year survival difference of 9.8% was detected in favor of an extended lymphadenectomy (TTE 49.8% versus THE 40.0%, p=0.57). Due to the relatively small number of T1bN1 patients (n=56), the fact that this difference did not reach statistical significance may represent a type-II error as other larger studies have found a relationship between the number of resected and positive lymph nodes on long-term outcome after esophagectomy for cancer.[16, 25, 26] In particular the subgroup analysis of a Dutch RCT, in which it was shown that in patients with one to eight positive lymph nodes a significant five-year locoregional disease-free survival advantage was obtained if operated through TTE, supports the present data indicating that T1bN1-patients (of whom the majority has only a limited number of positive lymph nodes) might benefit from an extended lymphadenectomy.[16] An other argument in favor of TTE may be the location of positive lymph nodes in this group: for example positive lymph nodes located high in the thorax that would not have been removed by a transhiatal procedure. Indeed, in 12 (34%) out of 35 N1-patients of the TTE-group, positive lymph nodes were located high in the mediastinum.

Despite major diagnostic advances pretreatment N-staging of early esophageal cancer is still unreliable. The accuracy of endosonographic staging for N-stage of early esophageal cancer has been reported 71-87%[27, 28], although the addition of fine needle aspiration (FNA) can improve these results.[29] This diagnostic uncertainty implicates a therapeutic dilemma in daily practice. Ideally, true N0-patients could be treated with organ-preserving endoscopic mucosal resection (EMR) or esophagectomy without lymphadenectomy, while N1-patients should undergo surgical resection, probably with extended lymphadenectomy. However, surgical resection remains the mainstay of submucosal esophageal cancer treatment because of the diagnostic uncertainty.

A limitation of the present study is its retrospective and non-randomized nature. Comparison of TTE with THE for submucosal esophageal cancer remains difficult due to the small number of studies and included patients[9], as well as the wide variety in definition of extended and limited lymphadenectomy. Moreover, studies often compare extensive and limited surgery for early esophageal cancer in general, considering T1a (mucosal) and T1b (submucosal) cancer as one clinical entity.[3, 8] Due to the relative scarcity of patients with T1bN1 disease, even in the current international multicenter study, the number of patients may not be sufficient to draw firm conclusions based on statistical evidence. Furthermore, the comparison of morbidity and severity of complications between the four participating hospitals is subject to bias because of variable definitions of complications and different scoring systems.

In conclusion, for submucosal esophageal cancer TTE with extended lymphadenectomy and THE with regional lymphadenectomy had similar short-term outcome and long-term survival in general. In the selected group of T1bN1 patients TTE may be the preferred operative technique due to a potential disease-free survival benefit; in patients with T1bN0 disease THE with en bloc dissection of the esophagus and regional lymph nodes offers an oncologically safe and less invasive treatment.

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Summary

The incidence of esophageal cancer is rising in the Western world, mainly due to the increase in adenocarcinomas of the esophagus and gastroesophageal junction over the past decades. Patients are usually diagnosed with esophageal cancer at an advanced stage of the disease, as symptoms such as dysphagia and odynophagia are experienced when the tumor is large and obstructing the esophageal lumen. Moreover, esophageal cancer is an aggressive disease with early lymphatic and hematogenous dissemination. Hence, less than half of the newly diagnosed patients are eligible for curative therapy due to tumor invasion into adjacent organs, or due to the presence of distant metastases.

The treatment of patients with esophageal cancer is highly complex and requires an interdisciplinary approach. New developments such as endoscopic treatment of early lesions and application of neoadjuvant chemo(radio)therapy have opened novel avenues towards the development of treatment strategies tailored to individual patients. However, surgery remains the mainstay in esophageal cancer treatment. It offers the best curative option, although it is associated with high morbidity and substantial mortality. Therefore, accurate preoperative staging and risk assessment is essential to select those patients who will benefit from surgery. Nevertheless, as five-year survival rates after potentially curative esophagectomy still rarely exceed 40%, ongoing research by means of both experimental (*e.g.* pathogenesis) and clinical (*e.g.* patient selection) studies is required.
This thesis included studies that address various aspects of esophageal cancer: pathophysiology, diagnosis, staging and treatment. The thesis consists of three parts: part A – pathogenesis of Barrett's adenocarcinoma, part B – optimization of staging, and part C – preoperative risk assessment and surgical treatment.

Part A – Pathogenesis of Barrett's adenocarcinoma

There is increasing evidence that a variety of human cancers is driven by a subset of cells, the so-called cancer stem cells (CSCs), which sustain tumor growth and underlie its malignant behavior. In **chapter 1** an overview is given of the current evidence for

the existence of CSCs in malignancies, and its potential implications for treatment of solid tumors. The CSC model opens new avenues with regard to the treatment of cancer. The development of tailor-made targeted therapies may entail great promises as several approaches can be developed to specifically target CSCs, such as direct CSC eradication by means of therapies directed at CSC-specific surface markers.

Several markers have been employed to isolate CSCs from various malignancies, though not from esophageal adenocarcinoma. In **chapter 2** we tested whether Barrett's esophagus and esophageal adenocarcinoma might serve as a model for the CSC concept. Transplantation assays were performed by injecting serially diluted bulk esophageal adenocarcinoma cells into immunodeficient mice to establish the presence and frequency of tumor-initiating cells. These were found to be present in approximately 1:64,000 cells, and the transplanted tumors fully recapitulated the primary lesions. Subsequently, a panel of CSC markers previously established in other solid tumor types was employed to sort the corresponding subpopulations of cancer cells and transplant them at low multiplicities into mice. However, no increased tumor-initiating capacity of sorted cells was observed upon transplantation. These results indicate that tumor-initiating cells are present in esophageal adenocarcinoma, thus reflecting a hierarchal organization. However, antibodies directed against novel surface antigens are needed to detect subpopulations enriched for CSCs.

In **chapter 3** an overview is given of the pathogenesis of Barrett's metaplasia and its progression towards esophageal adenocarcinoma. Gastroesophageal reflux disease (GERD) has been recognized as the most important risk factor for the onset of Barrett's metaplasia and esophageal adenocarcinoma. Environmental, dietary and genetic factors are also likely to play an important role. However, the exact mechanism underlying the transition from normal squamous epithelium towards metaplastic columnar epithelium has not been elucidated yet. The identification of stem cells in the normal squamous esophageal epithelium has lead to speculations about the contribution of these cells to the metaplastic process, as these cells are the only permanent residents of the epithelium.

Recently, a mouse model for Barrett's esophagus based on a zinc-deficient diet supplemented with deoxycholic bile acids has been published. **Chapter 4** comprises a short report in which the reproducibility of this non-invasive mouse model of

Barrett's esophagus has been investigated. In this study, all mice showed signs of considerable distress and discomfort already 4 weeks after the start of the modified diets, and the animals had to be euthanized before the first evaluation time point. Therefore, we were not able to reproduce the mouse model according to which Barrett's like lesions could be detected in animals fed with the zinc-deficient diet supplemented with deoxycholic bile acids. Notwithstanding their overall negative nature, these data are of relevance for the fine-tuning of this animal model especially as far as the role of dietary and environmental zinc is concerned.

Part B – Optimization of staging

In patients with resectable adenocarcinoma of the gastroesophageal junction (GEJ) preoperative staging will determine the therapeutic strategy with regard to type of surgical procedure and use of neoadjuvant therapy. Tumor location and lymph node status play a pivotal role in this tailored strategy. In **Chapter 5** we investigated in a prospective way the accuracy for determining the location of the primary tumor according to the Siewert classification as well as the lymph nodal status per station. The preoperative assessment by endoscopy/EUS and CT-scanning were compared with the histopathologic findings in the resection specimen (gold standard). However, given the frequent discrepancy between the endoscopic and pathologic location of the GEJ and the common problem of advanced tumors obscuring the landmarks used in the assessment of the Siewert classification, its usefulness in the assessment of tumor location appeared to be limited. Furthermore, the overall accuracy for EUS and CT in predicting the N-stage per station was only moderate.

In **chapter 6** a study on the feasibility of application of the sentinel node procedure in adenocarcinomas of the distal esophagus and GEJ was presented. It was concluded that detection of sentinel nodes is technically feasible during esophagectomy for cancer. However, given the relatively high false-negative rate and the high frequency of sentinel nodes in more than one nodal station, the clinical relevance of the sentinel node concept (through application of the blue dye technique) in the current treatment of patients with an adenocarcinoma of the distal esophagus or gastroesophageal junction seems limited.

In 2001 the Japanese Society of Esophageal Disease introduced its classification in which early cancers are subdivided in six successive layers of the mucosa (m1, m2, m3) or submucosa (sm1, sm2, sm3). Subdivision in six, rather than two categories has been shown helpful in directing patients to the most optimal treatment strategy. In **chapter 7** we attempted to address the question whether the presence of occult metastases in patients with m3 and sm1 adenocarcinomas could explain the variation in reported incidences in lymphatic dissemination in these patients. Indeed, lymphatic migration of tumor cells (isolated tumor cells or micrometastases) was found in conventionally node-negative m3- and sm1-adenocarcinomas of the esophagus (8.0% and 20.0%, respectively). However, the clinical relevance of these occult tumor cells (whether this could influence the decision to undertake an endoscopic or surgical resection) should become apparent from large series of endoscopically treated patients.

In **chapter 8** we evaluated the reproducibility of the Japanese classification of early esophageal cancer. The reproducibility was good in terms of inter- and intraobserver variability when grading early esophageal adenocarcinoma on surgical resection specimens. Nevertheless, dedicated gastrointestinal pathologists with broad experience are preferred when grading the resection specimens of patients with early esophageal cancer.

Part C – Preoperative risk assessment and surgical treatment

Individual preoperative risk assessment is essential for a proper patient selection in the preoperative phase. In **chapter 9** patients' preoperative risk assessment and the potential risk factors that can be taken care of both preoperatively and intraoperatively in order to prevent postoperative complications in patients with esophageal cancer have been reviewed. Age, pulmonary and cardiovascular condition, nutritional status, and application of neoadjuvant chemo- and/or radiotherapy have shown to be important predictive factors for postoperative morbidity and mortality. Both the surgeon (application of appropriate surgical techniques) and the anesthetist (adequate fluid management) play an important role in intraoperative risk reduction.

In order to improve the short-term outcome of esophagectomy, an optimal perioperative treatment strategy can be tailored for the individual patient.

In **chapter 10 and 11** the prognostic value of body mass index (BMI) and of delay in diagnostic work-up on short-term and long-term outcome after resection of esophageal cancer were studied, respectively. BMI was not of prognostic value with regard to short-term and long-term outcome in patients undergoing esophagectomy for cancer. Therefore, patients oncologically eligible for esophagectomy should not be denied surgery on the basis of their BMI class. A longer delay between endoscopic diagnosis and surgery resulted in worse patient's short-term outcome (higher overall morbidity and mortality rates), but not in a worse long-term outcome (overall survival). This may be explained by a more time-consuming diagnostic work-up in patients with a poorer physical status and not by tumor progression.

Furthermore, a recently developed nomogram predicting the occurrence and severity of complications in esophagectomy patients was validated externally in a large cohort of patients who underwent esophagectomy for cancer in an independent high-volume center. These results are presented in **chapter 12**: the model is applicable in other high-volume hospitals, but preoperative prediction of complications in individual patients remains difficult, most likely due to the complexity of mechanisms causing these complications.

Finally, an international multi-center study has been conducted in **chapter 13**, which focused on the best surgical treatment for early esophageal cancer with submucosal tumor infiltration (T1b). Outcome of patients who underwent esophagectomy for T1b cancer through a transthoracic approach (TTE) with extended lymphadenectomy was compared retrospectively to those of patients in whom a transhiatal esophagectomy (THE) was performed with regional lymph node dissection only. TTE with extended lymphadenectomy did not result in a worse short-term outcome (although more complications were graded as severe) and did not improve long-term survival in general. However, in the selected patient group of T1bN1 patients TTE might be the preferred operation technique due to a potential disease-free survival benefit; in all other cases THE will offer an oncologically safe and less invasive treatment.

Nederlandse samenvatting

In de westerse wereld neemt de incidentie van slokdarmkanker toe. Dit komt met name door de stijging van het aantal adenocarcinomen van de slokdarm en de slokdarm-maag overgang gedurende de laatste decennia. De diagnose slokdarmkanker wordt veelal laat gesteld, als de ziekte zich al in een vergevorderd stadium bevindt. Symptomen, zoals pijnlijk en moeizaam slikken, worden meestal pas ervaren als de tumor groot is en de slokdarm obstrueert. Tevens is slokdarmkanker een agressieve ziekte, waarbij uitzaaiingen via de lymfebanen en bloedvaten al vroeg in het ziekteproces kunnen voorkomen. Van de patiënten, bij wie de diagnose slokdarmkanker wordt gesteld, komt slechts minder dan de helft in aanmerking voor een behandeling waarbij genezing kan worden verwacht.

De behandeling van patiënten met slokdarmkanker is complex en vereist een multidisciplinaire aanpak. Nieuwe ontwikkelingen, zoals endoscopische behandeling van vroege tumoren en de toepassing van neoadjuvante chemo(radio)therapie, hebben de weg vrijgemaakt voor behandelingsstrategieën die gericht zijn op de individuele patiënt. Een operatieve ingreep blijft de belangrijkste peiler binnen de behandeling van slokdarmkanker, aangezien deze de grootste kans biedt op genezing. Een operatie gaat echter gepaard met een aanzienlijke kans op morbiditeit en mortaliteit. Daarom is het van belang om voorafgaand aan de operatie de patiënten te kunnen selecteren (door middel van tumorstadiëring en pre-operatieve risico-inschatting) die hierbij gebaat zullen zijn. Aangezien de vijfjaarsoverleving na een curatieve slokdarmresectie amper boven de 40% uitstijgt, is voortgaand onderzoek door middel van experimentele studies (bijvoorbeeld gericht op de ontstaanswijze van slokdarmkanker) en klinische onderzoeken (gericht op de patiënt) onontbeerlijk.

Dit proefschrift bevat onderzoeken die verschillende aspecten van slokdarmkanker belichten: pathogenese (ontstaanswijze), diagnose, stadiëring en behandeling van de ziekte. Het proefschrift bestaat uit drie delen: deel A – Pathogenese van het Barrett adenocarcinoom, deel B – Optimaliseren van tumorstadiëring, en deel C – Pre-operatieve risico-inschatting en chirurgische behandeling.

Deel A – Pathogenese van het Barrett adenocarcinoom

Er is steeds meer bewijs dat verschillende maligniteiten worden aangestuurd door slechts een subpopulatie aan cellen, de zogenaamde kankerstamcellen, die verantwoordelijk zijn voor de groei van de tumor en zijn maligne kenmerken. **Hoofdstuk 1** biedt een overzicht van de literatuur over het bestaan van kankerstamcellen en de mogelijke gevolgen voor de behandeling van tumoren. Het kankerstamcelmodel biedt volop nieuwe perspectieven voor de behandeling van kanker, aangezien hiermee nieuwe therapieën kunnen worden ontwikkeld, die erop gericht zijn deze kankerstamcellen direct aan te pakken. Een voorbeeld hiervan is het uitschakelen van kankerstamcellen door middel van een antilichaambehandeling gericht op antigenen, die zich op het celoppervlakte van kankerstamcellen bevinden. Meerdere celoppervlaktemarkers zijn geïdentificeerd om kankerstamcellen van verschillende maligniteiten te isoleren, maar nog niet van het slokdarm adenocarcinoom.

In **hoofdstuk 2** hebben we onderzocht of bij het slokdarm adenocarcinoom het kankerstamcelmodel ook van toepassing is. Er werden transplantatie-experimenten uitgevoerd, waarbij tumorcellen van verse humane slokdarm adenocarcinomen in verschillende hoeveelheden werden geïnjecteerd in immunodeficiënte muizen. Op deze wijze kon worden bepaald of tumorinitiërende cellen aanwezig zijn in het adenocarcinoom van de slokdarm. Uit deze experimenten is gebleken dat ongeveer 1 op de 64.000 cellen een tumorinitiërende cel is. Bovendien bleken de muis-tumoren histologisch identiek te zijn aan de primaire humane tumor. Vervolgens werd een panel van (bij andere maligniteiten) bekende kankerstamcelmarkers gebruikt om verschillende subpopulaties aan tumorcellen te isoleren en te injecteren in kleine aantallen. Er kon echter geen verhoogde tumorinitiërende capaciteit worden waargenomen voor een subpopulatie cellen verrijkt aan een specifieke marker. Deze resultaten suggereren dat tumorinitiërende cellen aanwezig zijn in het slokdarm adenocarcinoom, maar dat reeds bekende kankerstamcelmarkers niet de markers lijken te zijn voor dit type tumor. Antilichamen tegen nieuw te ontdekken oppervlakte-antigenen zijn nodig om celpopulaties te identificeren, die verrijkt zijn aan kankerstamcellen van het slokdarm adenocarcinoom.

Hoofdstuk 3 biedt een overzicht van de pathogenese van Barrett metaplasie (de voorloper van het adenocarcinoom) en de progressie naar het slokdarm adenocarcinoom. Gastro-oesofageale refluxziekte (GORZ) is de belangrijkste risicofactor voor het ontstaan van een Barrett slokdarm, waarbij de binnenbekleding of epitheel van de slokdarm van celtype verandert (metaplasie). Het exacte mechanisme dat ten grondslag ligt aan de omslag van het normale squameuze epitheel naar het metaplastische epitheel is echter nog onbekend. De identificatie van stamcellen in de basale laag van het normale squameuze slokdarmepitheel heeft tot de speculatie geleid dat stamcellen verantwoordelijk kunnen zijn voor deze omslag. Het zijn immers de enige cellen zijn die permanent aanwezig zijn in het epitheel.

Onlangs is een muismodel voor een Barrett slokdarm gepubliceerd, waarbij muizen een zinkdeficiënt dieet kregen, aangevuld met galzouten, en waarbij na enkele maanden veranderingen in de slokdarm konden worden geobserveerd, die veel gelijkenis vertoonden met een Barrett slokdarm. **Hoofdstuk 4** bestaat uit een zogeheten 'short report' waarin we de reproduceerbaarheid hebben onderzocht van dit non-invasieve muismodel. In ons onderzoek kregen alle muizen echter al na vier weken vanaf de start van het dieet zoveel ongemak, dat het experiment voortijdig moest worden afgebroken. Hierdoor waren wij niet in staat om het muismodel te reproduceren waarbij een Barrett slokdarm zou ontstaan na het introduceren van een zinkdeficiënt dieet aangevuld met galzouten. Ondanks het negatieve karakter van deze uitkomsten, zijn deze data relevant voor het aanpassen van het eerder beschreven model.

Deel B – Optimaliseren van tumorstadiëring

Bij patiënten met een resectabel slokdarm adenocarcinoom van de slokdarm of de slokdarm-maag overgang, zal de pre-operatieve tumor stadiëring het uiteindelijke behandelplan bepalen voor wat betreft de operatietechniek en de eventuele toepassing van pre-operatieve chemo- en/of radiotherapie. De locatie van zowel de tumor als de mogelijke betrokken lymfeklierstations speelt een centrale rol in deze

op maat gemaakte behandelingsstrategie. In **hoofdstuk 5** hebben we prospectief de betrouwbaarheid onderzocht van het bepalen van de tumorlocatie volgens de classificatie van Siewert en de lymfeklierstatus per klierstation. De pre-operatieve inschatting door de endoscopie/endo-echoscopie en de CT-scan werd vergeleken met het pathologische eindoordeel vanuit het chirurgische resectiepreparaat ('gouden standaard'). Uit dit onderzoek is gebleken dat door de vaak voorkomende discrepantie tussen de endoscopische en pathologische locatie van de slokdarm-maag overgang en door het frequente voorkomen van grote, stenotische tumoren die de markeringspunten van de Siewert classificatie maskeren, de bruikbaarheid van deze classificatie in de dagelijkse praktijk in twijfel kan worden getrokken. Daarnaast bleek de betrouwbaarheid in het voorspellen van de lymfeklierstatus per klierstation, door zowel de endo-echoscopie als de CT-scan, slechts matig.

In **hoofdstuk 6** staat het onderzoek beschreven waarin het gebruik van de zogeheten 'sentinel node procedure' (veelal gebruikt bij borstkanker) tijdens de operatie voor slokdarm adenocarcinomen werd onderzocht. Uit deze studie is gebleken dat deze poortwachtklieren ('sentinel nodes') inderdaad opgespoord kunnen worden tijdens de operatie met behulp van blauwe kleurstof. Echter, gezien de relatief hoge fout-negatieve uitslag en gezien het vaak voorkomen van meerdere poortwachtklieren op verschillende plaatsen, lijkt de klinische toepassing van dit concept bij de huidige behandeling van patiënten met een slokdarm adenocarcinoom beperkt.

In 2001 introduceerde de Japanse vereniging voor slokdarmkanker een nieuwe classificatie voor vroege tumoren van de slokdarm, waarbij deze vroege laesies worden onderverdeeld in zes opeenvolgende categorieën van de slokdarmmucosa (m1-, m2- en m3-tumoren) en -submucosa (sm1-, sm2- en sm3-tumoren). Deze onderverdeling in plaats van de gebruikelijke twee categorieën is zinvol gebleken tijdens het bepalen van de optimale behandelingsstrategie bij deze patiëntengroep.

In **hoofdstuk 7** hebben we geprobeerd de vraag te beantwoorden of de aanwezigheid van occulte tumorcellen (geïsoleerde tumorcellen of micrometastasen) in lymfeklieren bij patiënten met een m3- of sm1-slokdarmtumor de variatie kan verklaren in de gerapporteerde incidentie in lymfogene metastasering in deze subgroepen. Er bleken inderdaad in onze serie van m3- en sm1-patiënten, waarbij de lymfeklierstatus in eerste instantie als negatief was afgegeven, occulte tumorcellen aanwezig te zijn in de

lymfeklieren: 8% in m3-tumoren en 20% in sm1-tumoren. De klinische relevantie van deze tumorcellen (oftewel de mogelijke invloed op de beslissing om endoscopisch of chirurgisch te behandelen) zal echter in de toekomst duidelijk moeten worden uit een grote serie patiënten die endoscopisch zijn behandeld.

Hoofdstuk 8 beschrijft het onderzoek waarin de reproduceerbaarheid van de reeds genoemde Japanse classificatie voor vroege tumoren van de slokdarm wordt geëvalueerd. De reproduceerbaarheid was goed voor wat betreft de zogenaamde inter- en intra-observer variabiliteit bij het beoordelen van chirurgische resectiepreparaten op de tumor infiltratiediepte van vroege slokdarm adenocarcinomen. Toegewijde en ervaren pathologen blijven echter nodig om deze tumoren zorgvuldig te kunnen classificeren.

Deel C – Pre-operatieve risico-inschatting en chirurgische behandeling

Een individuele pre-operatieve risico inschatting is essentieel voor een goede selectie van patiënten die een operatie zullen ondergaan. In **hoofdstuk 9** worden alle mogelijke risicofactoren samengevat die zowel voor als tijdens de operatie beïnvloed kunnen worden om post-operatieve complicaties te voorkomen bij patiënten met slokdarmkanker. Leeftijd, hart- en longconditie, voedingstoestand en toepassing van pre-operatieve chemo- en/of radiotherapie, zijn belangrijke voorspellers voor post-operatieve morbiditeit en mortaliteit. Zowel de chirurg (toepassing van de juiste operatietechniek) als de anesthesist (handhaven van een adequate vochtbalans) spelen een belangrijke rol tijdens de operatie in het verkleinen van de kans op post-operatieve complicaties. Om de uitkomst op korte termijn na een slokdarmresectie te verbeteren, moet een optimaal behandelingsplan opgesteld worden voor de individuele patiënt.

In de **hoofdstukken 10 en 11** is onderzocht wat de voorspellende waarden van de parameters body mass index (BMI) respectievelijk de wachttijden voor een operatie zijn voor zowel de korte als de lange termijn na een slokdarmresectie. De BMI bleek niet van voorspellende waarde voor de uitkomst van een slokdarmoperatie: het aantal

en het soort post-operatieve complicaties en de overleving verschilden niet tussen patiënten met een lage en hoge BMI. Hierdoor werd uit deze studie de conclusie getrokken dat aan patiënten met slokdarmkanker een operatie niet onthouden mag worden op basis van een hoge BMI. Een langere wachttijd tussen het stellen van de diagnose tijdens endoscopie en de operatie was geassocieerd met een slechtere post-operatieve uitkomst op korte termijn (hogere morbiditeit en mortaliteit), maar was niet geassocieerd met een slechtere uitkomst op lange termijn (vergelijkbare overleving). Het is daardoor meer aannemelijk dat deze associatie tussen wachttijd en uitkomst op korte termijn verklaard kan worden door een intensievere en daardoor langere pre-operatieve voorbereiding bij patiënten die zich reeds in een slechtere lichamelijke conditie bevinden (en dus bij voorbaat al een grotere kans hebben op post-operatieve complicaties), dan dat tumorprogressie hiervoor verantwoordelijk is.

In **hoofdstuk 11** is een recent ontwikkeld nomogram (een grafisch model) beschreven dat het voorkomen en de ernst van complicaties bij patiënten die een slokdarmresectie ondergaan kan voorspellen en dat extern gevalideerd is in een groot onafhankelijk cohort patiënten van een ander hoogvolume centrum. Het blijkt dat het model toepasbaar is in andere ziekenhuizen, maar dat het pre-operatief voorspellen van complicaties voor een individuele patiënt moeilijk blijft. Dit is waarschijnlijk het gevolg van de complexe mechanismen die deze complicaties veroorzaken.

Ten slotte is een internationaal multi-center onderzoek uitgevoerd, dat zich richtte op de vraag welke chirurgische behandeling het beste is voor patiënten met een submucosaal ingroeiende slokdarmtumor (T1b-tumoren). In **hoofdstuk 13** zijn in retrospectie de uitkomsten van T1b-patiënten die een transthoracale slokdarmresectie ondergaan met een uitgebreide lymfeklierdissectie (TTE), vergeleken met de patiënten waarbij een transhiatale operatie werd uitgevoerd met een beperkte lymfeklierdissectie (THE). Een TTE leidde niet tot een slechtere uitkomst op korte termijn (alhoewel meer complicaties als ernstig werden gegradeerd), maar leverde ook geen overlevingswinst op voor deze patiënten. Echter, in de geselecteerde groep van T1bN1-patiënten, lijkt een TTE wellicht toch de operatietechniek van keuze door een mogelijk voordeel in ziektevrije-overleving. In alle andere gevallen zal een THE een oncologisch acceptabele en minder invasieve behandeling geven.

Future perspectives

In high-volume centers, the five-year survival rate (without standard application of neoadjuvant therapy) in patients with esophageal cancer after potentially curative esophagectomy rarely exceeds 40%. It is possible that a plateau in the effectiveness of surgery as primary therapy in esophageal cancer patients may have been reached. Therefore, further improvement in survival from this single modality approach seems unlikely. The care for patients with esophageal cancer will probably be increasingly individualized in the future, with the addition of new therapeutic targets. This paragraph describes novel options that might be implemented in the management of esophageal cancer, and on which future research should focus in both experimental and clinical settings.

Cancer stem cells

Obviously, further studies are needed in order to attempt to identify CSCs in esophageal cancer. The identification of CSCs has potentially important implications for the treatment of cancer, in specific the therapeutic promise of CSC-directed treatment strategies. Potential therapies which are selective to CSCs and not toxic to normal stem cells hold great promise for the effective and potentially curative therapy of many human cancers. CSC-targeting strategies may include (a) direct targeting of CSCs through antibodies directed at CSC-surface markers, (b) blocking the essential self-renewal signaling pathways, (c) reversal of CSC resistance to chemo- and/or radiotherapy and (d) induction of CSC differentiation.[1] Direct targeting of CSCs through their CSC-surface markers seems an obvious solution. However, most of these markers are also expressed in normal cells, thus finding a therapeutic window will be a challenge. Blocking essential self-renewal signaling pathways such as Wnt, Hedgehog and Notch is also a promising strategy. In colorectal cancer small-molecules that inhibit the Wnt- and Notch pathway have recently been identified as novel approaches.[2] Nevertheless, these pathways are mostly shared by normal stem cells, indicating the difficulty of this strategy. An other CSC-directed therapeutic strategy might be the targeting of signals that regulate CSC resistance to chemo- and/or radiotherapy. Recent findings in colon cancer further support the potential utility of CSC chemosensitizing agents: pretreatment of CD133+ colon CSCs with a neutralizing antibody enhanced apoptosis mediated by chemotherapy both *in vitro* and *in vivo*.[3]

Following the more recent findings suggesting the interaction between epithelial-mesenchymal transition (EMT) and CSCs, and more specifically the demonstration that the tumor microenvironment may regulate the stemness of cancer cells, a new treatment strategy can be proposed. Obviously, if non-CSCs can indeed give rise to CSCs, this plasticity would frustrate attempts to cure tumors by eliminating CSCs alone. Therefore, cancer therapy should include agents that focus on targeting both CSCs and non-CSCs. However, further research is first required to investigate the role of EMT in esophageal cancer, and its potential as a prognostic factor for metastases and poor survival like in colorectal cancer.[4]

Molecular tumor biology

The complete sequencing of the human genome in 2003 has enabled systematic approaches to identify cancer genome alterations. Molecular targets for therapy are becoming widely known and include epidermal growth factors, tyrosine kinases, vascular endothelial growth factors and intracellular signaling pathways. Microarray analysis to study gene expression is able to identify genes which can function as a guide for tailored therapy, *i.e.* prediction of response to neoadjuvant therapy. Recently, a four-gene signature has been discovered and validated that independently predicts survival of patients with resected adenocarcinoma of the esophagus or gastroesophageal junction.[5] Other examples of cancer-targeted therapy are Trastuzumab/Herceptin (used to treat Her2/neu overexpressing breast cancer) and Gefitinib/Iressa (used to treat lung cancers with EGFR mutations). Molecular characteristics of the tumor are becoming more and more important, with hopefully in the future the possibility to select the most appropriate treatment for the individual patient.

Neoadjuvant chemoradiotherapy

Improvement in survival may also be expected from neoadjuvant chemoradiotherapy. Neoadjuvant chemoradiotherapy is thought to improve primary tumor resectability (higher number of radical or R0 resections) and to eliminate (micro)metastatic disease. A meta-analysis published in 2007 showed a survival benefit for patients treated with neoadjuvant chemoradiotherapy followed by surgery compared to patients treated with surgery alone.[6] However, most included studies described mainly squamous cell carcinomas and had only small sample-sizes. In 2004 a multicenter randomized

phase-III trial (including 175 patients in each arm) was initiated in The Netherlands to evaluate the effect of neoadjuvant chemoradiotherapy (5-week scheme consisting of 41.4 Gray in 23 fractions of 1.8 Gray plus a weekly dosis of paclitaxel 50 mg/m2 and carboplatin AUC=2) followed by surgery compared to surgery alone in patients with potentially curable esophageal cancer.[7] Results of this study in terms of long-term survival are expected in 2011.

Patient selection

Better patient selection for esophageal surgery may lead to better survival. Improved pre-operative staging (*i.e.* dedicated gastroenterologists reporting all (tumor) landmarks during endoscopy and fine-needle aspiration of all potentially critical lymph nodes) will lead to well-considered decision-making with regard to operation technique and application of neoadjuvant therapy. Furthermore, preoperative risk assessment will supply important information for both patient and surgeon. Potential intervention of risk factors (*i.e.* preoperative respiratory muscle training in case of pulmonary dysfunction in order to reduce postoperative pulmonary complications, or routine prophylactic combination therapy such as beta-blocker, statin, and aspirin to prevent cardiovascular events) hold great promise.

Finally, collaboration between departments (surgery, gastroenterology, medical oncology, radiology and pathology) as well as cooperation between hospitals (nationwide and on an international basis) will be of utmost importance in future research in order to optimize our knowledge in pathogenesis, diagnostics, staging and treatment of patients with esophageal cancer. Otherwise, the disease will remain a miscellaneous mystery…

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Lief, ik houd met heel mijn hart van jou, op een zeer mooie toekomst samen!

Brechtje

Rotterdam, najaar 2010

PhD Portfolio

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Brechtje A. Grotenhuis Erasmus MC Department: Surgery / Pathology Research School: Molecular Medicine	PhD period: Oktober 2007 – June 2010 Promotors: Prof.dr. J.J.B. van Lanschot and Prof.dr. R. Fodde

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Laboratory animal science	2007	3.0 ECTS
- Safety in the laboratory	2007	0.3 ECTS
- Statistics and Methodology:		
* *Classical Methods for Data-analysis*	2008	5.7 ECTS
* *Modern Statistical Methods*	2008	4.3 ECTS
* *Survival Analysis for Clinicians*	2009	1.9 ECTS
Seminars and workshops		
- Erasmus Gastroenterology Day "Treatment of M1a-disease in patients with esophageal cancer"	2009	1.0 ECTS
Presentations		
- Various presentations at research meetings of the Department of Surgery and the Department of Experimental Pathology	2007 - 2010	
Presentations (inter)national conferences		
– SEOHS (Utrecht) "Kanker stamcel markers van het oesofagus adenocarcinoom"	2008	1.0 ECTS
– Chirurgendagen NVvH (Veldhoven) "Analyse naar kankerstamcelmarkers van slokdarmkanker"	2009	1.0 ECTS
– Chirurgendagen NVvH (Veldhoven) "Sentinel node procedure bij patiënten met slokdarmkanker"	2009	1.0 ECTS
– UEGW / Gastro (London) "Prognostic value of body mass index on short-term and long-term outcome after resection of esophageal cancer"	2009	1.0 ECTS
– UEGW / Gastro (London) "Surgical management of submucosal esophageal cancer: extended or limited lymphadenectomy?"	2009	1.0 ECTS

– UEGW / Gastro (London) "Analysis of cancer stem cell markers in esophageal adenocarcinoma"	2009	1.0 ECTS
– Najaarsvergadering NVvH (Ede) "De prognostische waarde van body mass index op de korte en lange termijn uitkomst na resectie voor slokdarmkanker"	2009	1.0 ECTS
– Voorjaarsvergadering NVGE (Veldhoven) "Lymphatic micrometastases in patients with early esophageal adenocarcinoma"	2010	1.0 ECTS
– Chirurgendagen NVvH (Veldhoven) "Validatie van een nomogram voor het voorspellen van postoperatieve complicaties bij patiënten met slokdarmkanker"	2010	1.0 ECTS
– Annual meeting European Surgical Association (Budapest) "Surgical management of submucosal esophageal cancer: extended or regional lymphadenectomy?"	2010	1.0 ECTS
– European Society of Surgical Research (Geneva) "Lymphatic micrometastases in patients with early esophageal adenocarcinoma"	2010	1.0 ECTS
– International Society for Diseases of the Esophagus (Japan) "Barrett's esophageal adenocarcinoma encompasses tumor initiating cells that do not express common cancer stem cell markers"	2010	1.0 ECTS
– International Society for Diseases of the Esophagus (Japan) "Preoperative assessment of tumor location in adenocarcinomas of the gastroesophageal junction according to the Siewert classification"		
Posterpresentations (inter)national conferences		
– UEGW (Vienna) "The sentinel node concept in esophageal adenocarcinoma"	2008	0.5 ECTS
– International Society Diseases of the Esophagus (Budapest) "Immunohistochemistry analysis of cancer stem cell markers in esophageal adenocarcinoma"	2008	0.5 ECTS
– Frontiers in Basic Cancer Research (Boston) "Barrett's esophagus and esophageal adenocarcinoma: a paradigm for the cancer stem cell model?"	2009	0.5 ECTS
– SEOHS (Nijmegen) "Micrometastasen bij het vroeg-carcinoom van de oesofagus"	2009	0.5 ECTS
– UEGW/Gastro (London) "Impact of delay in diagnostic work-up and treatment of patients with esophageal cancer"	2009	0.5 ECTS

2. Teaching

	Year	Workload (Hours/ECTS)
Other		
- Supervising second year medical students Elective "Kanker: van kliniek tot diagnostiek"	2009 / 2010	2 x 0.5 = 1.0 ECTS
- Supervising third year medical students Research elective	2008 / 2009	1.5 ECTS

Curriculum vitae

Brechtje Aleid Grotenhuis (April 5th 1980, Zeist) grew up in the neighborhood of the Utrechtse Heuvelrug. In 1998 she started her medical studies in Maastricht. Besides the challenge of the problem-based learning system propagated by the Maastricht University, several chances were offered to explore medicine abroad. Clinical rotations were conducted in Adelaide (Australia), Aruba (Netherlands Antilles) and Cape Town (South Africa). Her medical degree was obtained in 2005, after which she started working as a surgical resident at Tergooiziekenhuizen, Hilversum (supervisors: dr. J.W. Juttmann and dr. J.P. Eerenberg). After a year, she decided to take the chance to work abroad again, now as a surgical resident in Australia (Flinders Medical Centre, Adelaide; supervisors: prof. D.I. Watson and dr. B.P.L. Wijnhoven). Back from the Down Under experience, she started her PhD-project in October 2007 at the departments of surgery and experimental pathology of the Erasmus MC in Rotterdam (supervisors: prof.dr. J.J.B. van Lanschot and prof.dr. R. Fodde). In July 2010 she started her general surgical training at the Sint Franciscus Gasthuis in Rotterdam (supervisors: dr. A.J. Kerver and prof.dr. J.N.M. IJzermans).