ESOPHAGEAL ADENOCARCINOMA, MORE THAN ONE ENTITY?

A clinical and molecular analysis

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Esophageal Adenocarcinoma, More Than One Entity?

A clinical and molecular analysis.

Adenocarcinoom van de slokdarm, meer dan een entiteit?

Een klinische en moleculaire analyse.

Thesis

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Aan mijn ouders

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INTRODUCTION

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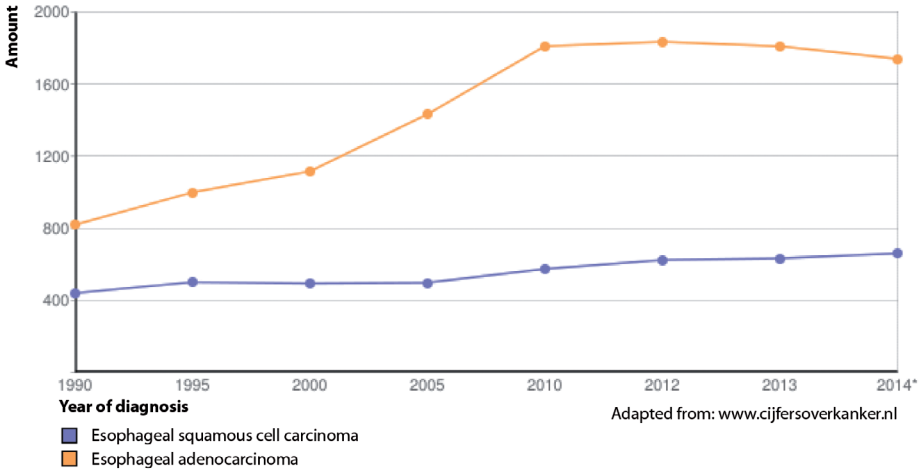
GENERAL INTRODUCTION

Worldwide, there has been a remarkable increase in the incidence of esophageal cancer, and presently it is the eight most common cancer and the sixth most common cancer related death in the world.[1] Primarily esophageal cancer is composed of two histological subtypes, esophageal adenocarcinoma (EAC) and squamous cell carcinoma (SCC). The increase in incidence is mainly attributed to an increase in EAC, especially in Western countries. [2-5] For example, in the Netherlands the incidence of EAC has increased from 819 new patients in 1990 to 1,740 new patients in 2014, while the incidence of SCC has roughly remained stable (www.cijfersoverkanker.nl) (Figure 1).

Figure 1: Incidence of esophageal cancer in the Netherlands (1990-2014)

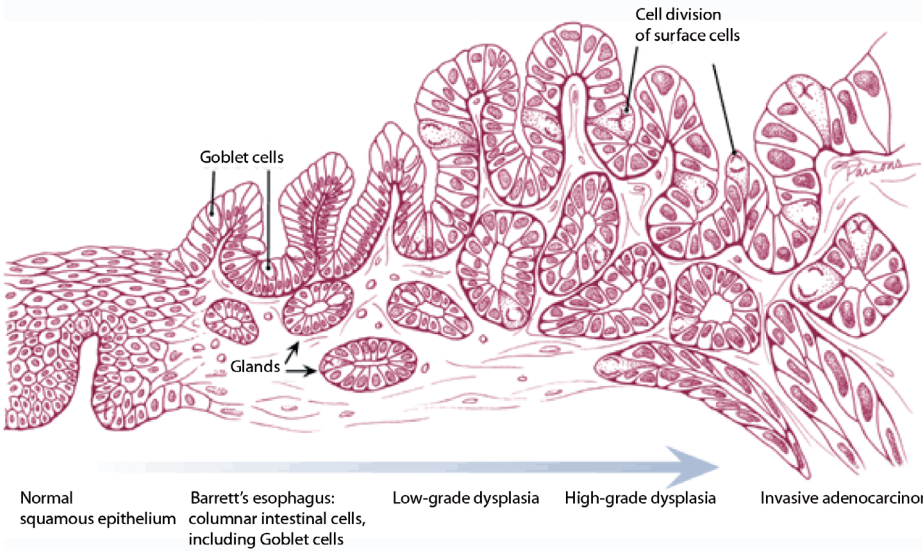


It is generally accepted that the premalignant lesion Barrett's esophagus (BE) is the major risk factor for EAC, in addition to age, male gender, Caucasian ethnicity, smoking, and obesity.[6-8] As a consequence of chronic gastroesophageal reflux (GER), the normal squamous epithelium of the lower esophagus can be replaced by columnar intestinal cells, including goblet cells, representing BE. The risk of developing EAC from BE is estimated at 0.12-0.5% per year[9,10] and follows a multimorphological sequence, in which metaplasia evolves to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and ultimately into invasive adenocarcinoma.[11] (Figure 2)-

EACs are typically diagnosed in white males with a median age of 70 years.[4] The most common symptom patients present with is dysphagia. Since dysphagia generally appears when the tumor has obtained already a significant size, patients frequently present with an advanced stage of disease. Hence, less than half of the newly diagnosed

patients, with limited locally advanced disease, are qualified for curative therapy, i.e. neoadjuvant chemoradiation (nCRT) followed by surgery. This combined treatment with curative intent results in a 5-year overall survival of approximately 50%.[12,13] A small proportion of patients with curable disease, however unfit for surgery are treated with definitive chemoradiation.[14] The remaining patients are not eligible for curative therapy and are therefore indicated for palliative treatment with chemotherapy, radiotherapy, endoscopic interventions (stent placement) and/or best supportive care,[14-16] resulting in a 2-year relative survival of nine percent.[4] Tumor staging is based on the tumor-node-metastasis (TNM) classification developed by the American Joint Committee on Cancer (AJCC), which takes into account the depth of tumor invasion, lymph node status, and the presence or absence of distant metastatic disease.[17]

Figure 2: Multimorphological sequence of the development of esophageal adenocarcinoma



Since the prognosis of advanced EAC is poor, patients diagnosed with BE are subjected to intensive endoscopic surveillance to identify patients with neoplastic progression in an early and curable stage. Current guidelines recommend endoscopic surveillance every 3-5 years in patients with non-dysplastic BE, every 6-12 months in patients with LGD and (endoscopic) treatment in patients with established HGD.[18] The histological diagnosis of LGD is currently the only accepted predictor for neoplastic progression, although it is hampered by sampling error and considerable interobserver variation. The cost-effectiveness of BE surveillance is under discussion given the relatively high prevalence of BE in the general population estimated at two percent,[19,20] the overall low incidence of neoplastic progression,[9,10] and the lack of discriminative tests for risk stratification. The

identification of additional risk factors, such as oncological biomarkers, might support the rationale for a BE surveillance program.

To identify oncological biomarkers that could serve as markers for risk stratification or as a target for therapy, an improved understanding of the molecular signature of BE and EAC is required. Therefore, there is a great urgency to clarify the genetic landscape of EAC. The availability of next-generation sequencing (NGS) has enabled examination of the genomes of human cancers at an exceptional scale. Large NGS studies on BE and EAC identified a broad mutational spectrum, with mutations most frequently discovered in the genes: *TP53, SYNE1*,[21-24] *CDKN2A, SMAD4, PIK3CA*,[23] *ARID1A*.[21,23,24] While, alterations in the *TP53* gene occur in the majority (approximately 75%) of EAC cases, and even an association between TP53 overexpression and an increased risk of developing EAC has been suggested,[25,26] only few other somatic alterations are shared between patients with EAC, representing substantial intertumor heterogeneity, which makes the discovering of a set of ubiquitous oncological biomarkers for EAC challenging. In addition, other studies suggest that even an extensive molecular variation is present within individual tumors, termed intratumor heterogeneity. For example, multiregion sequencing of clear-cell renal cell carcinomas revealed that 63-69% of the somatic mutations were heterogeneous and thus not detectable in every sequenced tumor region. Conceivably, intratumor heterogeneity may lead to an underestimation of the mutational spectrum of a tumor when using a single biopsy procedure and may present major challenges to personalized medicine and biomarker development.[27] Although the extent of intratumor heterogeneity in EAC has to be resolved, it might explain the difficulties in finding and validating clinically valuable oncological biomarkers.

To avoid the possible consequence of inter- and intratumor heterogeneity in the search for oncological biomarkers, patient specific biomarkers can be considered. Assessing the individual genetic susceptibility can help identifying high risk patients for malignant progression. From this perspective, the identification of single nucleotide polymorphisms (SNPs) is promising for identifying patients susceptible for invasive carcinoma. Recently, few genome-wide association studies (GWAS) were performed on BE and EAC samples. These studies revealed several SNPs to be associated with BE: rs9936833 (*FOXF1*), rs9257809 (*MHC*),[28] rs3072 (*GDF7*), and rs2701108 (*TBX5*).[29] Three SNPs were also found to be associated with EAC: rs10419226 (*CRTC1*), rs11789015 (*BARX1*), and rs2687201 (*FOXP1*). In addition, rs9936833 (*FOXF1*), previously associated with BE, was also associated with EAC, as well as the SNPs: rs2178146 and rs3111601 also located in *FOXF1*.[30] The identification of these SNPs provide evidence that the etiology of BE and EAC has a genetic component, although the exact role for SNPs has remained undisclosed. Inference of the underlying genes must be undertaken cautiously, and validation studies are required.

Another observation supporting the application of genetics in clinical decision-making is the reportedly increased risk for BE and EAC in patients with a positive family history. Although the vast majority of BE and EAC cases are sporadic and caused by somatic mutations, several families have been identified with clustering of BE and EAC. Previous studies introduced the definition familial BE (FBE), i.e. two or more first- or second-degree family members diagnosed with BE or EAC. It has been estimated that approximately seven percent of BE and EAC cases are considered familial.[31-33] Of all cancer types approximately five percent is caused by inherited factors. Regarding the concepts of inheritance, these familial cancer syndromes can be categorized as follows:

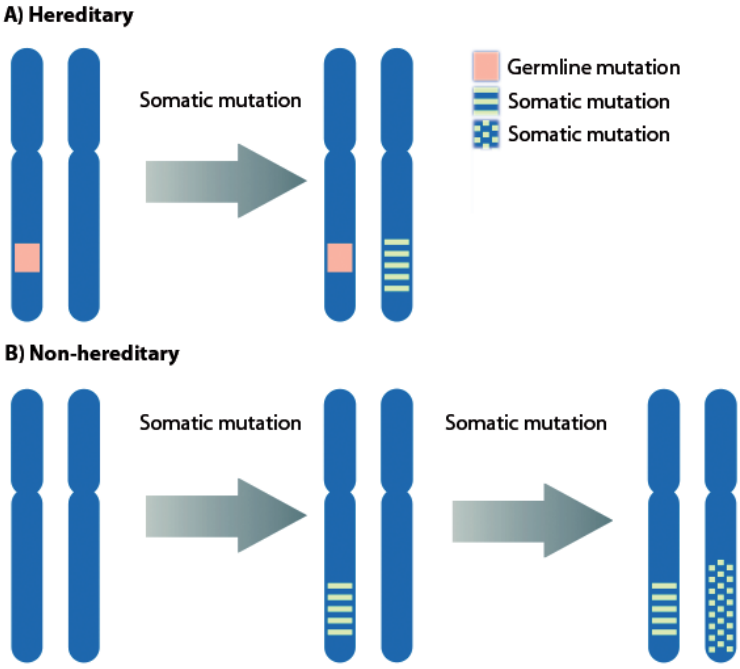
a) Autosomal dominant inheritance, in which a heterozygous germline mutation inherited from one of the parents, causes the cancer syndrome. For example, Multiple Endocrine Neoplasia Type 2 (MEN2) syndrome caused by a heterozygous germline mutation in the proto-oncogene *RET*.[34]
b) Autosomal recessive inheritance, which requires biallelic germline mutations inherited from both parents. For instance, biallelic germline mutations in MMR genes lead to constitutional mismatch repair-deficiency (CMMR-D).[35,36]
c) Biallelic inactivation of a tumor suppressor gene caused by one germline mutation inherited from one parent followed by a somatic second inactivating mutation in the wild type allele, based on the concept of the "Knudsons two hit" hypothesis (Figure 3) as described e.g. Lynch Syndrome (mismatch repair genes *MLH1*, *MSH2*, *MSH6*, *PMS2*)[37,38] in familial adenomatous polyposis (*APC*),[39-41] hereditary breast and ovarian cancer (*BRCA1*/*BRCA2*),[42,43] familial atypical multiple mole melanoma (P16),[44] and Li-Fraumeni syndrome (*TP53*).[45,46]

It can be hypothesized that the initiation of BE and EAC in several members of one family is caused by the presence of one or more inherited factors. The pattern of inheritance of most familial cancer syndromes is based on the concept of "Knudsons two hit" hypothesis, causing a phenotypic dominant inheritance pattern. It is likely that development of familial BE is caused by an inherited germline mutation in a (unknown) tumor suppressor gene followed by a somatic second inactivating mutation in the wild type allele causing biallelic inactivation.

Familial patients with BE and EAC are mostly diagnosed at a younger age,[32,33,47] which is in line with the presence of a germline mutation in a cancer-predisposing gene. However, of all patients diagnosed with EAC, regardless of the family history, a certain proportion of the patients are also diagnosed at a younger age, and since the rise in incidence has occurred among all age groups, EAC is no longer a disease exclusively of the elderly (www.cijfersoverkanker.nl), while the treating protocols are still based on the average cohort of EAC patients aged 70 years.[3] The effect of age on disease stage and survival of patients

with EAC is controversial [48-54] probably due to use of different definitions of young age, as well as the restriction of surgically treated EAC patients included in the studies. It can be anticipated that young EAC patients have a different clinical presentation as well as a different etiology of their tumor.

Figure 3: "Knudsons two hit" hypothesis



A) Most hereditary cancer syndromes are caused by an inherited germline mutation in a tumor suppressor gene followed by a somatic second inactivating mutation in the wild type allele. B) Most of the non-hereditary cancers are caused by two successive somatic mutations in a tumor suppressor gene.

Aims of the thesis

Esophageal cancer has traditionally been considered a sporadic disease of the elderly. However, recently it has become increasingly clear that this type of cancer comprises a relatively heterogeneous population of patients, including sporadic, hereditary or exceptionally young aged EAC cases. For these subgroups of patients with EAC, information regarding the clinicopathological characteristics, patient-tailored treatments and prognosis is currently lacking. It can be hypothesized that the molecular biology of these subgroups is distinct from a "conventional" EAC patient. Hence, the aim of this thesis was to elucidate the molecular biology of EAC by performing standard molecular analysis, i.e. whole-exome sequencing, targeted sequencing and Sanger sequencing on sporadic, familial and young EAC cases. As well as to describe the clinicopathological features of the familial and young subpopulations of EAC.

Outline of the thesis

Besides a general introduction (Part I, chapter 1) and a general discussion (Part V, chapter 10) this thesis is divided into three major parts: Part II: Molecular analysis of sporadic esophageal adenocarcinoma. Part III: Familial clustering of esophageal adenocarcinoma. Part IV: Young patients with esophageal adenocarcinoma.

Part II: Molecular analysis of sporadic esophageal adenocarcinoma
Besides the substantial intertumor heterogeneity identified in EACs by NGS, intratumor heterogeneity has been noticed in several other cancer types. In ***chapter 2*** the extent of intratumor heterogeneity in EACs is investigated. Based on the results of previous NGS studies on EACs, a panel of frequently mutated genes is designed and multiregional, targeted sequencing is performed on multiple tumor regions obtained from several EACs. As a consequence of intratumor heterogeneity the mutational spectrum of a tumor will be underrepresented, challenging the discovery of oncological biomarkers in EACs even more. Hotspot mutations in the promoter region of the *TERT* gene have been described in several cancer types. In ***chapter 3*** the occurrence of *TERT* promoter mutations in EAC is evaluated. Besides tumor specific biomarkers, patient specific biomarkers can be promising as well. SNPs, for example, can be helpful by identifying high risk patients for developing EAC from BE. In ***chapter 4*** the results of a validation study are presented, and the association between SNPs and the risk of EAC is studied in an independent case–control study.

Part III: Familial clustering of esophageal adenocarcinoma
Although the vast majority of BE and EAC cases are sporadic and caused by somatic mutations, over the last decades several families have been identified with clustering of BE and EAC. In ***chapter 5*** an overview is given of the occurrence and characteristics of patients from families with clustering of EAC. It can be hypothesized that the development of BE and EAC in more than two members of one family is caused by the presence of a germline defect. In ***chapter 6*** a family is described with clustering of BE and EAC, in which the germline DNA of an indicated index-patient is investigated by whole-exome sequencing and SNP array. In addition, potential germline variants are validated on germline and tumor DNA of the affected family members on a NGS platform.

Part IV: Young patients with esophageal adenocarcinoma.
Although EACs were previously thought to be a disease of the elderly, a small but clinically relevant group of younger patients suffering from EAC exists. The effect of age on disease stage and prognosis is controversial. In ***chapter 7*** and ***chapter 8*** the differences in clinicopathological characteristics, treatment and outcome between esophageal cancer patients (EAC and SCC) aged 50 years or younger and patients older than

50 years are evaluated in a retrospective analysis of a single center cohort study and a population-based study, respectively. Since it has been generally accepted that the transformation of a normal cell into a malignant cell and subsequently the outgrowth into a clinically manifest lesion takes several decades, it can be anticipated that cancers in young patients have gone through an accelerated transformation process. Hence, it can be hypothesized that esophageal cancer in young patients is a disease distinct from that in older patients. In ***chapter 9*** the differences in molecular biology, i.e. mutational load and profile, between young and older EAC patients aredescribed.
Finally, ***chapter 10*** includes a general discussion and the future perspectives, ***chapter 11*** is a summary of this thesis.

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PART II

MOLECULAR ANALYSIS OF SPORADIC ESOPHAGEAL ADENOCARCINOMA

Chapter 2

Molecular clonality analysis of esophageal adenocarcinoma by multiregion sequencing of tumor samples

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ABSTRACT

Background: Intratumor heterogeneity has been demonstrated in several cancer types, following a model of branched evolution. It is unknown to which extent intratumor heterogeneity is applicable to esophageal adenocarcinoma. Therefore, the aim of this study was to characterise intratumor heterogeneity in esophageal adenocarcinoma.

Methods: Multiregional targeted sequencing of four commonly altered genes was performed on 19 tumor regions collected from five esophageal adenocarcinomas. Alterations were classified as homogeneous or heterogeneous based on mutational and loss of heterozygosity analysis.

Results: Identical *TP53* mutations and homogeneously loss of heterozygosity of the *TP53* locus were identified in all separated tumor regions in each of five adenocarcinomas, and in the corresponding Barrett's esophagus and tumor positive lymph node of one primary tumor. Loss of heterozygosity of the *P16* locus was homogeneous among all tumor regions in four adenocarcinomas, and an identical pattern of loss of heterozygosity was present in the Barrett's esophagus. Loss of heterozygosity of the *SMAD4* and *APC* loci was observed in a heterogeneous pattern.

Conclusion: Known driver alterations, such as *TP53* and *P16* are homogeneously present within each adenocarcinoma, and therefore occur early during carcinogenesis and subsequently clonally expand throughout the entire tumor. However, loss of heterozygosity of the *SMAD4* and *APC* loci shows a heterogeneous pattern, indicating intratumor heterogeneity of esophageal adenocarcinoma.

INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) has been rising rapidly in Western countries over the last decades.[1-3] The major risk factor for EAC is Barrett's esophagus (BE),[4] a premalignant condition, in which the normal squamous epithelium of the distal esophagus has been replaced by columnar epithelium, including goblet cells. The risk of developing EAC from BE is estimated at 0.12-0.5% per year and follows a multimorphological sequence, in which metaplasia evolves into low-grade dysplasia (LGD), high-grade dysplasia (HGD) and ultimately into EAC.[5-7]

Previous results from whole-exome/genome sequencing of EACs demonstrated that these tumors bear a broad mutational spectrum, genes frequently altered are e.g. *TP53*, *P16, SMAD4* and *APC*.[8-10] Alterations in the *TP53* gene occur in the majority of EAC cases, however, only few other somatic alterations are shared between EACs, representing substantial intertumor heterogeneity. In addition, emerging evidence suggests that even an extensive molecular variation is present within individual tumors, termed intratumor heterogeneity.[11-13]

With the use of next-generation sequencing, intratumor heterogeneity has been demonstrated in several cancer types and even a model of branched evolution leading to intratumor heterogeneity has been advocated.[12,14] This model implicates that a single biopsy may not represent the total mutational burden of a tumor, which can explain the heterogeneous mutational spectrum described in EACs.[8] Conceivably, intratumor heterogeneity may lead to an underestimation of the mutational spectrum of a tumor using a single biopsy procedure, which might clarify the difficulties in finding and validating clinically valuable oncological biomarkers.

To date it is unknown to which extent this model of branched evolution for intratumor heterogeneity is applicable to EAC. Therefore, the aim of this study was to characterize intratumor heterogeneity in EAC, by multiregional targeted sequencing on a total of 19 tumor regions collected from five EAC patients, including adjacent BE in one of them, who underwent primary surgical resection with curative intent. To evaluate intratumor heterogeneity, DNA alterations were classified as homogeneous, i.e. present in all regions of the tumor, or heterogeneous defined as present in some regions or in only one region of the tumor.

METHODS

Patients and specimens

Resection specimens of distal esophageal or gastroesophageal junction adenocarcinomas were obtained from five patients treated between December 2001 and April 2002 at the Department of Surgery, Erasmus MC Cancer Institute, University Medical Center Rotterdam, The Netherlands. All patients underwent a transhiatal esophagectomy, and none of them received neoadjuvant chemo- and/or radiation therapy, which is part of the current standard treatment of EAC.[15]

Tissue samples, DNA isolation and immunohistochemistry

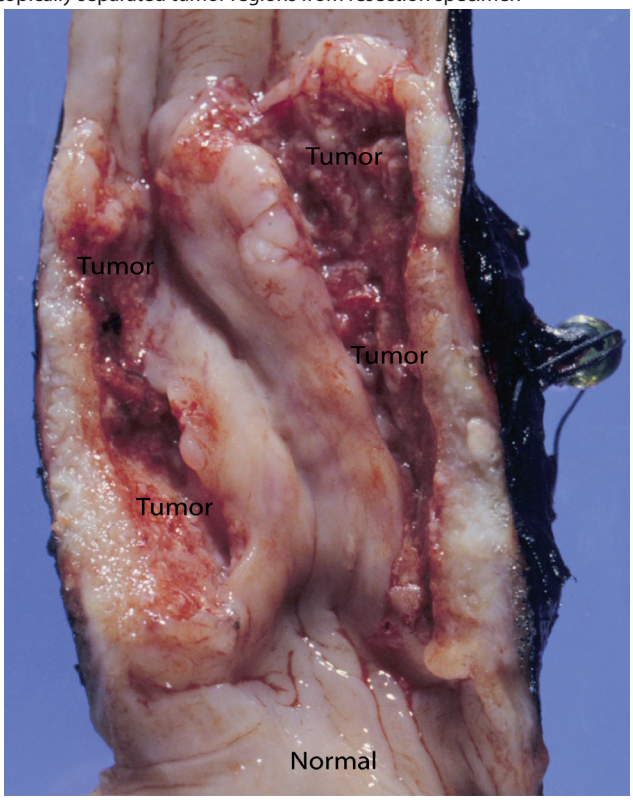
Tissue samples were derived from non-malignant and malignant areas in the fresh resection specimens and used according to the Code of Proper Secondary Use of Human Tissue in the Netherlands, as established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). In addition the study was approved by The Medical Ethical Committee of the Erasmus MC, University Medical Center, Rotterdam.

From each of the five EACs, multiple, macroscopically separated tumor regions were collected (Figure 1). In addition, from one resection specimen, samples from an area of premalignant BE (HGD) and from a tumor positive lymph node (TPLN) were available for study. Tumor tissue areas composed of at least 50% neoplastic cells (confirmed by a GI-pathologist) were manually microdissected from 10 to 15 hematoxylin-stained sections (4µm) of formalin-fixed paraffin-embedded tissue blocks. DNA was extracted using proteinase K and 5% Chelex 100 resin. TP53 immunohistochemistry was performed with the mouse monoclonal antibody Do-7 (Dako, Glostrup, Denmark), according to standard protocols.

Ion Torrent Personal Genome Machine

Ion semiconductor sequencing was performed on the Ion Torrent Personal Genome Machine (PGM) with a custom-made cancer panel on DNAs extracted from the macroscopically separated tumor regions according to the manufacturer's protocols. In short, libraries were made using the Ion AmpliSeq Library Preparation Kit. A template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion Sequencing Kit v2.0 on an Ion 316 chip. Data were analyzed with the Variant Caller v2.2.3-31149 (Life Technologies, Carlsbad, CA, USA). Variants were called when the position was covered at least 100 times. Sequences of all primers and probes are available on request.

Figure 1: Macroscopically separated tumor regions from resection specimen



Mutation analysis

The custom-made cancer panel contained the genes: *APC*, *P16*, *SMAD4* and *TP53*. Of all genes the total coding regions and the exon-intron boundaries were covered. Nonsynonymous somatic point mutations, insertions and deletions that change the protein amino acid sequence and splice site alterations were selected. In addition, variants present in the ESP6500si or 1000genomes databases in ≥1% were excluded. Variants present in at least 30% of the called reads were considered reliable and were validated by Sanger sequencing according to standard protocols.

Loss of heterozygosity analysis

To demonstrate loss of heterozygosity (LOH) of the genes of interest (*APC*, *P16*, *SMAD4*, *TP53*) amplicons for single nucleotide polymorphisms (SNPs, selected from dbSNP137) in these loci were added to the custom-made cancer panel. Per locus nine SNPs were selected, all with a minor allele frequency of ≥0.45 and located within the gene (three SNPs) and at positions ~300, ~600 and ~900 kb centromeric and telomeric of the gene. The SNPs were considered heterozygous if the percentage of the variant in the normal

DNA was within the range of 40-60%. When at least one SNP was present heterozygous in the normal DNA it was considered informative for LOH analysis. Variants present in the normal DNA in <10% were considered homozygous reference and therefore not informative for LOH analysis, as well as variants present in >90%, which were considered homozygous variant.

For tumor DNAs, of which the normal DNA was considered heterozygous, all SNPs with variants <40% or >60% were denoted as indicative for LOH. A sample was evaluated as having locus LOH when ≥50% of the informative SNPs in that locus demonstrated LOH (variant in tumor DNA <40% or >60%)[16]. In addition, identified tumor suppressor gene mutations also supplied information about possible LOH (loss of the wild type allele) by the relative frequency of the mutant DNA sequence compared to the normal wild type sequence in the tumor samples.

Intratumor heterogeneity analysis

Analysis of intratumor heterogeneity could be determined through mutation and LOH analysis. Homogeneity was signified by an identical mutation or the same pattern of LOH at a given gene identified in different tumor regions of the same tumor, whereas heterogeneity was signified by different mutations or various LOH patterns of a given gene in different regions of the same tumor.

RESULTS

Targeted sequencing with a custom-made cancer panel was performed on DNA isolated from 19 tumor regions derived from the resection specimen of five EAC patients, all male, with a median age at the time of diagnosis of 70 years (range 51-78 years). Two tumors were localised at the gastroesophageal junction and three in the distal esophagus within the background of BE. Four tumors were moderately differentiated, and one tumor was poorly differentiated. All resection margins were free of tumor cells. Three patients had tumor positive lymph nodes, without distant metastasis. Patient and tumor characteristics are listed in Table 1.

From one resection specimen, a region of BE (HGD) and a TPLN were analyzed with the custom-made cancer panel as well. Next-generation sequencing on the PGM and conventional Sanger sequencing revealed 21 mutations in the *TP53* gene, no mutations were identified in *APC*, *P16*, and *SMAD4*. Reliable data for the LOH analysis of *TP53*, *P16*, *SMAD4* and *APC* were obtained in 92%, 92%, 81% and 73% of the samples, respectively.

Table 1: Patient and tumor characteristics

Patient	Age (years)	Gender	Tumor type	Differentiation grade	Resection marge	TNM stage[$]	Tumor regions
EAC1	77	Male	GEJAC[*]	Moderate	R0[#]	pT3N1Mx	5
EAC2	62	Male	GEJAC	Moderate/Poor	R0	pT3N3Mx	4
EAC3	78	Male	BAC[^]	Moderate	R0	pT1N0Mx	3
EAC4	51	Male	BAC	Moderate	R0	pT3N1M0	3
EAC5	70	Male	BAC	Poor	R0	pT2N0Mx	4

* GEJAC = Gastroesophageal junction adenocarcinoma

^ BAC = Adenocarcinoma in Barrett's epithelium

R0 = Tumor free resection margin

$ According to the classification of the American Joint Committee on Cancer (AJCC) Staging Manual 7th edition.

All five EACs showed homogeneous *TP53* mutations: in each EAC the same *TP53* mutations (nonsynonymous somatic point mutations or splice site alterations) were identified in all investigated tumor regions, all mutations were previous described in esophageal cancer samples in the COSMIC database. In addition, the *TP53* mutation found in a primary EAC was also identified in the adjacent BE and TPLN samples. LOH of *TP53* occurred homogeneously in all informative regions of the EACs and in the paired EAC and BE samples with identical LOH patterns observed by SNP and/or mutation analysis (Figure 2). TP53 immunohistochemistry showed homogeneous and strong nuclear expression in all tumor regions and the BE sample of the four patients with *TP53* somatic missense mutations (Figure 3). The tumor cells of the four regions derived from the EAC with a *TP53* splice site mutation were all homogeneously negative for TP53 expression (EAC2).

LOH of *P16* occurred homogeneously in all regions of four informative tumors (EAC1, 2, 4, 5), and an identical LOH pattern of *P16* was observed in the paired tumor and BE samples (Figure 4). EAC1 and EAC3 showed homogeneous LOH of *SMAD4* in all tumor regions, two tumors showed different subclones with LOH or without LOH of *SMAD4* (EAC2 and 5), while in EAC4 no LOH of *SMAD4* was identified. Homogeneous LOH of *APC* was observed in two tumors (EAC1 and 2), different subclones with and without LOH of *APC* were observed in EAC3 and EAC4, whereas one tumor (EAC5) was not informative for the *APC* locus (Figure 2).

Figure 2: Overview of mutation and LOH analysis per tumor region of five EACs

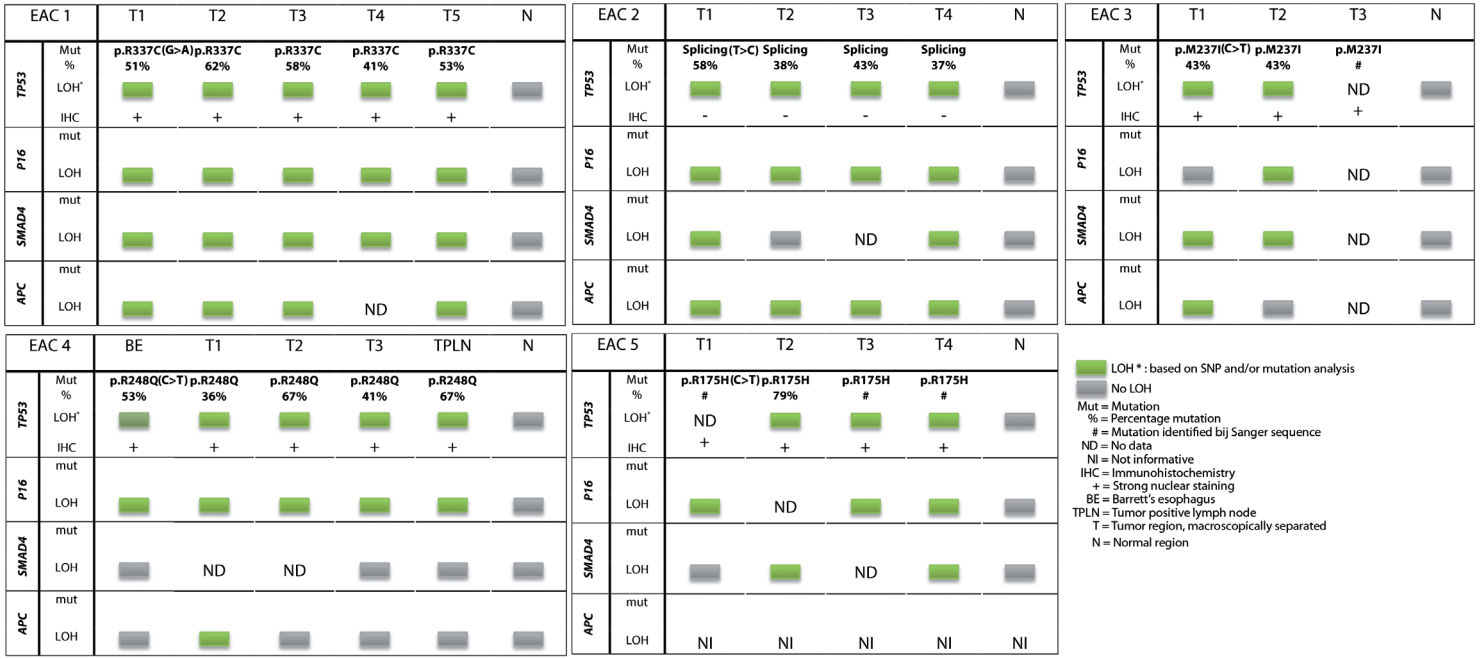
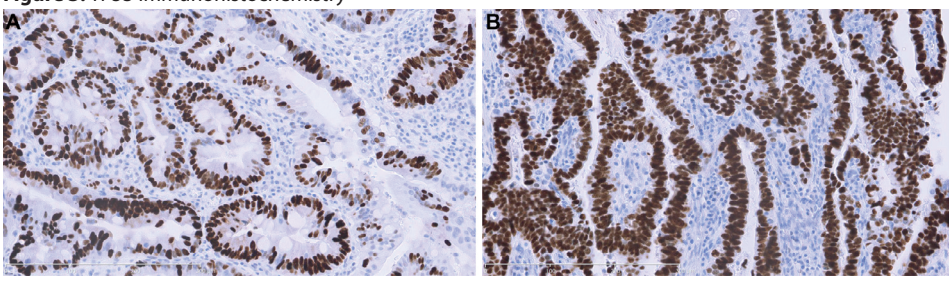


Figure 3: TP53 immunohistochemistry



TP53 immunohistochemistry showing strong and diffuse expression of TP53 in the premalignant Barrett epithelium (A) and the tumor region T3 (B) of EAC4, both samples bearing a nonsynonymous mutation: p.R248Q.

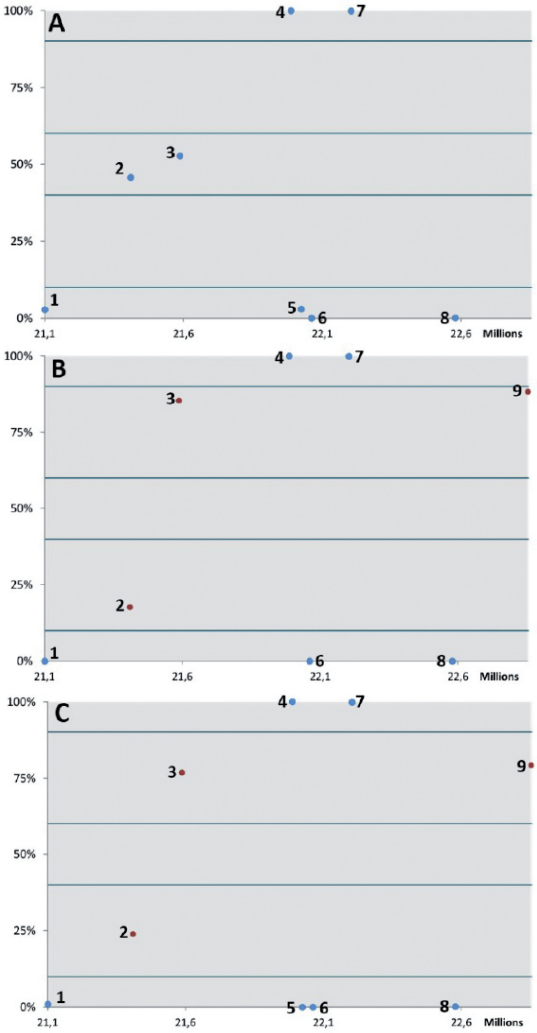


Figure 4: *P16* LOH analysis by the means of SNPs, which are represented as numbered dots.
On the horizontal axis the base position at chromosome 9 is denoted, and on the vertical axis the percentage of base variant. The SNPs in the normal sample (A) numbered with 2 and 3 have variant frequencies between 40-60% and are therefore considered heterozygous, the remaining SNPs with variant frequencies of <10% or >90% are considered homozygous, and not informative. In the premalignant Barrett epithelium (B) and the tumor region T5 (C) the SNPs 2,3 and 9 have variant frequencies between 10-40% and 60-90% and are therefore indicative for LOH. In addition, the pattern of LOH in the premalignant Barrett epithelium (B) and the tumor region T5 (C) is identical.

DISCUSSION

Recent data on next-generation sequencing of several tumor DNAs were supportive for the model of branched tumor evolution leading to intratumor heterogeneity.[11,13] This model describes a tumor as a tree structure, with the trunk representing early molecular alterations, which clonally expand and therefore are homogeneously present throughout the entire tumor, reflecting a process involved before and during tumor initiation and early development. The branches of the tree represent later molecular alterations, which as a result are only present in different subclones of the tumor, contributing to intratumor heterogeneity and shaping the genome during tumor maintenance and progression.[12,14] The extent of this branched evolution model leading to intratumor heterogeneity in EACs is as yet unknown.

Therefore, the aim of the current study was to characterize intratumor heterogeneity in EACs, by performing multiregion, targeted sequencing on 19 tumor regions derived from five surgical resected EACs. Targeted sequencing of the commonly altered genes *TP53*, *P16*, *SMAD4* and *APC* was performed. This analysis revealed a clonal origin of *TP53* alterations: all five EACs where homogeneous with regard to *TP53* mutations and LOH of the *TP53* locus, in addition the same mutation and pattern of LOH in *TP53* was observed in a paired TPLN of one EAC. These results indicate that *TP53* mutation and LOH of the *TP53* locus are relatively early events in EAC tumorigenesis and clonally expand throughout the entire tumor. In concordance with this is the finding of the same *TP53* mutation, identical pattern of LOH, and comparable strong nuclear TP53 expression in the paired primary tumor and BE case.

Mutations in the *P16* gene were not observed in this study, however LOH of the *P16* locus was identified in all EACs. Four EACs had a homogeneous *P16* locus LOH pattern, while one EAC was heterogeneous for *P16* LOH. In addition, an identical pattern of *P16* locus LOH was present in the paired primary EAC and BE case, suggesting that LOH of *P16* is an early alteration, clonally expanding throughout the tumor. No mutations were identified in *SMAD4* and *APC*, but LOH of these genes was observed in a heterogeneous pattern within the EACs. Some EACs showed homogeneous LOH of *SMAD4* and/or *APC*, one EAC showed no LOH of *SMAD4* at all, while the remaining EACs showed different subclones: some with LOH of *SMAD4* and/or *APC* and others without LOH of these loci. In addition, no LOH of *SMAD4* and *APC* was found in the BE sample, indicating a late occurrence of these alterations, reflecting intratumor heterogeneity of EACs.

Taken together, the results of the current study suggest that both homogeneous and heterogeneous intratumoral molecular alterations are present in EACs. A homogeneously

present *TP53* mutation and LOH of the *TP53* locus as well as LOH of the *P16* locus in the primary tumor were also found in the adjacent BE sample, indicating that the earliest molecular alterations can already be present in the premalignant lesion (BE) and from there on clonally expand throughout the entire tumor. Temporarily, no multiregion sequencing of EACs was performed before. Alterations in *TP53* were described previously in the sequential of BE (HGD) and EAC, and in addition were found to be present in a major clone, which also indicates clonal expansion of *TP53* as an early alteration.[17]

The presence of alterations in BE is in accordance with previous studies, showing that multiple BE crypts contain different clones with alterations competing with each other,[18,19] of which one progenitor clone with a selective growth advantage will expand clonally and will create a field in which other (pre)malignant alterations might arise.[19] Several studies on *P16* have reported clonal expansion of *P16* alterations in all regions of BE segments, and it has been suggested that expansion of *TP53* alterations occur only in a background of *P16* altered clones.[20] However, others described progenitor clones containing *TP53* alterations alone or in combination with *P16* alterations present at many levels of BE segments.[21] Leedham et al.[18] concluded that BE was genetically heterogeneous, however they observed identical *TP53* mutations in multiple BE crypts, indicating widespread and far-reaching clonal expansion as a consequence of the strong selective advantage that absence of *TP53* function supposedly provide. Recently, a study on cytochrome c oxidase (CCO) deficient cells in BE confirmed the concept of clonal expansion of BE, probably by fission of BE glands.[22]

Conceivably, intratumor heterogeneity can cause tumor sample bias using single biopsy approaches, since it may underestimate the mutation spectrum of a tumor. This could contribute to difficulties in identifying and validating biomarkers, which are desirable to identify BE patients with a high risk for neoplastic progression. Furthermore, intratumor heterogeneity may contribute to therapy resistance: if the actionable target of treatment is only present in a subclone of the tumor, than targeting this genetic alteration may not have an impact on the entire tumor. This concept might explain the diversity in responders and non-responders after neoadjuvant chemoradiotherapy (nCRT) in EACs.[23]

CONCLUSIONS

Recent studies on EAC reveal extensive intertumor heterogeneity concerning molecular alterations with different studies showing different recurrently mutated genes. Therefore, the genes found frequently mutated in several studies were investigated in the current study.[8-10,17] Even though the sample size is small, and targeted sequencing of

only commonly mutated genes was performed, the current study provides evidence that although intratumor heterogeneity is present in EACs, known driver alterations in *TP53* and *P16* were homogeneously present in all five primary EACs, indicating clonal cellular expansion. Studies with larger cohorts and extensive genome sequencing are needed to fully characterize intratumor heterogeneity in EACs and in addition, to understand the impact of intratumor heterogeneity on current clinical outcome of both BE and EAC patients.

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Chapter 3

Letters to the editors: Absence of *TERT* promoter mutations in esophageal adenocarcinoma

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DEAR EDITORS:

Telomerase reverse transcriptase (*TERT*) is the catalytic subunit of telomerase, which maintains the telomere length at the end of the chromosomes.[1] Recently, promoter mutations in the *TERT* gene have been described in melanomas, bladder cancer, gliomas, and thyroid cancers.[2] The two most common mutations, 1,295,228 C>T (C228T) and 1,295,250 C>T (C250T), have been demonstrated to confer increased transcriptional activity on the *TERT* promoter and probably represent a novel mechanism of telomerase activation in human tumorigenesis.[3] Three additional mutations: 1,295,228 C>A (C228A), 1,295,228/1,295,229 CC>TT (C228T/C229T) and 1,295,242/1,295,243 CC>TT (C242T/C243T) have been rarely described.[2,4]

Zhoa et al. reported a very low frequency (1.6%) of *TERT* promoter mutations in esophageal squamous cell carcinomas (ESCC),[5] which is the most common histological type of esophageal cancer in East Asian countries. Conversely, the incidence of ESCC in the Western world is declining while that of esophageal adenocarcinoma (EAC) is remarkably increasing. This rising incidence together with a poor prognosis of EAC represents a growing health concern.[6] Hence, there is great urgency to clarify the genomic alterations underlying EAC to better comprehend the pathogenesis of these tumors and to encourage development of new diagnostic, prognostic, therapeutic and prevention strategies. Previous studies have identified frequent mutations in *TP53* and somatic mutations in *CDKN2A*, *ARID1A*, *PIK3CA* and *SMAD4*,[7] but widespread use of these markers in clinical practice has not taken place. To investigate whether mutations in the *TERT* promoter play a role in EAC tumorigenesis, the occurrence of *TERT* promoter mutations in EAC was evaluated.

This study included 90 EAC samples. The tumor tissue samples were obtained from the resection specimens of patients with distal EAC or gastroesophageal junction adenocarcinoma and were used according to the Code of Proper Secondary Use of Human Tissue in the Netherlands established by the Dutch Federation of Medical Scientific Societies. Of each patient the clinical information was accessible (Table 1). Ten to fifteen hematoxylin-stained sections (4 µm) from formalin-fixed paraffin-embedded tissue blocks were used for manual microdissection of tissue areas composed of a high percentage (at least 60%) of neoplastic cells. DNA was extracted using proteinase K and 5% Chelex 100 resin. In addition, ten human EAC cell lines, which were authenticated by DNA short tandem repeat profiling, were included in the study,[8] as well as four bladder tumor samples, which were previously tested positive for the mutations C228T, C228A, C242T/C243T and C250T.[4]

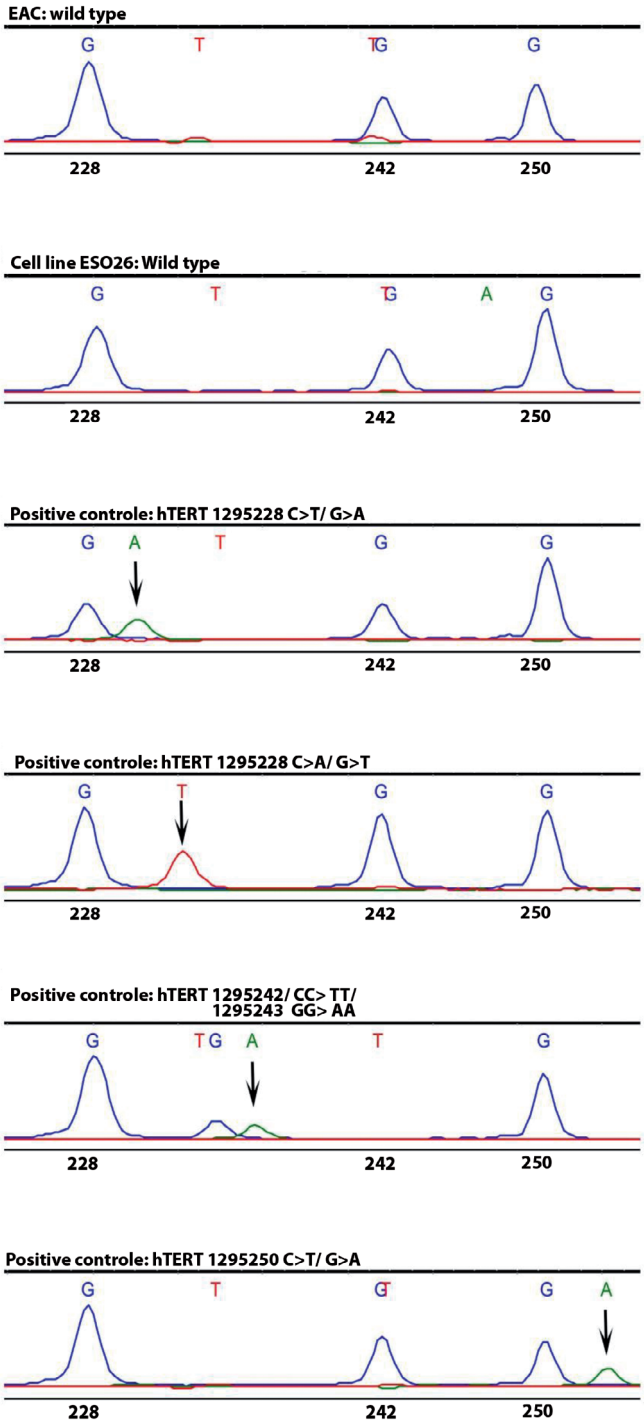
Table 1: Patients characteristics

Patients characteristics	Total (%)
All cases	90
Mean Age (sd)	63.23 (11.21)
Sex	
Male	75 (83.3)
Female	15 (16.7)
Tumor grade	
Well	4 (4.4)
Moderate	48 (53.3)
Poor / undifferentiated	38 (42.2)
TNM-stage	
IA	13 (14.4)
IB	4 (4.4)
IIA	1 (1.1)
IIB	22 (24.4)
IIIA	18 (20.0)
IIIB	14 (15.6)
IIIC	16 (17.8)
IV	2 (2.2)

Primers were used to amplify the regions of the previously described *TERT* promoter mutations: C228T, C228A, C242T/C243T, and C250T (Hg19). The amplified fragments of the tumors were analyzed by SNaPshot using the ABI Prism SNaPshot Multiplex Kit (Life Technologies).[4] Data analysis was performed using GeneMarker Analysis Software version 2.4.0 (Softgenetics). *TERT* promoter mutations were successfully evaluated in 90 EAC samples, ten verified human EAC cell lines, and four positive bladder tumor samples, the *TERT* promoter mutations C228T, C228A, C242T/C243T, and C250T were not identified in the 90 EAC samples and neither in the ten verified human EAC cell lines. However, the mutations C228T, C228A, C242T/C243T and C250T were verified properly in the positive bladder tumor samples (Figure 1).

Previous studies support the hypothesis that *TERT* mutations are mainly associated to tumors derived from tissues with relative low rates of self-renewal under normal circumstances, such as melanomas, bladder cancer, gliomas and thyroid cancers.[2,9] Cancers that originate from tissues that constantly self-renew, such as cancers of the epithelia of the gastrointestinal tract, would be unlikely to harbor telomere-maintaining mutations, since telomerase is already activated in their precursor cells.[9] In addition, it has been suggested that these *TERT* mutations can result from environmental factors such as ultraviolet radiation and chemical carcinogens, which also could explain the high frequency of *TERT* mutations in melanoma, bladder cancers and squamous cell carcinoma of the tongue.[2]

Figure 1: Results of SNaPshot analysis



In summary, our results demonstrate that *TERT* promoter mutations do not occur in EAC. Obviously, these tumors have alternative mechanisms to maintain telomere lengthening, and therefore would be less likely to benefit from activating mutations in *TERT*. This suggests that *TERT* promoter mutations are not likely to play a crucial role in the development and progression of EAC.

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Chapter 4

Single nucleotide polymorphisms in *CRTC1* and *BARX1* are associated with esophageal adenocarcinoma

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ABSTRACT

Objective: Recently, single nucleotide polymorphisms (SNPs) associated with esophageal adenocarcinoma (EAC) and Barrett's esophagus (BE) were identified: rs10419226 (*CRTC1*), rs11789015 (*BARX1*), rs2687201 (*FOXP1*), rs2178146 (*FOXF1*), rs3111601 (*FOXF1*), and rs9936833 (*FOXF1*). These findings indicate that genetic susceptibility could play a role in the initiation of EAC in BE patients. The aim of this study was to validate the association between these previously identified SNPs and the risk of EAC in an independent and large case–control study.

Design: Six SNPs found to be associated with EAC and BE were genotyped by a multiplex SNaPshot analysis in 1,071 EAC patients diagnosed and treated in the Netherlands. Allele frequencies were compared to a control group derived from the Rotterdam Study, a population-based prospective cohort study (n = 6,206). Logistic regression analysis and meta-analysis were performed to calculate odds ratios (OR).

Results: Rs10419226 (*CRTC1*) showed a significantly increased EAC risk for the minor allele (OR = 1.17, p= 0.001), and rs11789015 (*BARX1*) showed a significantly decreased risk for the minor allele (OR = 0.85, p= 0.004) in the logistic regression analysis. The meta-analysis of the original GWAS and the current study revealed an improved level of significance for rs10419226 (*CRTC1*) (OR = 1.18, p= 6.66×10^{-10}) and rs11789015 (*BARX1*) (OR = 0.83, p= 1.13×10^{-8}).

Conclusions: This independent and large Dutch case–control study confirms the association of rs10419226 (*CRTC1*) and rs11789015 (*BARX1*) with the risk of EAC. These findings suggest a contribution of the patient genetic make-up to the development of EAC and might contribute to gain more insight in the etiology of this cancer.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is one of the rapidly rising cancers in the Western world.[1-3] Despite improvements in multimodality treatment, the prognosis for EAC remains disconcerting.[4] The major risk factor for EAC is the premalignant lesion Barrett's esophagus (BE), in addition to age, male gender, and Caucasian ethnicity.[5] As a consequence of gastroesophageal reflux (GER), the normal squamous epithelium of the lower esophagus can be replaced by columnar intestinal cells, including goblet cells, representing BE. Per year 0.12–0.5% of the patients diagnosed with BE will develop EAC, following a multimorphological sequence, in which intestinal metaplasia evolves to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and ultimately to invasive adenocarcinoma.[6-8]

The prevalence of BE in the general population is estimated at two percent,[9] and among patients with GER even at ten percent.[10] Since the prognosis of advanced EAC is relatively poor,[3] patients with BE are subjected to intensive endoscopic surveillance with biopsy sampling to identify those patients with neoplastic progression at an early stage.[11] However, because the annual risk of developing EAC from BE is relatively low, most BE patients will not progress to cancer and do not benefit from this surveillance.[6,7]

It can be anticipated that genetic susceptibility could play a role in the initiation of EAC in BE patients. From this perspective the identification of single nucleotide polymorphisms (SNPs), which identifies high risk patients, could make the surveillance of BE patients more cost-effective and could be helpful by diagnosing patients with EAC in an early and curable stage, which will increase the prognosis remarkably.

Recently, the first Genome-wide association study (GWAS) on EAC and the premalignant lesion BE was published. This study revealed three SNPs associated with EAC: rs10419226 (*CRTC1*), rs11789015 (*BARX1*), and rs2687201 (*FOXP1*).[12] In addition, evidence was found that rs9936833 (*FOXF1*), previously associated with BE,[13] was also associated with EAC and that the SNPs: rs2178146 and rs3111601 near rs9936833 had even a stronger association with EAC.[12] Rs9936833 was first identified in a GWAS on BE performed by Su et al. in 2012, simultaneously rs9257809 (*MHC*) was found to be associated with BE.[13] The aim of the present study was to validate the association between these six previously identified SNPs and the risk of EAC in an independent and large case–control study.

MATERIALS AND METHODS

Study population

Patients diagnosed with an adenocarcinoma of the distal esophagus or esophagogastric junction (EGJ) and treated at the Department of Surgery, Erasmus MC Cancer institute, University Medical Centre, Rotterdam, between January 1996 and December 2013 (n = 761) were selected for the study. In addition, patients treated in the Academic Medical Centre at the university of Amsterdam, between 1994 and 2004 were included as well (n = 310). All patients underwent an esophagectomy with curative intention.

The control group was derived from the Rotterdam Study, a population-based prospective cohort study. In brief, this is an ongoing large population-based cohort study, which started in January 1990.[14] All inhabitants, who were aged 55 years and older, living in Ommoord, a district in Rotterdam, The Netherlands, were invited to participate. This population-based control group provided reference groups of allele frequencies, which reflect the local general European population. Individuals in the control group diagnosed with EAC were excluded. Patients and controls were of European descent.

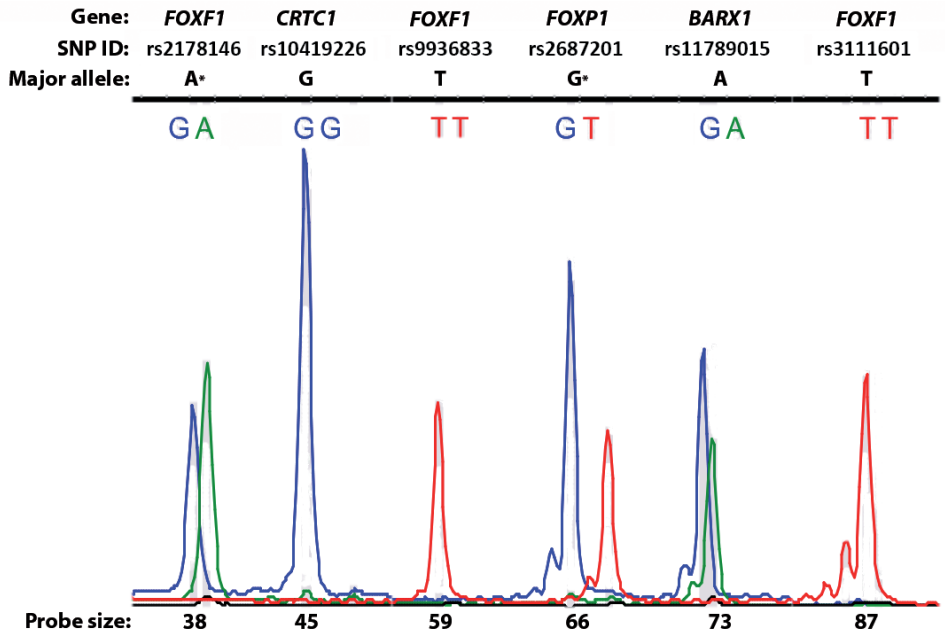
DNA isolation

For cases, the tissue samples were obtained from the resection specimens and used according to the Code of Proper Secondary Use of Human Tissue in the Netherlands established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). Non-malignant tissue from the resection specimen; tumor negative lymph nodes or tumor negative resection margins, confirmed by an experienced GI-pathologist, were macrodissected from microscopic sections of fresh frozen-or formalin-fixed paraffin -embedded tissues. DNA was extracted using proteinase K and 5% Chelex 100 resin. For controls, genomic DNA was extracted from whole blood samples using standard methods.[15]

Genotyping

For cases, a multiplex SNaPshot assay was designed: multiplex polymerase chain reaction was used to amplify the regions of the SNPs: rs2178146 (*FOXF1*), rs10419226 (*CRTC1*), rs9936833 (*FOXF1*), rs2687201 (*FOXP1*), rs11789015 (*BARX1*), and rs3111601 (*FOXF1*) (Hg19). The amplified fragments of the normal DNAs were analyzed by SNaPshot using the ABI Prism SNaPshot Multiplex Kit (Life Technologies, Carlsbad, CA, USA). Data analysis was performed using GeneMarker Analysis Software version 2.4.0 (Softgenetics, State College, PA, USA) (Figure 1).[16] Primers and probes sequences are listed in Supplementary Table 1.

Figure 1: Results of SNaPshot analysis



Results of SNaPshot analysis of DNA from a patient with esophageal adenocarcinoma: SNPs rs2178146, rs2687201, and rs11789015 are heterozygous, all other SNPs are homozygous for the major allele.
* Reverse probes used.

For controls, genome-wide SNP genotyping was performed using Infinium II assay on the HumanHap550 and Human 660-quad Genotyping BeadChips (IlluminaInc, San Diego, CA, USA). Approximately 30 million SNPs were imputed using 1000G Phase 1 v3 populations as reference.[17] The imputations were performed using MACH software (http://www.sph.umich.edu/csg/abecasis/MACH/). All variants tested here had an imputation quality of 0.9 or higher, suggesting near perfect imputation. Best-guess genotypes were used for the analyses.

Statistical analysis

Departures from Hardy–Weinberg equilibrium were tested for using the goodness-of-fit χ^2 test. Logistic regression analysis were used to calculate odds ratios (OR) with 95% confidence intervals (95% CI). The major allele homozygous was set as a reference and was compared with the minor allele. P values were corrected for multiple testing using Bonferroni correction ($p = 0.05/6 = 0.008$) before considered significant. Logistic regression was performed with SPSS version 20.0 (SPSS, Chicago, IL, USA) and meta-analysis of the original GWAS,[12] and the current study was performed using R library rmeta.[18] A fixed-effects inverse-variance meta-analysis was performed.

RESULTS

Study population

A total of 1,071 cases were initially analyzed, due to technical failure of 972 cases reliable data were obtained that was compared with 6,206 controls regarding the six previously mentioned SNPs. Clinical data were available of 550 cases from Rotterdam. The median age of these patients was 63 years (Range: 19–84 years) and 80% was male. Almost half of the patients received some form of neoadjuvant therapy and all patients underwent esophagectomy. Ninety percent was diagnosed with an invasive adenocarcinoma, of which 35% arose clearly from BE. Most tumors were located in the distal esophagus (41.3%) or at the EGJ (40.5%). The majority of the tumors were moderately or poorly differentiated (36.5% and 45.1%, respectively). The most common pathological tumor stage (pT) was pT3 (53.8%) and half of the patients appeared to have positive lymph nodes (pN1-3), whereas four patients had distant metastasis (pM) according to the TNM-classification of the American Joint Committee on Cancer Staging Manual 7th edition. After surgery, 40.4% of the cases developed recurrence of disease (locoregional disease or distant metastasis). The mean overall survival of the 550 cases was 53.8 months (95%CI: 49.5–58.2) and the 5-year overall survival was estimated at 38.2%.

Allelic association analysis

The distribution of genotype frequencies for the investigated SNPs was consistent with Hardy–Weinberg equilibrium ($p > 0.05$), except for *FOXF1* rs3111601 ($p = 0.013$). The allelic association of the six SNPs with EAC showed significantly increased risk for the minor allele of rs10419226 (*CRTC1*) (OR = 1.17, $p = 0.001$) and significantly decreased risk for the minor allele of rs11789015 (*BARX1*) (OR = 0.85, $p = 0.004$). None of the other four SNPs were significant in the currently studied population, although direction and effect size were consistent with previous GWAS results. The meta-analysis of the original GWAS and the current study revealed more accurate effect estimate and improved level of significance for rs10419226 (*CRTC1*) (OR = 1.18, $p = 6.66 \times 10^{-10}$) and rs11789015 (*BARX1*) (OR = 0.83, $p = 1.13 \times 10^{-8}$), and in addition for rs2178146 (*FOXF1*) (OR = 0.87, $p = 9.37 \times 10^{-7}$), while there was no significant allelic association with EAC in the currently studied population (Table 1).

Genotypic association analysis

Genotypic association analysis showed a dose effect for the significantly associated SNPs. The GT genotype for rs10419226 (*CRTC1*) increased the risk of EAC in comparison with the GG genotype (OR = 1.07, $p = 0.428$), which became significant for the TT genotype (OR = 1.39, $p = 0.001$). The rs11789015 (*BARX1*) AG genotype decreased the risk of EAC in comparison with the AA genotype (OR = 0.95, $p = 0.456$), this decrease in risk became significant for the genotype GG (OR = 0.57, $p = 3.68 \times 10^{-4}$) (Supplementary Table 2).

Table 1: Logistic regression and meta-analysis.

							Current		Levine et al. 2013		Combined	
SNPID	chr	position	gene	Major	Minor	MAF	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
rs2178146	16	86463695	*FOXF1*	T	C	0.43	0.91 (0.83-1.00)	0.060	0.85 (0.79-0.91)	$4.37*10^{-6}$	0.87 (0.82-0.92)	**$9.37*10^{-7}$**
rs10419226	19	18803172	*CRTC1*	G	T	0.45	1.17 (1.07-1.29)	**0.001**	1.19 (1.11-1.27)	$8.35*10^{-7}$	1.18 (1.12-1.25)	**$6.66*10^{-10}$**
rs9936833	16	86403118	*FOXF1*	T	C	0.35	1.03 (0.93-1.14)	0.589	1.16 (1.05-1.27)	$2.06*10^{-3}$	1.10 (1.03-1.18)	0.006
rs2687201	3	70928930	*FOXP1*	C	A	0.32	1.07 (0.97-1.18)	0.193	1.20 (1.12-1.29)	$5.76*10^{-7}$	1.15 (1.09-1.22)	$1.76*10^{-6}$
rs11789015	9	96716028	*BARX1*	A	G	0.29	0.85 (0.76-0.95)	**0.004**	0.81 (0.75-0.88)	$1.80*10^{-7}$	0.83 (0.77-0.88)	**$1.13*10^{-8}$**
rs3111601	16	86400081	*FOXF1*	T	C	0.29	1.01 (0.91-1.13)	0.813	1.16 (1.08-1.24)	$8.49*10^{-5}$	1.12 (1.06-1.18)	$1.34*10^{-4}$

MAF: minor allele frequency, OR (95% CI): odds ratio (95% confidence interval). In bold: p-value passing multiple testing correction (current) or improving compared to original GWAS

DISCUSSION

In this study two SNPs, rs10419226 (*CRTC1*) and rs11789015 (*BARX1*), were replicated to be associated with EAC. In the performed meta-analysis including the data of the EAC cohort from the original GWAS,[12] the level of significance was improved compared with the original findings of the GWAS, confirming the association of *CRTC1* and *BARX1* with EAC. This was expected, since the present case–control study revealed a significantly increased risk of EAC for the minor allele T of rs10419226 (*CRTC1*) and a significantly decreased risk of EAC for the minor allele G of rs11789015 (*BARX1*). Both SNPs showed a dose-effect in the genotypic analysis:two minor alleles gave a stronger effect than one minor allele.

In addition, rs2178146 (*FOXF1*) showed an improved level of significance in the meta-analysis while it did not reach significance in the allelic analysis of the present study cohort. This could be explained by a smaller sample size compared to the population used for the original GWAS resulting in a decreased power to detect the association. However, because all cases were retrieved clinically, and controls with EAC were excluded, no attenuation had taken place. Only two of the six previously identified SNPs, appeared to be significantly associated with EAC in the current study, however, a consistency of the direction of effect and effect size was seen for all variants, suggesting that all these variants may play a role in EAC.

Rs10419226 is an intronic variant in the *CRTC1* gene, which is encoding for CREB-regulated transcription co-activator that has been found previously to be associated with the oncogenic activity. The down-regulation or loss of *LKB1*, a tumor suppressor kinase, activates *CRTC1* signaling and the transcriptional activity of the downstream targets of *CRTC1*. In addition, altered *LKB1*/*CRTC1* signaling has been demonstrated to induce a migratory and invasive phenotype in esophageal cancer cell lines.[19,20]

Rs11789015 is located in an intron of *BARX1*, a homeobox transcription factor. The homolog of *BARX1* has been found to be associated with the differentiation of the esophagus and trachea in developing mouse embryos and in addition to be associated with the down-regulation of the Wnt pathway in stomach morphogenesis and differentiation.[21]

Identifying these SNPs associated with EAC suggests that genetic susceptibility might play a role in the initiation of EAC and could be of importance for the surveillance of BE patients. Since the prevalence of BE patients is valued at two percent in the general population,[9] and the annual risk of developing EAC from BE is estimated at 0.12–0.5%,[6,7] most patients with BE will not benefit from endoscopy surveillance. However because of

the relatively poor prognosis of EAC,[3] it is of utmost importance to diagnose EAC patients in an early and curable stage of the disease. Therefore, it could be of additional value to incorporate SNPs associated with EAC in the surveillance program of BE, in order to only select the high risk patients for developing EAC.

CONCLUSIONS

This independent and large Dutch case–control study replicated the association of rs10419226 (*CRTC1*) and rs11789015 (*BARX1*) with the risk of EAC. These findings indicate a possible genetic contribution to the development of EAC and might contribute to gain more insight in the etiology of this cancer. In addition, SNPs associated with EAC could be helpful by identifying patients at increased risk for malignant progression during surveillance and/or screening programs aimed to improving the survival of these patients by diagnosing EAC in an early and curable stage.

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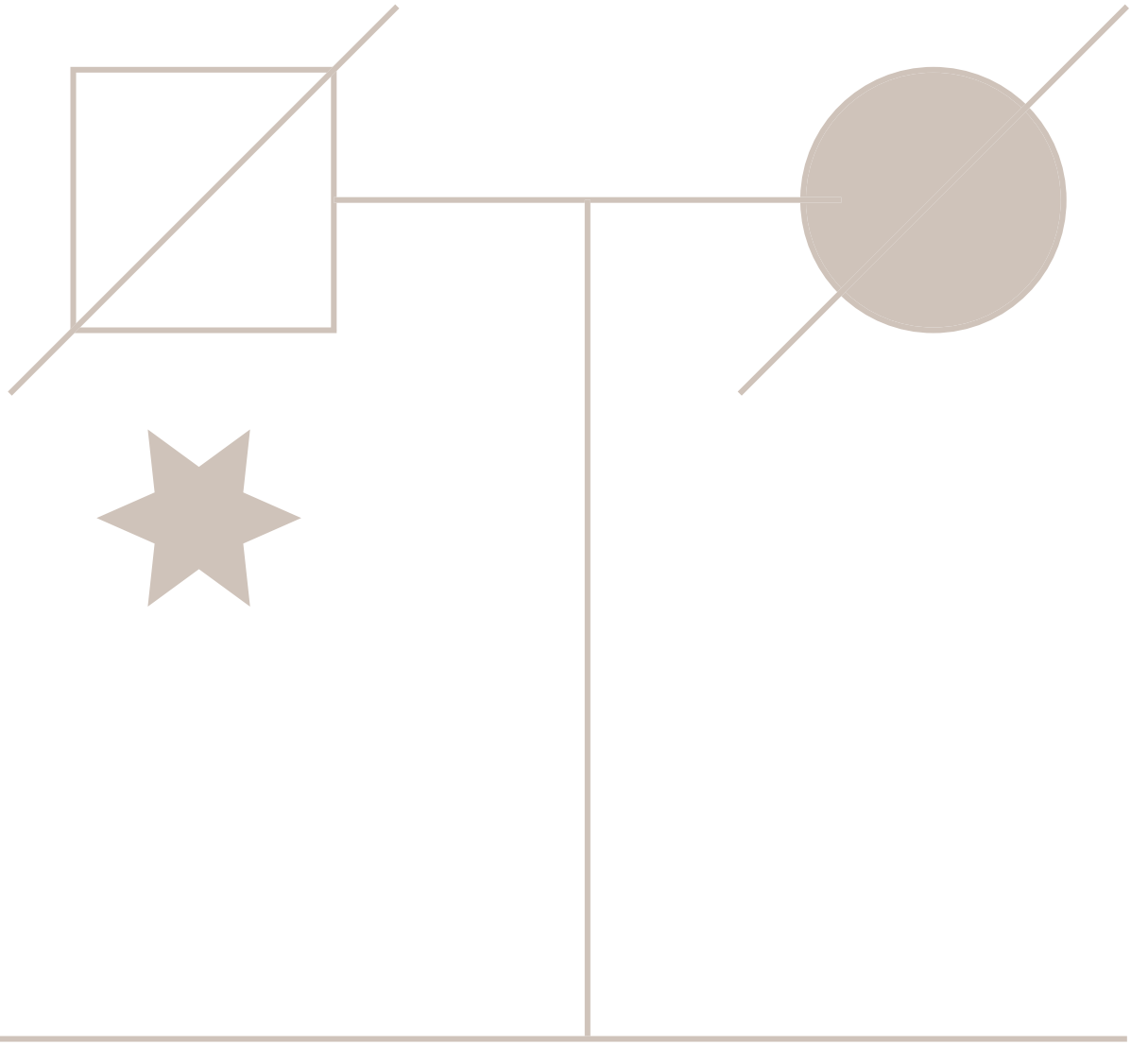
Supplementary Table 1: Primers and probes

Gene	SNP ID	Forward primers	Reverse primers	Forward probes	Reverse probes	Size
FOXF1	Rs2178146	5′-ATAGGGAGGTGCTCGGCAG-3′	5′-TCTTGATTGTTAGGGCAGGC-3′	5′-CCGTGAGTGTGGTCAACTG-3′	5′-CAGGTAAGCAGGAAGGCC-3′	38
CRTC1	Rs10419226	5′-CTGGTGCTACAGGTTCTGTCAG-3′	5′-ATCATATTGATGACGGTGAGGG-3′	5′-GCCACTGGCTAAAGTCACAAAT-3′	5′-ACCACAAAGTGAGGGGCATT-3′	45
FOXF1	Rs9936833	5′-CGATAAACTCAGATTGGAACACAG-3′	5′-AATGGAAATTGTTCAGGATCATCTAC-3′	5′-GAGGGTGGTAGAGAGTGGCA-3′	5′-CTTTAACAAAACAGAAGTCAAAAGCA-3′	59
FOXP1	Rs2687201	5′-CACCTACCAGCAGGTTCTCC-3′	5′-TCTCCCCTTTACCACTGCAC-3′	5′-CTCCAGTGACAGTGACAGATTCTAT-3′	5′-GACCACTGTGGTCTTTTCCAT-3′	66
BARX1	Rs11789015	5′-CGGAAATTCCAAAGTACCCTG-3′	5′-CGGGCACACAATCATTTTAGG-3′	5′-CTGGAAATTATTGTTCACGTTTTCT-3′	5′-ATGGGGAAGCGTCTGAAAA-3′	73
FOXF1	Rs3111601	5′-CCCAGCTAAATATTTTAAGAACATAATTTC-3′	5′-CCTAAAAATGGCAATTACATAAATAACTAG-3′	5′-CTCACCATACAAAGCATTTACTTG-3′	5′-AAATAAAGGATGGGCCATATCC-3′	87

Supplementary Table 2: Genotypic association analysis

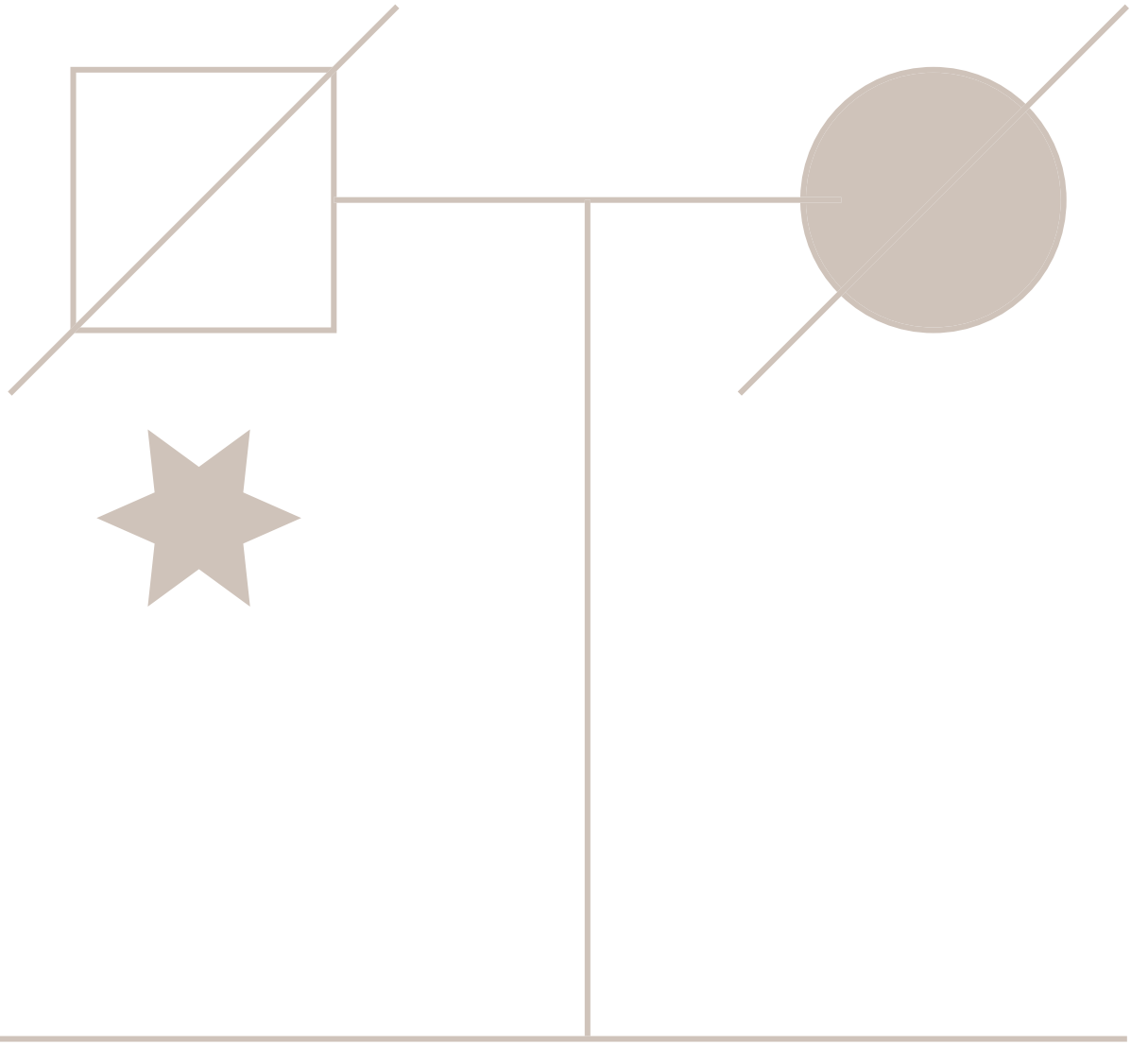
	EAC N=972 (%)	Controls N=6,206(%)		
SNPID			OR (95%CI)	*p*-value
rs2178146				
TT	351 (36.1)	2,054 (33.1)	1	
TC	459 (47.2)	3,022 (48.7)	0.89 (0.765-1.033)	0.123
CC	162 (16.7)	1,130 (18.2)	0.84 (0.687-1.024)	0.085
rs10419226				
GG	260 (26.7)	1,845 (29.7)	1	
GT	469 (48.3)	3,117 (50.2)	1.07 (0.908-1.256)	0.428
TT	243 (25.0)	1,244 (20.0)	1.39 (1.147-1.675)	0.001
rs9936833				
TT	399 (41.0)	2,609 (42.0)	1	
TC	453 (46.6)	2,847 (45.9)	1.04 (0.900-1.202)	0.591
CC	120 (12.3)	750 (12.1)	1.05 (0.840-1.303)	0.687
rs2687201				
CC	426 (43.8)	2,847 (45.9)	1	
CA	437 (45.0)	2,720 (43.8)	1.07 (0.930-1.239)	0.331
AA	109 (11.2)	639 (10.3)	1.14 (0.908-1.431)	0.258
rs11789015				
AA	521 (53.9)	3,138 (50.6)	1	
AG	403 (41.5)	2,560 (41.3)	0.95 (0.824-1.091)	0.456
GG	48 (4.9)	508 (8.2)	0.57 (0.417-0.776)	$3.68*10^{-4}$
rs3111601				
TT	484 (49.8)	3,060 (49.3)	1	
TC	401 (41.3)	2,653 (42.7)	0.96 (0.829-1.102)	0.531
CC	87 (9.0)	493 (7.9)	1.12 (0.871-1.429)	0.385

EAC: Esophageal adenocarcinoma, OR (95% CI): odds ratio (95% confidence interval).



PART III

FAMILIAL CLUSTERING OF ESOPHAGEAL ADENOCARCINOMA



Chapter 5

Hereditary factors in esophageal adenocarcinoma

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ABSTRACT

Background: The vast majority of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) cases are sporadic and caused by somatic mutations. However, over the last decades several families have been identified with clustering of EAC. Here, we review data from the published literature in order to address the current knowledge on familial EAC.

Summary: Although familial EAC comprises a relatively small group of patients, it is a clinically relevant category due to the poor prognosis of this type of cancer. Efforts should be made to identify specific genetic risk factors for familial EAC to enable identification of relatives at risk, since endoscopic surveillance can diagnose preneoplastic or early neoplastic lesions leading to early treatment, with improved outcome.

Key Message: Although familial EAC comprises a relatively small group of patients, this is a clinically relevant category due to the poor prognosis. Efforts should be made to identify specific genetic risk factors for familial EAC in order to facilitate the identification of other family members with a predisposition for this type of cancer.

Practical Implications: Approximately seven percent of BE and EAC cases are considered familial. Age at diagnosis is generally lower for patients with familial EAC as compared to sporadic cases, while other known risk factors for EAC, such as male gender and Caucasian ethnicity, do not differ between the two groups. In several described families with clustering of EAC the pattern of inheritance seems to be consistent with a rare autosomal dominant genetic trait. However, some association has been found with (attenuated) familial adenomatous polyposis, mismatch repair deficiency and recently with the genes *MSR1*, *ASCC1* and *CTHRC1*. Nevertheless, no specific genetic predisposition has yet been identified.

INTRODUCTION

During the last decades there has been a dramatic increase in the incidence of esophageal adenocarcinoma (EAC) in Western countries.[1-3] Despite improvements in multimodality therapy, the prognosis of patients with EAC remains poor.[4] Barrett's esophagus (BE) is the predominant risk factor for EAC in addition to age, male gender and Caucasian ethnicity.[5] BE is a premalignant condition in which the normal squamous epithelial lining in the lower esophagus is replaced by columnar intestinal cells.[6] BE is considered a long-term complication of severe chronic gastroesophageal reflux (GER). The susceptibility for GER may in turn be influenced by factors such as obesity, alcohol consumption and nicotine abuse.[7]

The vast majority of BE and EAC cases are sporadic and caused by somatic mutations,[8] i.e. mutations that may occur in any cell of the body except for germ cells. However, over the last decades several families have been identified with clustering of EAC.[9-14] This observation suggests that one or more inherited factors might play a role in the initiation of EAC in these families. Familial clustering of cancer is important thanks to the success of implementing genetic testing and screening methods for cancer syndromes.[15-17] However, for cancers that are not covered in familial risk management guidelines, such as EAC, awareness of the true familial risk is needed to provide rational advice.[17] In terms of clinical genetics, for a true familial risk, the number of affected family members needs to be higher than is to be expected by chance alone.[18]

The purpose of the present study was to review the literature on the occurrence and characteristics of familial EAC. Previous studies introduced and persevered the definition familial BE, i.e. two or more first- or second-degree family members diagnosed with BE, EAC or gastroesophageal junction adenocarcinoma (GEJAC).[19] These studies considered familial BE and familial EAC to be part of the same genetic trait, because EAC appears to arise from BE and both conditions share the same epidemiological risk factors. We hypothesize that familial EAC can be distinct from most familial BE. Since BE is much more prevalent among the common population, familial BE does not necessarily have to be the underlying condition of familial EAC. If two or more first-degree family members are diagnosed with EAC, it is unlikely that this can be explained by chance alone, based on the absolute risk of 0.12-0.5% for malignant transition of BE into EAC.[20,21] Therefore, familial EAC might be the result of accelerated malignant progression from familial BE, or familial EAC might arise without familial BE as the premalignant condition. In both scenarios involvement of specific germline mutations driving familial EAC can be envisaged.

Prevalence of familial EAC

In the literature familial EAC has been grouped with familial BE and has been termed familial BE, which is defined as two or more family members diagnosed with BE, EAC or GEJAC.[19] Two studies estimated the prevalence of familial BE by reporting the proportion of patients diagnosed with BE, EAC or GEJAC that had at least one other family member diagnosed with BE, EAC or GEJAC. Chak et al.[22] reported that about seven percent fulfill the criteria of familial BE, which is in line with the six percent reported by Ash et al.[23] Including GEJAC in familial BE can be criticized, since a tumor present on the gastro-esophageal junction can be originated either from the esophagus or the gastric cardia. In the last instance the tumor probably did not arose from BE.

Risk of GER, BE and EAC for familial EAC

BE is generally accepted as the premalignant lesion for EAC. BE is a complication of chronic GER. The prevalence of BE in the common population is estimated at two percent,[24] while the prevalence of BE among patients with GER is approximately ten percent.[25] The annual risk of developing EAC from BE is estimated to be between 0.12 and 0.5%.[20,21] Two case-control studies about the prevalence of GER among relatives of BE patients suggested a familial predisposition for GER in these families.[26,27] Among 27 and 47 relatives of patients diagnosed with BE or EAC, the prevalence of BE was reported to be 18% (n=5) and 28% (n=13), respectively.[28,29] More importantly, the prevalence of EAC described among 20 families with a strong familial expression of GER, BE, and EAC was estimated at 31%.[14] This finding suggests a true familial risk of EAC in these families. In contrast, a Swedish population-based, nationwide case-control study did not find an association between a positive history of esophageal cancer among first-degree relatives and the risk of EAC.[30] In addition, an Italian case-control study revealed no difference in prevalence of cancer in general between patients diagnosed with BE and patients with reflux esophagitis plus healthy controls. However, relatives of patients diagnosed with esophageal or gastric cancer had an increased risk of BE, particularly if the affected relative was younger than 50 years at time of diagnosis.[31] It should be noted that the prevalence of familial BE is relatively low, hence in a randomly taken cohort of patients with BE only a few patients will be part of family with clustering of BE and/or EAC. Although the previously mentioned studies are based on relatively small samples sizes, the prevalence of EAC in these families with clustering of EAC appears to be distinctly higher than in the common population, and this also accounts for the prevalence of BE.

Risk factors and patient characteristics

Known risk factors for the malignant transition of BE into EAC are increasing age, male gender and Caucasian ethnicity.[7] No differences in gender, ethnicity, and in addition in nicotine abuse and alcohol consumption were reported between patients with familial

BE and/or EAC and sporadic cases.[22,23,28,32] Contradictory results were observed regarding the prevalence of obesity, with some studies reporting no difference in prevalence,[28,32] while others reported a lower body mass index for patients with familial BE and/or EAC compared to sporadic cases.[22,23]

Several studies reported a lower age at diagnosis for patients with familial BE and/or EAC compared with sporadic cases.[12,13,23,33,34] Other studies could not confirm these observations.[22,28,32] In a study on 20 families with a strong familial expression of GER, BE, and EAC, the age at diagnosis of EAC appeared to be five to ten years younger when compared to sporadic cases.[14] This finding is consistent with the concept of the presence of a germline mutation, which generally results in a lower age at disease onset when compared with sporadic cases. This concept is based on the "Knudsons two hit" hypothesis for the complete (biallelic) inactivation of tumor suppressor genes, i.e. individuals born with already one inactivating germline mutation are likely to develop the second somatic inactivating hit earlier in life than individuals without an inherited predisposition, who have to develop both somatic hits during life in the same cell before tumorigenesis occurs. The second hit may be influenced by environmental factors and/or by other genetic factors.[35]

Pattern of inheritance

Since 1978 there have been several case reports on families with clustering of BE and EAC.[9-14,34] All studies suggest a pattern of inheritance consistent with an autosomal dominant genetic trait,[10-14] which likely reflects genetic predisposition to the disease. Nevertheless, it can be anticipated that familial EAC can also be caused by common environmental exposures in family members or by a combination of both. However, the segregation analysis (i.e. an analysis to determine whether a certain gene is involved in the distribution of a phenotypic trait) of Sun et al.[36] provided the first epidemiologic evidence in support of a genetic etiology for familial BE, EAC or GEJAC. The pattern of inheritance was found to be consistent with a rare autosomal dominant genetic trait.

Genetic alterations and molecular markers

Hereditary tumors are generally caused by the presence of germline mutations, which are present in the reproductive cells of one or both of the parents of the affected offspring. Germline mutations are therefore present in all the cells of the affected offspring and can be transmitted from one generation to the next. Colorectal cancer has well-defined familial syndromes due to specific gene mutations. For example, patients with Lynch syndrome have germline mutations in mismatch repair (MMR) genes and attenuated familial adenomatous polyposis, familial adenomatous polyposis (FAP) and Gardner's syndromes are all caused by germline mutations in the adenomatous polyposis coli (*APC*) gene.[37]

Esophageal lesions are rarely described in these hereditary colorectal cancer syndromes. However, several studies reported the occurrence of BE and/or EAC in patients with (attenuated) FAP or Gardner's syndrome, which are both caused by mutations in the *APC* gene. Gupta et al.[38] described a family with a father who tested positive for exon 4 deletion in *APC*. All three of his sons were diagnosed with attenuated familial adenomatous polyposis based on the presence of multiple polyps (<100) throughout the entire colon. The middle son (41 year old) was additionally diagnosed with EAC in the background of BE. A biopsy of the father revealed BE with low-grade dysplasia. In the youngest son BE was also confirmed in histopathological biopsies. The oldest son had endoscopic findings compatible with esophagitis without metaplastic changes. Although the simultaneous occurrence of these two potentially inherited disorders in a single family may be due to chance alone, it is also possible that the disorders are linked. This suggests that deletions of the *APC* gene could play a role in the pathogenesis of familial BE and subsequently familial EAC. In another study with 36 FAP patients, six patients were additionally diagnosed with BE (16.7%); interestingly, the average age at diagnosis of BE was 20 years younger than that in the non-FAP patients with BE.[39] In (attenuated) FAP, *APC* gene mutations lead to increased nuclear β-catenin levels and activation of the Wnt pathway. In EAC, although through a different mechanism of Wnt activation, nuclear β-catenin is also frequently increased.[40] It can be anticipated that the mutation in *APC* in patients with (attenuated) FAP might increase the chance of developing EAC.
EAC has not been associated with Lynch syndrome. However, three to five percent of EAC appears to have MMR deficiency.[41] It is unknown, however, whether these tumors arise as part of Lynch syndrome or whether they are due to somatic MMR gene mutations.

Orloff et al.[42] sought to identify genes associated with BE and EAC predisposition. Germline mutations in three candidate genes were identified in approximately 11% of patients diagnosed with BE and EAC, the most commonly affected gene being macrophage scavenger receptor 1 (*MSR1*), followed by activating signal cointegrator 1 complex subunit 1 (*ASCC1*) and collagen triple helix repeat containing 1 (*CTHRC1*). However, further studies are needed to determine the role of these genes in the development of familial EAC.

CONCLUSIONS

Clustering of EAC in a family is rare but may be caused by an autosomal dominant genetic trait. In view of the poor prognosis of EAC, efforts should be made to identify a genetic predisposition for EAC in these families. Finding a genetic predisposition in familial EAC would facilitate identifying non-affected family members with the predisposition for this type of cancer. Endoscopic surveillance of relatives with the predisposition may lead to

the detection of preneoplastic or early neoplastic stages of EAC. Because patients diagnosed with earlier stages of EAC have a better chance for definite cure, this strategy could probably improve outcome.[4]

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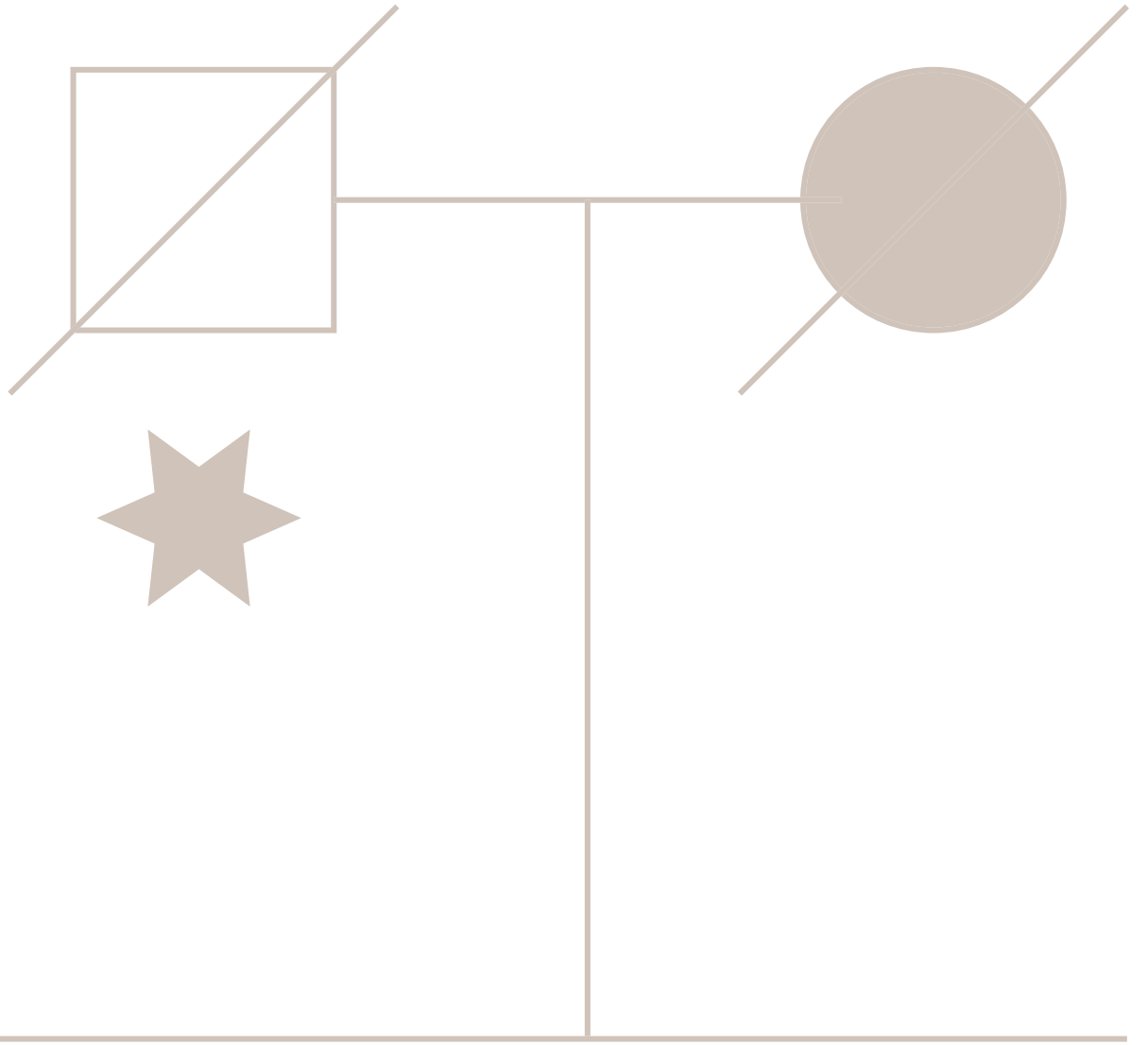

Chapter 6

Germline variant in *MSX1* identified in a Dutch family with clustering of Barrett's esophagus and esophageal adenocarcinoma

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ABSTRACT

Introduction: The vast majority of esophageal adenocarcinoma cases are sporadic and caused by somatic mutations. However, over the last decades several families have been identified with clustering of Barrett's esophagus and esophageal adenocarcinoma, defined as familial Barrett's esophagus. This observation suggests that one or more hereditary factors may play a role in the initiation of Barrett's esophagus and esophageal adenocarcinoma in these families.

Methods: A Dutch family with clustering of Barrett's esophagus and esophageal adenocarcinoma was identified. Normal DNA obtained from an indicated proband diagnosed with Barrett's esophagus was analyzed with SNP array and exome sequencing. A custom-made panel consisting off potential germline variants was verified in the normal DNA of the affected family members. In addition, the respective tumors were analyzed for somatic loss of the wild type allele or the presence of an inactivating somatic mutation in the wild type allele.

Results: Exome sequencing revealed 244 candidate variants in the normal DNA of the proband, of which 206 variants were verified successfully on the Ion Torrent Personal Genome Machine and six variants by Sanger sequencing. After the normal DNA of the affected family members was analyzed for the presence of the 212 potential germline variants and subsequently the respective tumors, only one potential germline variant in *MSX1* showed loss of the wild type allele in both the tumor DNAs of the affected family members.

Conclusion: A germline variant in *MSX1* was identified in a Dutch family with clustering of Barrett's esophagus and esophageal adenocarcinoma. This finding indicates that the germline defect in *MSX1* may be associated with Barrett's esophagus and cancer in this particular family.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is a histopathological subtype of esophageal cancer, of which the incidence is rising rapidly over the last decades in Western countries.[1-3] The presence of Barrett's esophagus (BE), as a consequence of chronic gastroesophageal reflux (GER), is generally accepted as the predominant risk factor of EAC. Other risk factors are high age, male gender, Caucasian ethnicity,[4] and obesity.[5] The risk of developing EAC from BE is estimated at 0.12-0.5% per year[6,7] and follows a multimorphological sequence, in which metaplasia evolves to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and ultimately into invasive adenocarcinoma.[8]

Although the vast majority of BE and EAC cases are sporadic and caused by somatic mutations, over the last decades several families have been identified with clustering of BE and EAC.[9-12] Previous studies introduced the definition familial BE (FBE), i.e. two or more first- or second-degree family members diagnosed with BE or EAC. It has been estimated that approximately seven percent of BE and EAC cases are considered familial.[13-15] When compared to sporadic cases, familial cases of BE and EAC are mostly diagnosed at a younger age.[14-16] These findings are consistent with the presence of a germline mutation in a cancer-predisposing gene. Theoretically, a germline mutation can be present in a proto-oncogene or in a tumor suppressor gene. The pattern of inheritance suggested for FBE[17] and for most familial cancer syndromes (familial adenomatous polyposis, hereditary breast and ovarian cancer, familial atypical multiple mole melanoma, Li-Fraumeni syndrome) is consistent with the concept of "Knudsons two hit" hypothesis, and is caused by biallelic inactivation of a tumor suppressor gene.

It can be anticipated that the development of BE and EAC in several members of one family is caused by the presence of a germline defect. Orloff et al.[18] identified germline mutations in the genes *MSR1*, *ASCC1*, and *CTHRC1* with the use of a linkage analysis on affected siblings diagnosed with BE or EAC. However, information about the presence of identical germline mutations in affected siblings is lacking and the role of these genes in the development of FBE is unknown. Extensive candidate gene and linkage researches have to date been unsuccessful in identifying genetic variants that are associated with the risk of FBE.

Here, we describe a family, of whom two members were diagnosed with BE and three with EAC. To identify a possible germline defect in the affected family members, we investigated the normal DNA of a proband with a SNP array and exome sequencing. Subsequently, we validated the potential germline variants identified in the proband in the normal and tumor DNA of the other affected family members on a next-generation sequencing platform.

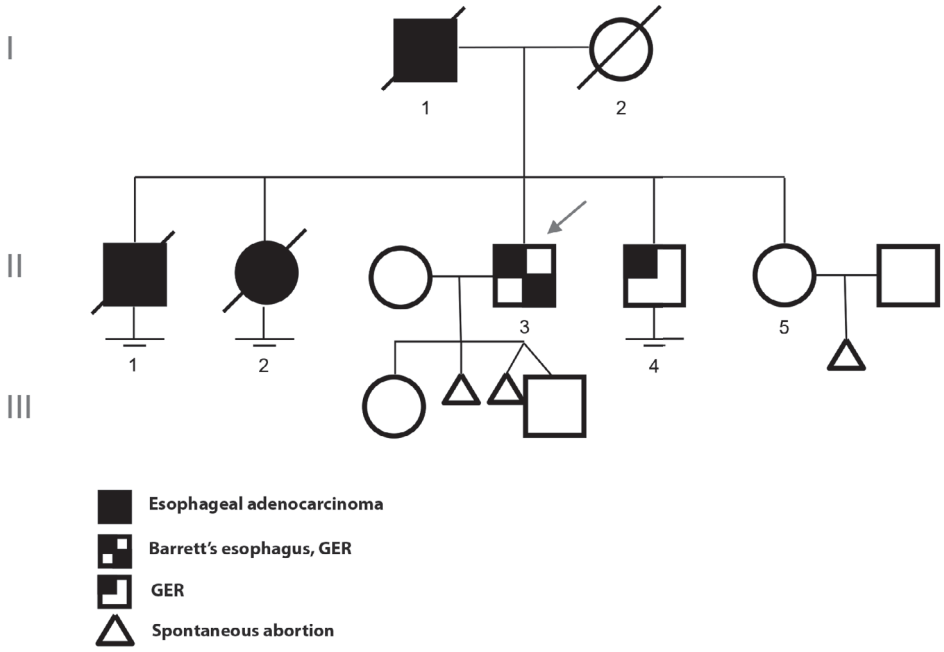
METHODS

This study was approved by the Erasmus MC – University Medical Center Rotterdam Institutional Review Board. Formal written informed consent was obtained from the living family members, whom are therefore included in the study. All tissues investigated in this study were used in accordance with the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" as established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

Family presentation

The first family member (further referred to as proband), who came to our attention is a male patient of 45 years (Figure 1: II-3). He has been suffering from pyrosis for several years and was diagnosed with BE (LGD) based on histopathological examination of multiple biopsies obtained during upper gastrointestinal endoscopy. In addition, in his family there is a high incidence of BE and EAC (Figure 1). His father (I-1) was diagnosed with EAC at the age of 50 years and died in the same year of the consequences of the disease (no further information available). The oldest brother of the proband (II-1) was diagnosed with EAC at the age of 49 years. He underwent neoadjuvant chemoradiotherapy followed by an

Figure 1: Pedigree of investigated family



Black symbols indicate esophageal adenocarcinoma. Partly blocked symbols indicate Barrett's esophagus and/or gastroesophageal reflux (GER). The proband is indicated by a red arrow.

esophagectomy. Pathological examination of the resection specimen revealed a vital adenocarcinoma within the background of BE located at the distal esophagus, Mandard score III-IV, pT3N0. He died one month later at the age of 50 years due to postoperative complications. At autopsy, liver metastases were identified. Furthermore, the older sister of the proband (II-2) was diagnosed with a poorly differentiated adenocarcinoma at the gastroesophageal junction at the age of 45 years and died one year later of the consequences of the disease. The younger brother of the proband (II-4) has been suffering from pyrosis for several years, and was diagnosed with BE (intestinal metaplasia, no dysplasia) based on histopathological examination of multiple biopsies obtained during upper gastrointestinal endoscopy at the age of 40 years. He was prescribed proton pump inhibitors and included in the surveillance program for BE, in the biopsies taken during the latest control no intestinal metaplasia was observed any longer. The youngest sister of the proband (II-5) did not give informed consent, and was therefore not included in the study.

SNP array and exome sequencing

The proband (II-3) was subjected to genetic testing, i.e. SNP array and exome sequencing performed at the Erasmus MC Center for Biomics, Rotterdam, the Netherlands. After informed consent was obtained during a counselling session at the Department of Clinical Genetics, blood was drawn and DNA was isolated using a Chemagic DNA Blood Kit according to standard procedures. To identify copy number variations (CNVs) in the germline DNA of the proband (II-3), the Genome-wide human SNP array 6.0 was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Data were analyzed using Nexus Copy Number tm V4 software (Biodiscovery, Hawthorne, CA, USA). Exome sequencing was performed on the Hiseq2000, using the Agilent version 4 capture kit, according to the Illumina TruSeq v3 protocol. Reads were aligned against the human reference genome build 19 (hg19) using Burrows-Wheeler Aligner,[19] and the NARWHAL pipeline.[20] Subsequently, genetic variants were called using tools from the genome analysis toolkit.[21] The resulting VCF files were processed with a custom variant annotation tool that determines the variant effects. Germline variants identified by exome sequencing were selected to cause amino-acid changes or splice site alterations, in addition variants present in the dbSNP135 database or with a frequency of >1% in ESP6500 and the 1000 Genomes databases were excluded. To confirm these data on a different platform, a custom-made panel was designed by the Ion AmpliSeq Designer V2.0 for targeted sequencing on the Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA, USA). In short, libraries were made using the Ion AmpliSeq Library Preparation Kit. A template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion Sequencing Kit v2.0 on an Ion 316 chip. Data were analyzed with the Variant Caller v2.2.3-31149 (Life Technologies, Carlsbad, CA,

USA). For the variants that could not be validated by the PGM, primers were designed for PCR amplification and Sanger sequencing on an ABI 3730 sequencer (Life Technologies, Carlsbad, CA, USA) according to the BigDye Terminator v3.1 Cycle Sequencing Kit Protocol. DNA sequences were visualized using the Mutation Surveyor software (Softgenetics, State College, PA, USA), which aligned the sequences to annotated GenBank reference files.

Tissue samples

To test whether the validated germline variants in the proband (II-3) could also be identified in the normal and tumor DNA of the family members, formalin-fixed paraffin-embedded (FFPE) tissue blocks were reclaimed from several pathology archives in the Netherlands. Tumor tissue (II-1, II-2) composed of at least 50% neoplastic cells (confirmed by a GI-pathologist) and areas of non-malignant esophageal cells (II-1, II-2, II-4) were manually microdissected from 10 to 15 hematoxylin-stained sections (4µm). Subsequently, DNA was extracted using proteinase K and 5% Chelex 100 resin.

RESULTS

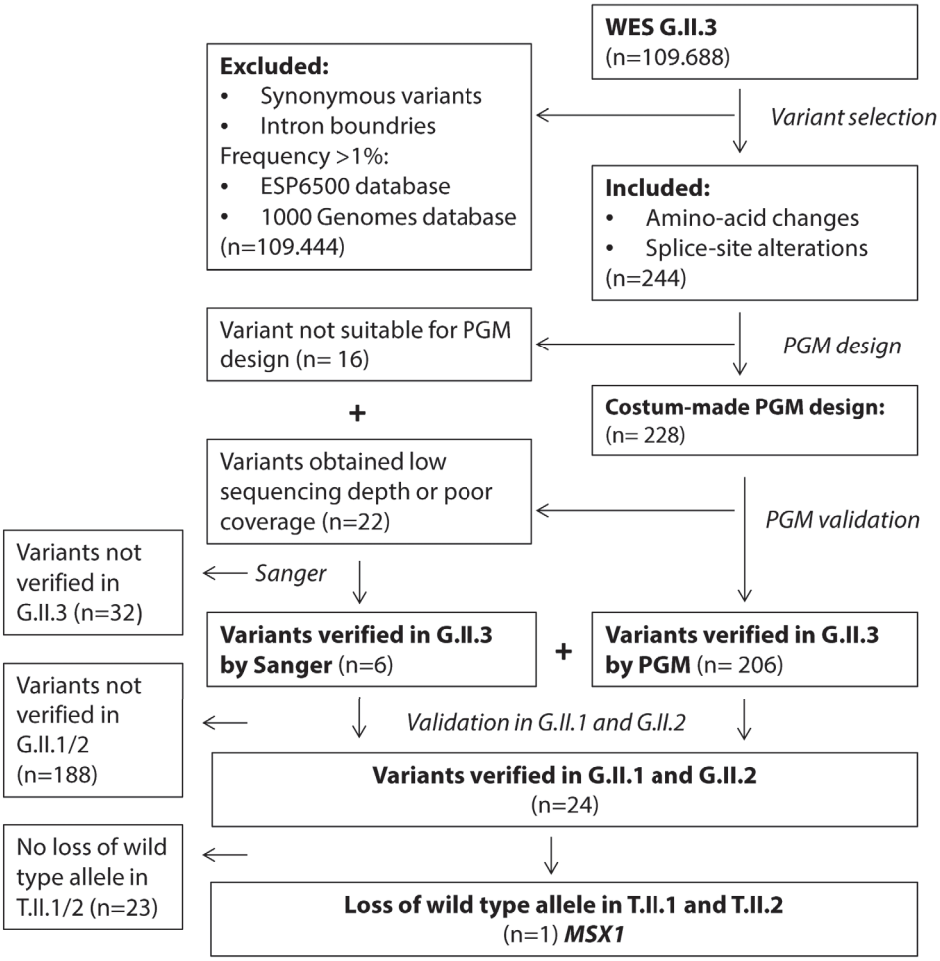
The SNP array revealed a known CNV with no clinical relevance and a CNV in an unknown gene (data not shown). Exome sequencing performed on the normal DNA of the proband (II-3) revealed after the selection procedure 244 candidate germline variants (Table 1). For 228 variants custom-made primers could be designed to confirm the data on a different platform. After sequencing on the PGM, 206 variants were verified successfully in the normal DNA of the proband (II-3). The remaining 22 variants obtained either low sequencing depth or poor coverage and therefore did not qualify for further analysis on the PGM. For the 38 variants, which could not be validated by the PGM, primers were designed for PCR amplification and Sanger sequencing. Of the 38 variants, six were validated in the normal DNA of the proband (II-3) by Sanger sequencing. Leaving 212 potential germline variants of interest, which were validated in the family members (Figure 2).

Table 1: Mutations identified in normal DNA of the proband by exome sequencing.

	Amount N=244 (%)
Nonsynonymous	164 (67.2)
Stop gains	3 (1.2)
Frameshift indels*	14 (5.7)
Non-frameshift indels*	44 (18.0)
Splice site variants	12 (4.9)
Unknown	7 (2.9)

* Indels = insertions and deletions

Figure 2: Flowchart of sequencing pipeline

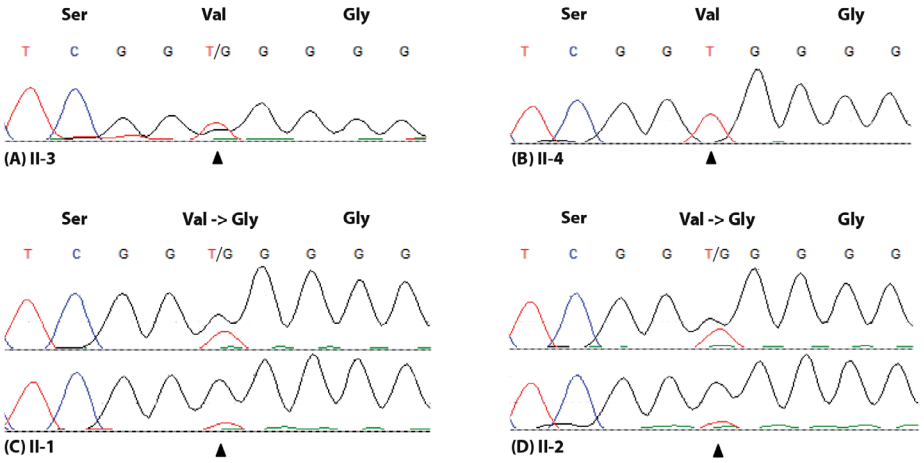


WES: Whole-exome sequencing
PGM: Ion Torrent Personal Genome Machine
Sanger: Sanger Sequencing
G: Germline DNA
T: Tumor DNA

The custom-made panel for the PGM, containing the germline variants identified in the proband (II-3), was extended to two other affected members of the family (II-1, II-2). Normal DNA of the affected family members (II-1, II-2) was analyzed for the presence of the 212 potential germline variants identified in the proband (II-3). In addition, the respective tumors of the family members (II-1, II-2) were analyzed for somatic loss of the wild type allele or the presence of an inactivating somatic mutation in the wild type allele. Twenty-four of the potential germline variants were also identified in the normal

DNA of the family members II-1 and II-2. Only one potential germline variant, *MSX1* (chr4: 4861985 T>G, c.359T>G, p.V120G, NM_002448), showed loss of the wild type allele in both the tumor DNA of family members II-1 and II-2. In the normal and metaplastic DNA of the youngest brother (II-4) no variant in *MSX1* was identified (Figure 3).

Figure 3: Sanger sequencing results of *MSX1*



(A) Sanger sequencing of normal DNA of the proband (II-3) confirmed the presence of the heterozygote variant (c.359T>G) in *MSX1*.

(B) Sanger sequencing of normal DNA of youngest brother of the proband (II-4) reveal no variant in *MSX1*.

(C) Sanger sequencing of normal DNA (upper panel) and tumor DNA (lower panel) of the oldest brother of the proband (II-2) confirmed the presence of the heterozygote variant (c.359T>G) in *MSX1* in normal DNA and loss of the wild type allele in tumor DNA, which changed the codon 120 from Valine into Glycine (p.V120G).

(D) Sanger sequencing of normal DNA (upper panel) and tumor DNA (lower panel) of the oldest sister of the proband (II-2) confirmed the presence of the heterozygote variant (c.359T>G) in *MSX1* in normal DNA and loss of the wild type allele in tumor DNA, which changed the codon 120 from Valine into Glycine (p.V120G).

DISCUSSION

For the first time a germline variant in the *MSX1* gene was identified in a family with clustering of BE and EAC with the aid of exome sequencing. In addition, the investigated tumors of two affected family members showed somatic loss of the wild type allele. The germline variant in *MSX1* changed codon 120 from Valine into Glycine. Although not reported in the Cosmic database and in dbSNP135, the variant was described before on a very low frequency (allele frequency: 0.00052),[22] suggesting that this is a rare variant. This finding indicates that the germline defect in *MSX1* may be associated with the high occurrence of BE and EAC in this Dutch family.

MSX1 encodes a homeobox protein and is involved in multiple epithelial-mesenchymal interactions. In addition, MSX homeobox genes are able to interact with bone morphogenetic proteins (BMPs), in particular to the closely related BMP-2 and BMP-4. Recent studies established e.g. BMP-4 signaling as important interconnected regulatory pathways that contribute to the early stage of the transformation of the epithelial cells of the distal esophagus from the normal stratified squamous mucosa to an intestinal columnar cell type. BMP-4 was found to be present in inflamed squamous epithelium but not in normal squamous mucosa.[23] *MSX1* may be involved in the malignant progression of BE into EAC for familial cases as well as for sporadic cases, however somatic mutations in *MSX1* were identified in a very low frequency by Dulak et al.[24] (p.R158W, p.F151L, p.P153P), suggesting a prominent role of this germline defect in the development of BE and EAC in this particular family. In addition, mutations in *MSX1* have also been reported in families with dominantly inherited congenital absence of several permanent teeth, called oligodontia or hypodontia, with or without cleft lip and/or palate,[25-28] no oligodontia or hypodontia was observed in the proband.

In one of the family members (II-4) the *MSX1* variant was not observed, although he did suffer from pyrosis and was diagnosed with intestinal metaplasia at the first histopathological examination. However, the most recent biopsies taken during upper gastro-intestinal endoscopy revealed no signs of intestinal metaplasia. It can therefore be hypothesized that family member II-4 is a phenocopy. Since the prevalence of BE in the common population is estimated at two percent,[29,30] it can be anticipated that the initial metaplasia in this family member developed as a consequence of an environmental factor, instead of within the context of an inherited genetic defect.

Family member II-3 was indicated as the proband and since he was only diagnosed with BE without invasive carcinoma, it has to be taken into account that *MSX1* may only be associated with the development of BE and not necessarily with EAC. In addition, it was not possible to test the presence of the *MSX1* variant in the father of proband (I-1), also diagnosed with EAC, since no tissues blocks were present.

In conclusion, a germline variant in *MSX1* was identified in the normal DNA of three affected members of a family with clustering of BE and EAC, in addition the investigated tumors showed somatic loss of the wild type allele, consistent with biallelic inactivation of a tumor suppressor gene. This germline defect may be associated with the development of BE and EAC in this family. However, functional studies have to be performed to prove any effect of this germline defect.

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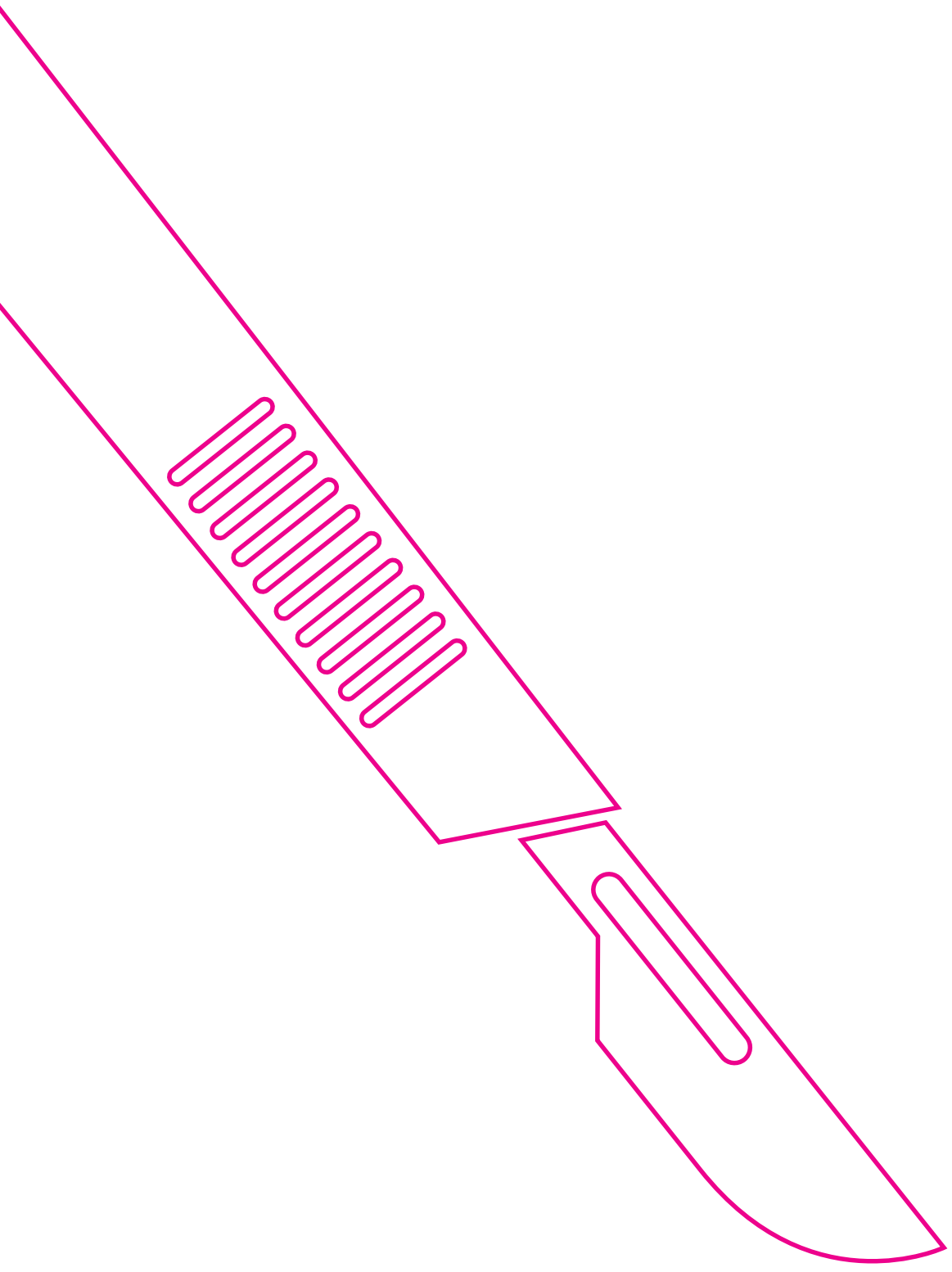
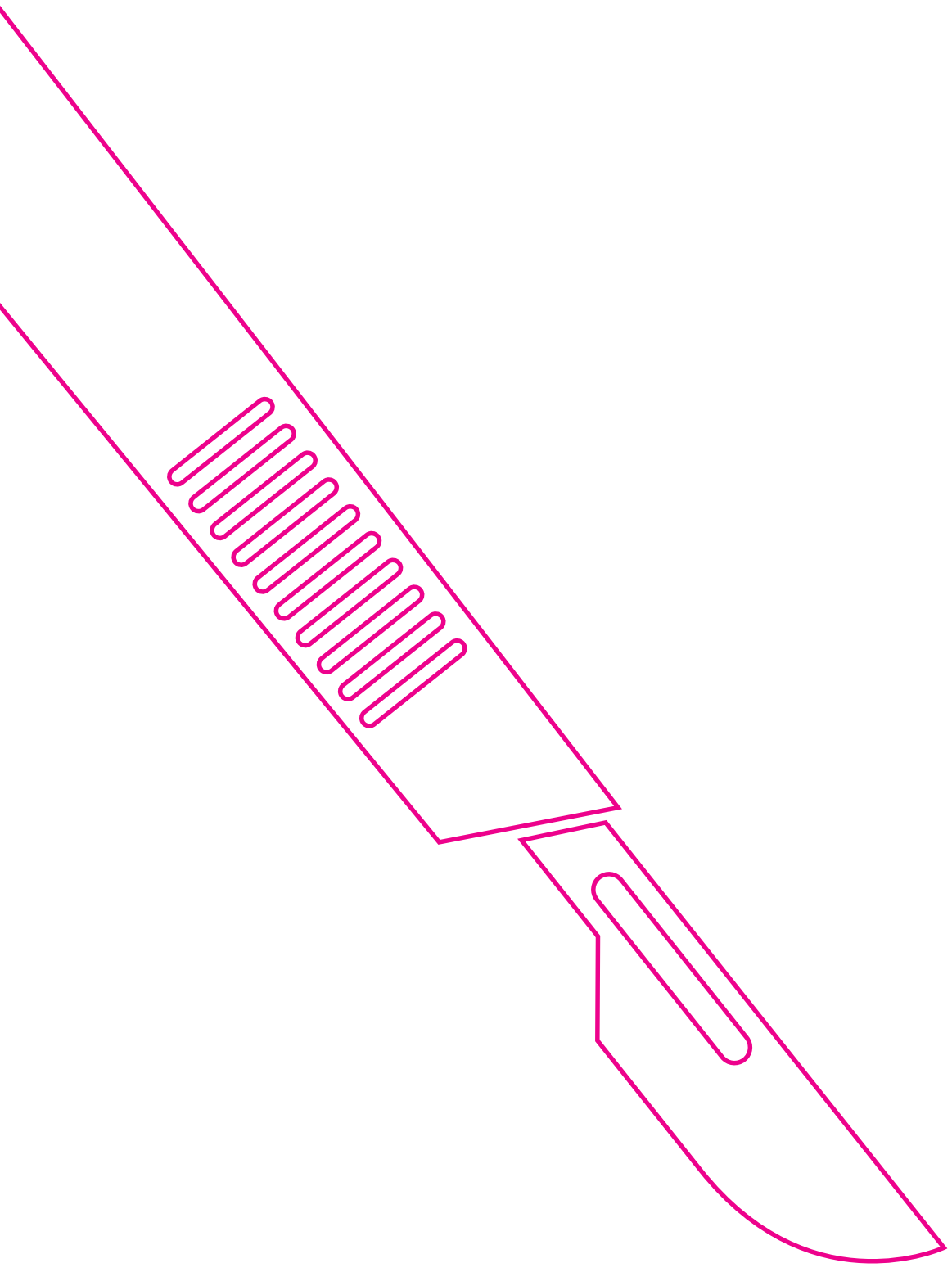


PART IV

YOUNG PATIENTS WITH ESOPHAGEAL CANCER



Chapter 7

The influence of young age on outcome after esophagectomy for cancer

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ABSTRACT

Background: The incidence of esophageal cancer has risen among all age groups. Controversy exists about the clinical presentation and prognosis of young patients. The aim of this study was to compare the clinicopathological characteristics and outcome after surgery between patients with esophageal cancer, who were ≤50 years of age and those >50 years of age.

Methods: Patients diagnosed with esophageal carcinoma, who underwent esophagectomy between January 1990 to January 2011 in a single institution were selected from a prospective database. Patients aged ≤50 years at diagnosis (n=163) were compared with those >50 years (n=1,151) with respect to clinicopathological stage and oncological outcome.

Results: Younger patients had less co-morbidities (p<0.001). There were no significantly differences in tumor localization, histology, differentiation or TNM stage in the two groups. In both groups, 37% of the patients underwent neoadjuvant chemo(radio) therapy. One or more nonsurgical complications developed in 53% of the older group versus 42% in the younger group (p=0.012). In-hospital mortality was 6.3% for patients >50 years compared to 1.8% for younger patients (p=0.021). The 5 -year overall survival was significantly better for the younger patients than for those <50 years (41 vs. 31%, p<0.001), but median disease-specific and disease-free survival did not differ between the groups (37 vs. 30 months, p=0.140 and 49 vs. 28 months, p=0.079 respectively). Multivariate analysis identified moderate, poorly, and undifferentiated tumors; tumor-positive resection margins (pR1-2) and TNM stage IIB- IV as independent predictors of disease-specific survival.

Conclusion: A considerable proportion (12%) of patients diagnosed with resectable esophageal carcinoma were ≤50 years. Phenotypic tumor characteristics and disease-specific survival were comparable for the two age groups.

INTRODUCTION

Over the last decades there has been a marked increase in the incidence of esophageal cancer, which is mainly attributed to an increase in esophageal adenocarcinoma (EAC) in developed countries.[1-3] This rise has occurred among all age groups, which makes esophageal cancer no longer a disease of the elderly,[4] although esophageal cancer is still relatively rare in patients ≤50 years of age.

Controversy exists about the biological behaviour and prognosis of young patients with EAC. Some studies suggest that younger patients present with more advanced disease but receive more aggressive treatment and therefore attain a survival comparable to that of their older counterparts.[5-7] Others found that younger patients did not differ from their older counterparts with regard to clinicopathological characteristics or survival.[8-11] The aim of this retrospective analysis was to compare clinicopathological characteristics and short- and long-term follow-up of patients with esophageal cancer, who were ≤50 years of age and those >50 years.

METHODS

Patients

All patients who were diagnosed with cancer of the esophagus or gastroesophageal junction (GEJ) and underwent esophagectomy with curative intent between January 1990 and January 2011 were included in the study. Patients with an unresectable tumor and those with a tumor of the GEJ, who underwent a total gastrectomy were excluded. Neoadjuvant chemotherapy was given to a cohort of patients with squamous cell carcinoma (SCC) of cT1-3N0-1M0, who participated in a randomized controlled trial.[12] Preoperative and postoperative chemotherapy was given to patients with proximal gastric cancer infiltrating into the distale esophagus after the results of the MAGIC-trial became available.[13] None of the patients received adjuvant chemoradiationtherapy. Induction chemotherapy was used in patients with gross celiac lymph node involvement, who were not eligible for primary surgical therapy.[14] From 2003 untill 2009, a number of patients with an operable tumor received neoadjuvant chemoradiation as participants in a randomized controlled trial.[15] Neoadjuvant chemoradiation has become our preferred treatment since early 2010.

Clinicopathological characteristics of patients, treatment, and follow-up were prospectively collected by a dedicated database manager, who closely collaborated with the medical team and attended the weekly multidisciplinary conferences. Also attending

the meetings were one or more surgeons, a gastroenterologist, a medical oncologist, a radiation therapist and a pathologist. At these meetings, patient characteristics, clinical staging, details on treatment, postoperative morbidity and mortality, and pathological staging were discussed. They were reported thereafter in the database.[16]

The young aged group was defined as patients ≤50 years of age at the time of diagnosis. This group was compared with patients >50 years of age. The cut-off point of 50 years was arbitrary chosen and based on data in other articles.[6,8] The 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual was used for describing the tumor, the node and metastasis classifications, and staging.[17]

Surgery

Most of the patients underwent 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. 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 local 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 from the neck to the abdomen with a vein stripper. No formal cervical or mediastinal lymph node dissection was carried out. Esophagogastrostomy (hand-sewn or by using a circular stapler) was performed in the neck.

Right posterolateral thoracotomy was the first step in transthoracic resection with extended lymphadenectomy. 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 aortopulmonary 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 part of the transthoracic procedure was identical to that of the transhiatal procedure.

Statistical analysis

Data analysis was performed with SPSS version 18.0 (SPSS, Chicago, IL, USA). Two-sided p-values <0.05 were considered significant. Categorical variables were summarized using frequencies and percentages. Proportions were compared using χ^2 test or Fisher's exact tests for the categorical variables. Survival estimates were calculated using Kaplan-Meier

curves, and differences were tested with a log-rank test. Survival time was measured from the date of surgery to the date of an event (defined as death or recurrence of disease). The cause of death was obtained from the database. When it was unknown, the patient's general practitioner was contacted to retrieve the information. Patients were censored at the date of last follow-up. Survival data are expressed as medians with interquartile ranges (IQR). Separate models for overall survival (OS), disease-specific survival (DSS) (excluded: in-hospital mortality and 30 day mortality), and disease-free survival (DFS) were created. Univariate analysis were performed to identify prognostic variables associated with DSS and OS after esophagectomy. Cox proportional hazard models were used to identify independent predictors of DSS and OS. Variables were entered in the multivariate Cox proportional hazard model if they reached p-value of <0.10 in the univariate analysis.

RESULTS

Patients

A total of 1,589 patients with cancer of the esophagus or cancer of the GEJ were retrieved from the database. Patients with the bulk of the tumor being located in the cardia (n=37), patients with proof of local irresectability and/or distant dissemination [n=192: 25 (13.0%) ≤50 years, 167 (86.9%) >50 years], patients who underwent total gastrectomy with distal esophagectomy (n=43), and patients who did not undergo esophagectomy because of intraoperative complications (n=3) were excluded. The remaining 1,314 patients underwent esophagectomy with curative intent.

The mean ± SD age of the study population was 62.2 ± SD 9.824 years. Some 1,019 patients (77.5%) were men and 295 (22.5%) were women. Among these patients, 163 (12.4%) were ≤50 years of age (median: 47 years, range: 19-50 years), and 1,151 (87.6%) were >50 years (median: 64 years, range: 51-90 years).

Patient characteristics are shown in Table 1. Patients >50 years had significantly more co-morbidities, in particular cardiac, respiratory, and vascular diseases. Localization of the tumor did not differ between the younger and older patients.

Tumor characteristics are shown in Table 2. Among the patients, adenocarcinoma was the most prevalent histologic type (67.8%) and was found to be more common among the patients ≤50 years of age. There were no differences between the two groups with regard to grade of differentiation, pTNM stage and radicality of the resection.

Table 1: Patients characteristics

	Age≤ 50 years n=163 (%)	**Age> 50 years n= 1,151 (%)**	**p-value**
Age (median)	47 (19-50)	64 (51-90)	
Sex			0.186
Male	133 (81.6)	886 (77.0)	
Female	30 (18.4)	265 (23.0)	
Co-morbidity	50 (30.7)	675 (58.6)	<0.001
Cardiac	14 (8.6)	322 (27.9)	
Respiratory	14 (8.6)	199 (17.3)	
Vascular	5 (3.1)	167 (14.5)	
Neurological	5 (3.1)	63 (5.5)	
Diabetes Mellitus	6 (3.7)	110 (9.6)	
Mental	3 (1.8)	6 (0.5)	
Other malignancy	8 (4.9)	101 (8.8)	
Other	10 (6.1)	98 (8.5)	
Localisation tumor			0.400
Cervical	1 (0.6)	3 (0.3)	
Upper 1/3 thoracic	4 (2.5)	16 (1.4)	
Middle 1/3 thoracic	15 (9.2)	163 (14.2)	
Lower 1/3 thoracic	67 (41.1)	484 (42.1)	
Gastroesophageal junction	75 (46.0)	481 (41.8)	
Unknown	1 (0.6)	4 (0.3)	

Table 2: Tumor characteristics

	Age≤ 50 years n=163 (%)	**Age> 50 years n= 1,151 (%)**	**p-value**
Tumor type			0.066
Squamous cell carcinoma	39 (23.9)	346 (30.1)	
Adenocarcinoma	116 (71.2)	775 (67.3)	
High-grade dysplasia	6 (3.7)	15 (1.3)	
Other	2 (1.2)	15 (1.3)	
Differentiation			0.373
Unknown	21 (12.9)	106 (9.2)	
Good	13 (8.0)	78 (6.8)	
Moderate	69 (42.3)	487 (42.3)	
Poor/Undifferentiated	60 (36.8)	480 (41.7)	
pT*			0.171
0	10 (6.1)	75 (6.5)	
is	10 (6.1)	29 (2.5)	
1	28 (17.2)	167 (14.5)	
2	23 (14.1)	186 (16.2)	
3	91 (55.8)	687 (59.7)	
4	1 (0.6)	7 (0.6)	
pN*			0.651
0	69 (42.3)	454 (39.4)	
1	32 (19.6)	274 (23.8)	
2	31 (19.0)	183 (15.9)	
3	21 (12.9)	165 (14.3)	
pM*			0.778
0	152 (93.3)	1062 (92.3)	
1	1 (0.6)	14 (1.2)	

Table 2: Tumor characteristics (continued)

	Age≤ 50 years n=163 (%)	Age> 50 years n= 1,151 (%)	p-value
Stage grouping*			0.373
No evidence of primary tumor	11 (6.7)	74 (6.4)	
0 (HGD)	7 (4.3)	25 (2.2)	
IA	14 (8.6)	84 (7.3)	
IB	17 (10.4)	94 (8.2)	
IIA	5 (3.1)	74 (6.4)	
IIB	38 (23.3)	260 (22.6)	
IIIA	24 (14.7)	226 (19.6)	
IIIB	24 (14.7)	138 (12.0)	
IIIC	22 (13.5)	162 (14.1)	
IV	1 (0.6)	14 (1.2)	
Resection**			0.729
R0	124 (76.1)	842 (73.2)	
R1	36 (22.1)	284 (24.7)	
R2	3 (1.8)	25 (2.2)	

* According to AJCC, American Joint Committee on Cancer 7th edition[17]

** R0 = tumor free resection margin, R1= vital tumor present at 1 mm from the proximal, distal or circumferential resection margin, R2= residual macroscopic tumor

Treatment

Treatment details are summarized in Table 3. In both groups, 37% of the patients underwent neoadjuvant therapy. The percentage of transhiatal esophagectomy was higher in the younger patients than in the older patients (82.8 vs. 75.2%, p=0.032). In 89.2% of all patients who underwent transhiatal esophagectomy, the tumor was localized in the lower one-third of the esophagus or at the GEJ, and 73.7% of the tumors were EACs. Adjuvant therapy was rarely used. It was applied more often in the younger patients (chemotherapy 1.8 vs. 1.2%, radiotherapy 8.0 vs. 1.9%; p<0.001).

Table 3: Treatment details

	Age≤ 50 years n=163 (%)	Age> 50 years n= 1151 (%)	p-value
Neoadjuvant therapy			
Chemotherapy only	36 (25.2)	262 (25.7)	0.896
Chemoradiation	20 (12.3)	127 (11.0)	0.639
Type of surgery			
Transthoracic esophagectomyy	28 (17.2)	286 (24.8)	0.032
Transhiatal esophagectomy	135 (82.8)	865 (75.2)	
Adjuvant therapy			
Chemotherapy	3 (1,8)	14 (1.2)	<0.001
Radiotherapy	13 (8.0)	22 (1.9)	

Morbidity and mortality

Postoperative complications are shown in Table 4. Surgical complications did not differ between the two groups, but nonsurgical complications were seen more often in the older patients (p=0.012). In-hospital mortality was 1.8% for the younger patients compared to 6.3% for the older patients (p=0.021). The 30 day mortality rates for the younger and the older patients was 1.0 and 4.3%, respectively (p=0.069).

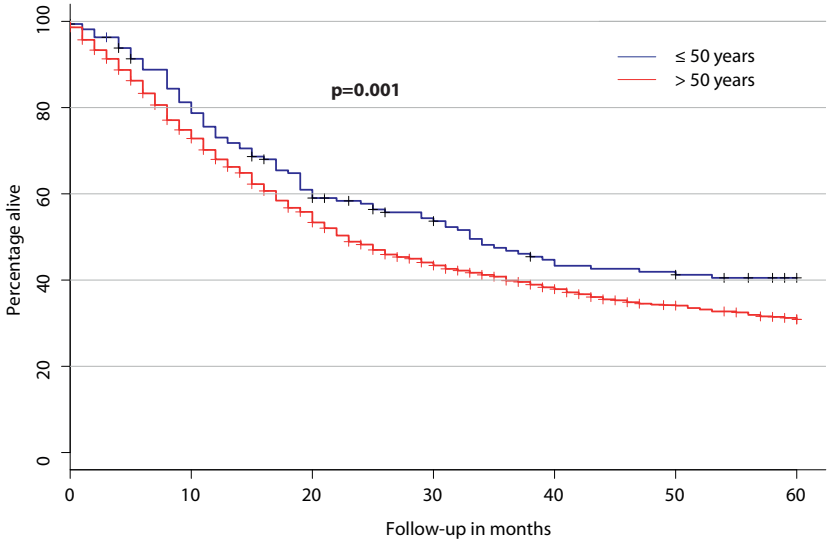
Table 4: Morbidity and mortality

	Age≤ 50 years n=163 (%)	**Age> 50 years n= 1,151 (%)**	**p-value**
Surgical complications	67 (41.1)	444 (38.6)	0.535
Hemorrhage	4 (2.5)	41 (3.6)	
Chylothorax	8 (4.9)	48 (4.2)	
Suture leak	17 (10.4)	106 (9.2)	
Jejunum fistula leak	1 (0.6)	10 (0.9)	
Unilateral recurrence nerve palsy	26 (16.0)	153 (13.3)	
Bilateral recurrence nerve palsy	3 (1.8)	27 (2.3)	
Wound infection	11 (6.7)	56 (4.9)	
Graft necrosis	2 (1.2)	37 (3.2)	
Other	10 (6.1)	127 (11.0)	
Nonsurgical complications	69 (42.3)	608 (52.8)	0.012
Pneumonia or atelectasis	50 (30.7)	384 (33.4)	
ARDS	4 (2.5)	56 (4.9)	
Myocardial infarction	0 (0.0)	15 (1.3)	
Urinary tract infection	0 (0.0)	24 (2.1)	
Sepsis	4 (2.5)	44 (3.8)	
Thrombosis or pulmonary embolism	4 (2.5)	40 (3.5)	
Other	25 (15.3)	319 (27.7)	
Mortality			
In-hospital	3 (1.8)	73 (6.3)	0.021
30 days	1 (1.0)	36 (4.3)	0.069

Survival

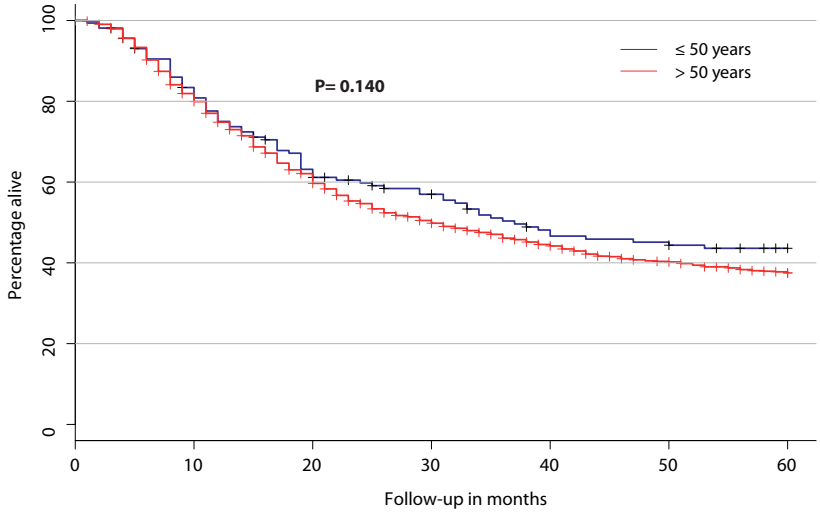
The median OS was 33 months (IQR 12-134 months) for patients ≤50 years of age and 23 months (IQR 9-86 months) for the patients >50 years. The 5-year OS was 40.5% for the younger patients versus 31.0% for the older patients (p= 0.001) (Figure 1). The DSS did not differ between the two groups, with a median survival for the younger group at 37 months (IQR 12-134 months) and for the older group at 30 months (IQR 12-185 months). The younger group versus older group 5-year survivals were 43.6 and 37.4%, respectively (p=0.140) (Figure 2). There was no significant difference between the younger and older group in regard to the percentage of patients who developed locoregional and/or distant recurrences: 48.5 versus 50.2%, respectively (p= 0.676). The median DFS was 49 months (IQR 10-109 months) for the young patients and 28 months (IQR 10-147) for the older patients, with corresponding 5-year survival rates of 49.2 and 39.8% (p=0.079) (Figure 3).

Figure 1: Overall survival according to age group



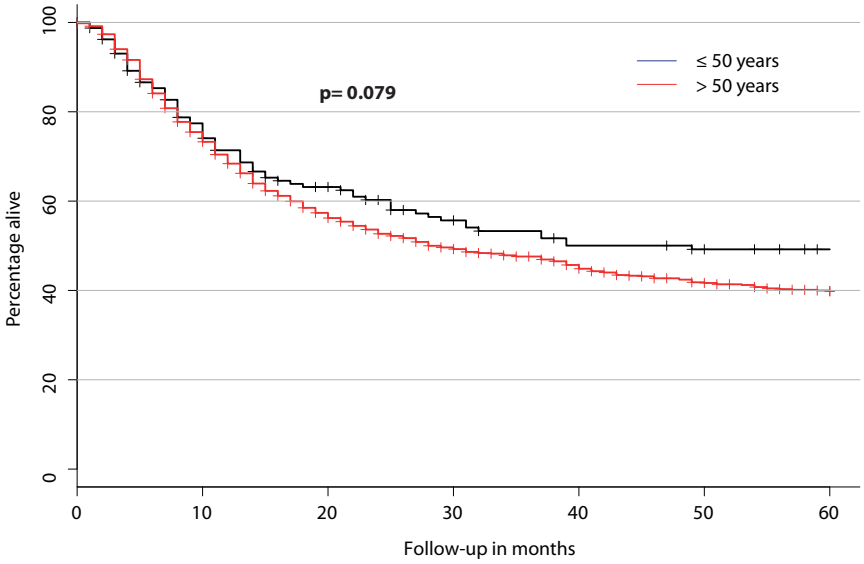
Overall survival	**Age ≤ 50 years (n= 163)**	**Age > 50 years (n= 1,151)**
Median survival (IQR)	33 (12-134)	23 (9-86)
Mean survival (95% CI)	93.8 (76.8-110.8)	63.6 (58.1-69.1)
5-year survival	40.5%	31.0%

Figure 2: Disease-specific survival according to age group



Disease-specific survival	**Age ≤ 50 years (n= 159)**	**Age > 50 years (n= 1,041)**
Median survival (IQR)	37 (12-134)	30 (12-185)
Mean survival (95% CI)	105.7(87.5-123.8)	92.1 (84.7-99.5)
5-year survival	43.6%	37.4%

Figure 3: Disease-free survival according to age



Disease-free survival	**Age ≤ 50 years (n= 163)**	**Age > 50 years (n= 1,151)**
Median survival (IQR)	49 (10-109)	28 (10-147)
Mean survival (95% CI)	119.2 (100.1-138.4)	99.8 (92.0-107.6)
5-year survival	49.2%	39.8%

Prognostic factors

Factors predicting OS in the univariate analysis are shown in Table 5. Independent predictors of OS were age, presence of co-morbidity, occurrence of surgical complications, poorly and undifferentiated tumors, tumor-positive resection margins (pR1-2), and TNM stage IIB- IV(Table 6). Multivariate analysis identified moderately, poorly, and undifferentiated tumors, tumor positive resection margins (pR1-2), and TNM stage IIB- IV as independent predictors of DSS.

Table 5: Cox proportional hazard analysis for overall survival (univariate)

Factor	Hazard ratio (95% CI)	p-value
Co-morbidity	1.277 (1.122-1.454)	<0.001
Surgical complications	1.365 (1.198-1.555)	<0.001
Nonsurgical complications	1.123 (0.987-1.276)	0.078
Differentiation*		
Good	1.520 (0.993-2.326)	0.054
Moderate	3.083 (2.235-4.249)	<0.001
Poor/undifferentiated	4.292 (3.113-5.918)	<0.001
Neoadjuvant Chemotherapy	0.949 (0.813-1.108)	0.511
Neoadjuvant Chemoradiation	0.635 (0.489-0.823)	0.001
R1/2**	2.840 (2.468- 3.268)	<0.001
Sex: Male	1.146 (0.980-1.340)	0.088
Stage***		
0 - IIA	1.798 (1.152-2.806)	0.010
IIB - IIIB	4.527 (2.955-6.936)	<0.001
IIIC - IV	9.332 (5.984-14.553)	<0.001
Age ≤50 years	0,718 (0,584-0,882)	0.002
Transthoracic esophagectomy	1.194 (1.021-1.397)	0.026

* With reference to not differentiated

** With reference to R0

*** With reference to Tis (HGD)

Table 6: Cox proportional hazard analysis for overall survival (multivariate)

Factor	Hazard ratio	p-value
Age ≤50 years	0.761 (0.616-0.939)	0.011
Co-morbidity	1.233 (1.079- 1.407)	0.002
Surgicalcomplications	1.296 (1.133- 1.483)	<0.001
Nonsurgical complications	1.074 (0.940-1.228)	0.293
Differentiation*		
Good	0.973 (0.582-1.627)	0.917
Moderate	1.463 (0.935-2.288)	0.096
Poor/undifferentiated	1.635 (1.037-2.577)	0.034
R1-2**	1.988 (1.710-2.311)	<0.001
Stage***		
0-IIA	1.296 (0.729-2.303)	0.378
IIB-IIIB	2.563 (1.412-4.654)	0.002
IIIC-IV	4.581 (2.475-8.478)	<0.001
Sex: Male	1.049 (0.894-1.232)	0.556
Neoadjuvant Chemoradiation	1.032 (0.788-1.352)	0.819
Transthoracic esophagectomy	1.223 (1.079-1.407)	0.788

* With reference to not differentiated

** With reference to R0

*** With reference to Tis (HGD)

DISCUSSION

There is an on going debate about the relations between patient's age and the clinical presentation and prognosis of esophageal cancer. Because survival is highly correlated with tumor stage at presentation, early diagnosis is important. To identify young patients at an early stage and to define the best treatment strategy for young patients, more insight in the oncological nature and prognosis of young patients is needed. This study shows that EAC is by far the most common type of cancer among all age groups. Also, there is a trend towards a higher prevalence of EAC in younger patients. These younger patients have less co-morbidities than their older counterparts. Contrary to others, there was no difference shown between the age groups regarding tumor localization, tumor grade, TNM stage, or resection margin. Survival analysis demonstrated a significantly better overall 5-year survival for the younger patients. However, DSS and DFS did not differ significantly between the two groups.

Another study from our institution showed that the increased incidence of EAC has occurred parallel to the increase of Barrett's esophagus, the precursor lesion of EAC.[18] This rise in incidence represents a real increase in the burden of the disease.[19] This implies a shift in the prevalence of causal exposure. Gastroesophageal reflux (GER) is thought to be the primary causal factor of EAC.[20-24] Obesity and overweight are associated with GER and show an upward trend in prevalence among all ages.[25] Therefore, it is conceivable that the downward shift in age at the onset of EAC is due to an increase in the prevalence of obesity and overweight among younger individuals. Unfortunately, we do not have detailed information on anthropomorphic characteristics of our patients. Donohoe et al. reported no difference between patients ≤50 versus >50 years in regard to body mass index at the time of diagnoses. They also observed that younger patients were more likely to be current smokers.[8]

The transformation of a normal cell into a tumor cell is caused by 10 to 20 mutations in a specific gene, and the time necessary for this to occur is one to several decades. Provided that specific inherited predispositions are absent, cancer is a disease that occurs after several decades of human life and is thought to be uncommon before the age of 50 years. Moreover, the average age of patients with esophageal cancer is around 72 years.[26] Patient and doctor's awareness is relatively low at a young age, and therefore the diagnosis in young patients with specific symptoms is often delayed. Previous studies concluded that younger patients with esophageal carcinoma present with more advanced disease because of a delay in diagnosis, but they attain a survival comparable to that of their older counterparts because of more aggressive treatment or the lower prevalence in co-morbidities and larger physiological reserve.[5-7] Our study also dem-

onstrated that younger patients present with fewer co-morbidities, undergo adjuvant therapy more often, and have a lower in-hospital mortality rate. Contrary to our expectations, a transhiatal surgical approach was used more often in the younger patients rather than transthoracic esophagectomy. Nowadays, we prefer transhiatal esophagectomy for tumors at the GEJ,[27] but localization of the tumor did not differ between the two groups. A higher percentage of the patients >50 years were diagnosed with SCC, which is invariably treated with a transthoracic approach.

Although OS was significantly better for the younger group, there were no differences in clinicopathological characteristics or the DSS rate between the groups. These findings suggest that patients >50 years die of causes other than recurrent cancer, such as cardiac, respiratory and vascular events. Other studies have reported no difference between younger and older patients regarding clinicopathological characteristics or survival,[8-11] which is in line with the results of this study. Taken together, younger patients do not present with more aggressive disease than their older counterparts, which suggests no difference in biological behaviour of the tumor. Because of excluding patients with an unresectable tumor in this study, this conclusion cannot yet be drawn.

A limitation of the study is that the duration of symptoms and disease stage at presentation were not evaluated in patients who were not eligible for surgery and were treated with palliative intent. It could be that a larger or smaller percentage of patients aged ≤50 years had metastatic disease and did not undergo treatment with curative intent. Two studies included operated and non-operated patients with esophageal cancer,[6,8] and showed that younger patients present with TNM stage and tumor grade similar to those of their older counterparts. However, younger patients more often underwent curative treatment including neoadjuvant therapy. Hashemi et al.[6] showed identical OS for both age groups, whereas Donohoe et al.[8] described improved OS and DSS for the younger patients. Interestingly, patients aged ≤35 years had a more advanced TNM stage and a poorer survival. Our study included only eight patients aged 35 years. Hence, a subgroup analysis was not feasible. Second, tumor grade and TNM stage were based on the pathological staging of the resected specimen and resected lymph nodes. In patients responding to neoadjuvant therapy, the pathologic TNM stage may be more favourable. However, a comparable percentage of patients underwent neoadjuvant treatment in the two groups.

Treatment regimes and peri-and postoperative management certainly have changed over the last decades. Improvements made in the diagnosis and treatments of esophageal cancer are likely independent of patient's age and histological type. We therefore think that it does not lead to bias when comparing outcomes of the two age groups.

Next, because patients seen in a tertiary hospital mostly have a resectable tumor, there may have been referral bias. All patients underwent a curative esophagectomy demonstrating no differences in TNM stage or resection margins between the two groups. This translates into similar DSS and the DFS for the young and old patients.

A considerable proportion (12%) of the patients diagnosed with esophageal carcinoma and qualified for curative resection are aged ≤50 years. Younger patients presented with fewer co-morbidities, resulting in a lower incidence of morbidity and mortality after esophagectomy. Although OS is better compared with their older counterparts, DSS and DFS are comparable for both age groups of patients with resectable tumors.

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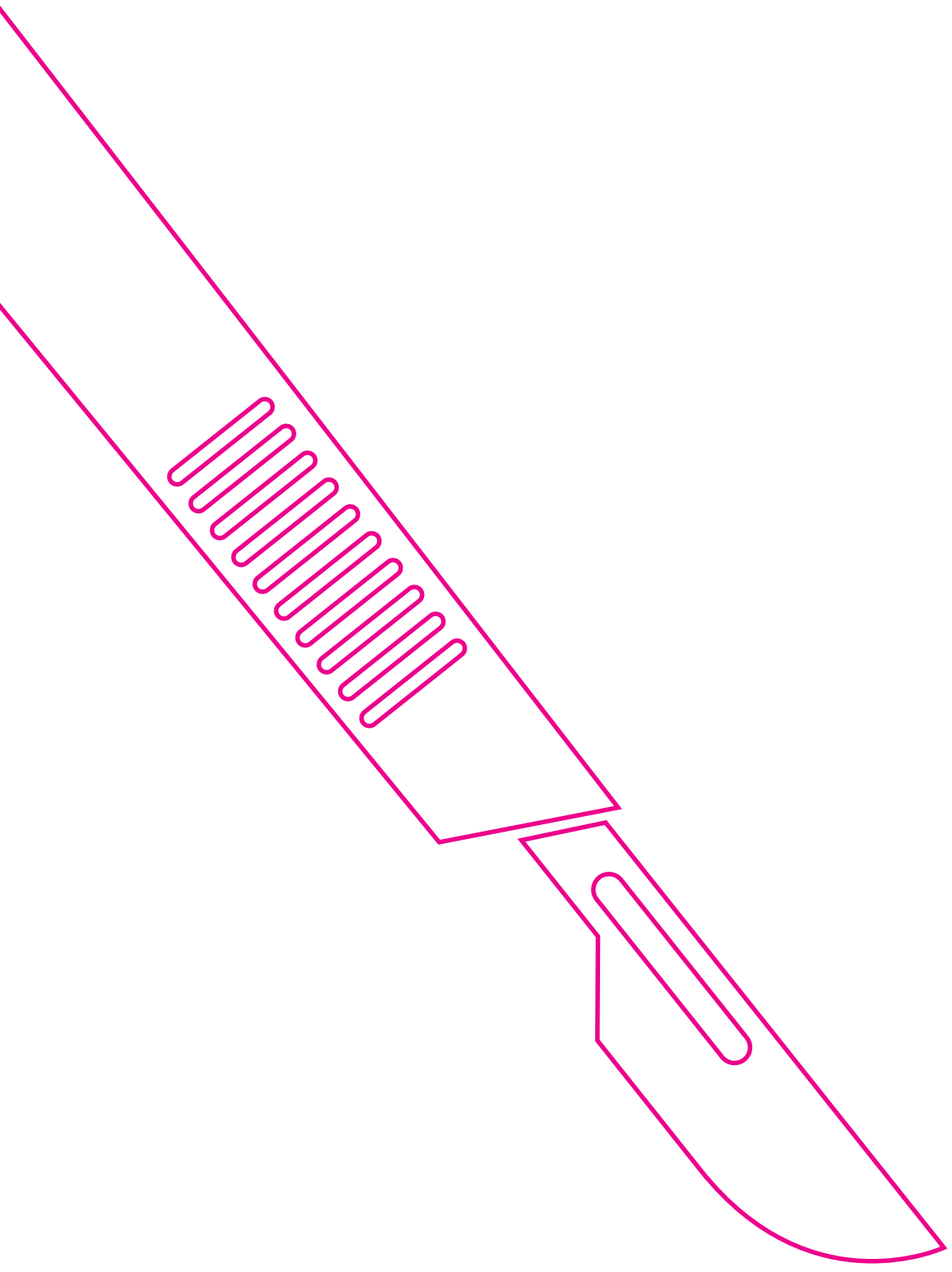

Chapter 8

Treatment and outcome of young patients with esophageal cancer in the Netherlands

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ABSTRACT

Background: Esophageal cancer is increasingly recognized in younger patients. We compared clinicopathological characteristics, treatment, and survival of patients aged ≤50 years with patients aged >50 years diagnosed with esophageal cancer in the Netherlands.

Methods: From the nationwide Netherlands Cancer Registry we identified all patients diagnosed with esophageal cancer between January 2000 and January 2011. Proportions were compared using the χ^2 test for categorical variables. Overall and relative survival was calculated.

Results: Eleven percent of the patients (n=1,466) were aged ≤50 years and adenocarcinoma was the most common tumor type (73.6%). Grade of tumor differentiation was comparable between both age groups (p=0.460) as well as T-stage (p=0.058). Younger patients presented more often with positive lymph nodes (70.1 vs. 66.4 %, p=0.010) and distant metastasis (50.5 vs. 44.7 %, p<0.001) but had surgery more often as compared to older patients: 40.6 vs. 37.9 %, p=0.047. There was no significant difference in the 5-year relative survival between both age groups: 18.1 vs. 17.2%, p>0.05. A subgroup analysis among patients diagnosed with adenocarcinoma revealed similar results.

Conclusions: Young patients with esophageal cancer present with more advanced disease stage and received more often treatment. However, they show comparable relative survival rates with their older counterparts.

INTRODUCTION

There has been a remarkable increase in the incidence of esophageal cancer over the last decades. This rise is attributed to an increase in adenocarcinomas.[1-3] Esophageal cancer was thought to be a disease of the elderly, with a peak incidence in patients aged 60-70 years. [4] However, recent studies have shown a rise in incidence of adenocarcinomas, also among patients aged <50 years. [2,3,5]

The effect of age on disease stage and survival of patients with esophageal cancer is controversial. Given the rarity of esophageal cancer in young patients, it is reasonable to assume that this disease is more easily misdiagnosed, and therefore diagnosis might be delayed.[6-9] Some studies suggest that patients under 50 years present with more advanced disease but at the same time receive more aggressive treatment and have a similar prognosis compared to older patients.[6-8] Others found that younger patients did not differ from their older counterparts with regard to clinicopathological characteristics or survival,[10-13] which is in agreement with a previous study from our institute.[14]

Previous studies about age differences used institutional databases and included only patients who underwent surgery.[8,10,12] However, less than 50% of esophageal cancer patients receive a potentially curative treatment and the majority has incurable disease at the time of diagnosis. To optimize treatment and to improve survival for younger patients, a clearer picture of all patients with esophageal cancer, including those who are not eligible for surgery, is needed.

The aim of this population-based study was to compare the clinicopathological characteristics, treatment, and outcome of esophageal cancer patients aged less than 50 years with those older than 50 years.

METHODS

Data collection

Population-based data from the nationwide Netherlands Cancer Registry (NCR), which was started in 1989 and is maintained and hosted by the Comprehensive Cancer Center, were used[4]. The NCR is based on notification of all newly diagnosed malignancies in the Netherlands by the automated pathological archive (PALGA). Additional data sources are the national registry of hospital discharge diagnoses, which accounts for up to eight percent of new cases.[4] Information on patient characteristics such as gender and date of birth, as well as tumor characteristics such as date of diagnosis, subsite (International

Classification of Diseases for Oncology (ICD-O-3),[15] histology, stage (Tumor Lymph Node Metastasis (TNM) classification)[16] and grade are obtained routinely from the medical records about nine months after diagnosis. The quality of the data is high, thanks to thorough training of the specialized registrars and computerized consistency checks at regional and national levels. Completeness is estimated to be at least 95%.[17] Follow-up of vital status of all patients was complete up to January 2012. In addition to passive follow-up via the hospitals, date of death is also retrieved from the Municipal Personal Records Database.[4]

For the present study we selected all patients diagnosed with invasive primary esophageal carcinoma or gastroesophageal junction carcinoma (C15-C16) between January 2000 and January 2011. Patients diagnosed with a tumor of the esophagus defined as epithelial neoplasm (ICD-O codes: 8010-8033) or not otherwise specified neoplasms (ICD-O codes: 8046, 8082-8084, 8123) were excluded from the study. Patients diagnosed with a tumor of the esophagus defined as squamous cell carcinoma (ICD-O codes: 8051, 8052, 8070-8078), adenoma or adenocarcinoma (ICD-O codes: 8140-8145, 8200, 8201, 8210, 8211, 8230, 8244, 8246, 8255, 8260, 8263, 8310), cystic, mucinous or serous neoplasm (ICD-O codes: 8480,8481,8490), ductal, lobular or medullary neoplasm (ICD-O codes: 8510) or complex epithelial neoplasm (ICD-O codes: 8560,8570,8573-8576) were included in the study. Patients aged ≥75 years at the time of diagnosis were excluded from the study, because of the high prevalence of co-morbidities and complications after treatment, which can cause a distortion of the results.[14,18-20]

The TNM-classification of the American Joint Committee on Cancer (AJCC) Staging Manual 6th edition was used for tumor staging.[21] For patients who underwent surgery TNM stage was based on pathological examination of the resected specimen (pTNM). For patients who did not undergo a resection, TNM stage was based on the clinical staging (cTNM).

Statistical analysis

The young group was defined as patients aged ≤50 years at time of diagnosis. This group was compared with the group of patients aged >50 years. The cut-off point of 50 years was arbitrarily chosen and based on previous studies.[9,14,22]

Patient- and tumor characteristics as well as details on treatment were first described with regard to the whole study population and subsequently for patients diagnosed with esophageal adenocarcinoma. Categorical variables were described using frequencies and percentages. Proportions were compared using the $\chi 2$ test for categorical variables. Two-sided p-values <0.05 were considered significant.

Survival estimates were calculated using Kaplan-Meier curves, and differences were tested with a log-rank test. Survival time was measured from the date of surgery or the date of diagnose to the date of death. Patients were censored at the time when they were lost to follow-up or when they left the study before the end of follow-up time. Survival data were expressed as medians with interquartile ranges (IQR). Univariable and multivariable Cox proportional hazard models were used to identify independent predictors of overall survival. Variables were entered in the multivariable analysis independent of the p-value reached in the univariable analysis, since all variables were considered to be clinically relevant.

Relative survival was used as an estimation of disease-specific survival. It reflects survival of cancer patients, adjusted for survival in the general population with the same structure for age and gender. Relative survival was calculated as the ratio of the observed rates in cancer patients to the expected rates in the general population. Expected survival was calculated from population life tables from the Netherlands, according to the Ederer II method.[23] Data analysis was performed with SPSS version 20.0 (SPSS, Chicago, IL, USA) and SAS system 9.3 (SAS Institute, Cary, NC, USA).

RESULTS

Study population

A total of 18,118 patients with cancer of the esophagus and the gastroesophageal junction were identified in the NCR database. Patients diagnosed with a tumor of the esophagus defined as epithelial neoplasm (n=552) or with a tumor not otherwise specified (n=66) were excluded, as well as patients aged ≥75 years at time of diagnosis (n=4,169). The remaining 13,331 patients were included in this study (Figure 1).

Patient and tumor characteristics

The mean age of the patients was 61.4 ±8.5 years (median age ≤50 years: 47.0 years, range 18-50 years, median age >50 years: 64.0 years, range 51-74 years). Eleven percent was aged ≤50 years and almost 80% of all patients were male. Esophageal adenocarcinoma was the most common histological type, especially in the younger age group. Younger patients also presented more often with positive regional lymph nodes (N+) and distant metastasis (M+) (70.1 vs. 66.4 %, p=0.010 and 50.5 vs. 44.7 %, p<0.001, respectively). Tumor grade was comparable between both age groups (p=0.460) (Table 1).

Figure 1: Flowchart

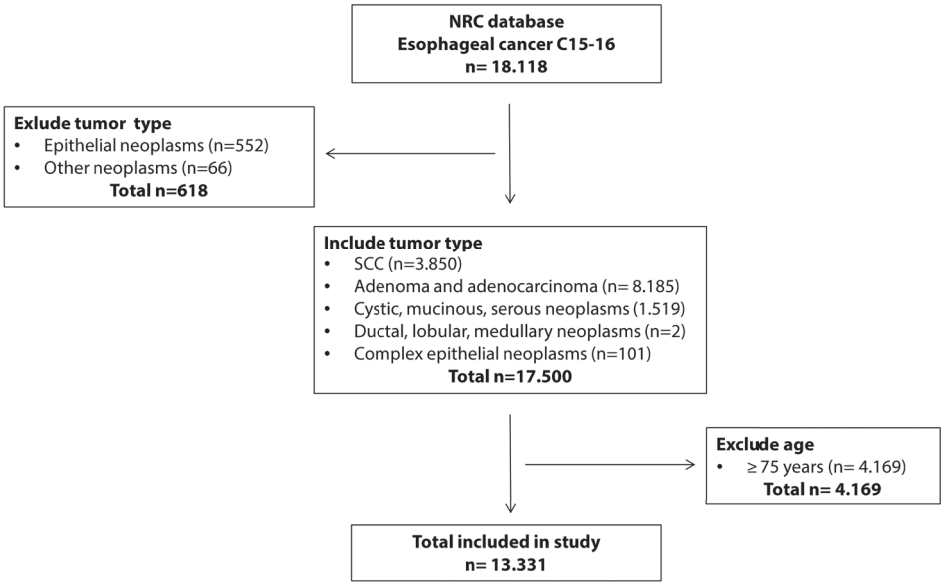


Table 1: Patient and tumor characteristics of the study population

	≤ 50 years N=1,466 (%)	> 50 years N=11,865 (%)	Total N=13,331 (%)	p
Gender				0.916
Male	1,148 (78.3)	9,277 (78.2)	10,425 (78.2)	
Female	318 (21.7)	2,588 (21.8)	2,906 (21.8)	
Histology				<0.001
Adenocarcinoma	1,140 (77.8)	8,667 (73.0)	9,807 (73.6)	
Squamous cell carcinoma	326 (22.2)	3,198 (27.0)	3,524 (26.4)	
T stage				0.058
No tumor + HGD*	1 (0.1)	25 (0.2)	26 (0.2)	
T1	115 (7.8)	987 (8.3)	1,102 (8.3)	
T2	242 (16.5)	2,081 (17.5)	2,323 (17.4)	
T3	499 (34.0)	4,151 (35.0)	4,650 (34.9)	
T4	208 (14.2)	1,585 (13.4)	1,793 (13.4)	
Unknown	391 (26.7)	3,033 (25.6)	3,424 (25.7)	
N stage				0.010
N0	262 (17.9)	2,499 (21.1)	2,761 (20.7)	
N+	1,027 (70.1)	7,881 (66.4)	8,908 (66.8)	
Unknown	177 (12.1)	1,485 (12.5)	1,661 (12.5)	
M stage				<0.001
M0	634 (43.2)	5,682 (47.9)	6,316 (47.4)	
M+	740 (50.5)	5,300 (44.7)	6,040 (45.3)	
Unknown	92 (6.3)	883 (7.4)	975 (7.3)	

Table 1: Patient and tumor characteristics of the study population (continued)

	≤ 50 years N=1,466 (%)	> 50 years N=11,865 (%)	Total N=13,331 (%)	p
Grade				0.460
Good	45 (3.1)	363 (3.1)	408 (30.6)	
Moderate	379 (25.9)	2,965 (25.0)	3,344 (25.1)	
Poor/ undifferentiated	590 (40.2)	4,603 (38.8)	5,193 (39.0)	
Unknown	452 (30.8)	3,934 (33.2)	4,386 (32.9)	
Resection margin				0.777
R0[#]	382 (26.1)	3,196 (26.9)	3,578 (26.8)	
R1[##]	65 (4.4)	540 (4.6)	605 (4.5)	
R2[###]	11 (0.8)	70 (0.6)	81 (0.6)	
Unknown	1,008 (68.8)	8,059 (67.9)	9067 (68.0)	

[*]HGD = High-grade dysplasia
[#]R0 = Tumor free resection margin
[##]R1 = Vital tumor present within 1 mm from the proximal, distal, or circumferential resection margin
[###]R2 = Residual macroscopic tumor

Treatment

Some 5,093 patients underwent surgery. Despite more advanced tumor stage, patients aged ≤50 years underwent more often surgery with or without neoadjuvant therapy as compared to patients aged >50 years: 40.6 vs. 37.9 %, p=0.047 (Table 2). For patients who only received palliative treatment (61.8%), younger patients received chemotherapy more often (44.8 vs. 32.7 %, p<0.001). Radiotherapy for palliation was applied more frequently in older patients (27.4 vs. 31.2 %, p=0.003) (Table 2).

Table 2: Treatment details of the study population

	≤ 50 years (N=1,466) (%)	> 50 years (N=11,865) (%)	Total (N=13,331) (%)	p
Surgery	595 (40.6)	4,498 (37.9)	5,093 (38.2)	0.047
Neoadjuvant therapy[*]	212 (14.5)	1,441 (12,1)	1,653 (12.4)	
Palliative treatment				
Chemotherapy	657 (44.8)	3,884 (32.7)	4,541 (34.1)	<0.001
Radiotherapy	402 (27.4)	3,705 (31.2)	4,107 (30.8)	0.003

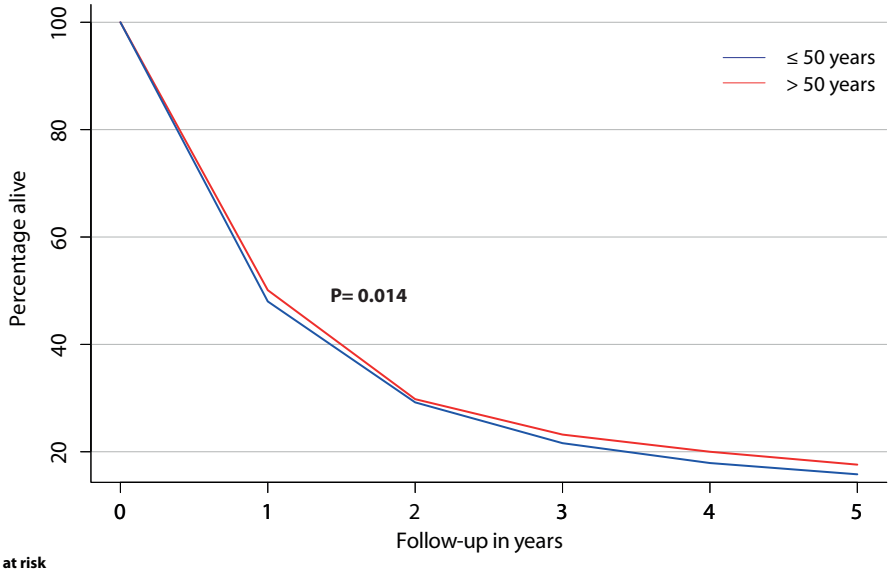
[*]Neoadjuvant chemo-, radio- or chemoradiation therapy followed by surgery

Survival

The median overall survival for patients aged ≤50 years was 12.1 months (IQR: 5.5-32.7 months) and 11.3 months (IQR: 4.8-29.4 months) for patients aged >50 years. The 5-year overall survival was 17.6 % for the younger patients versus 15.8% for the older patients (p=0.014) (Figure 2). Multivariate survival analysis showed that male gender, patients aged >50 years, squamous cell carcinoma, poorly differentiated tumors and advanced T-, N- and M stage and a tumor-positive resection margin were independently associated

with a higher risk of dying. Patients who underwent surgery had a lower risk of dying (Table 3).

Figure 2: Overall survival according to age



No at risk						
≤ 50 years	1,466	735	437	340	293	258
> 50 years	11,865	5,695	3,465	2,563	2,124	1,875
5-year survival						
≤ 50 years	17.6% (17.58-17.62)					
> 50 years	15.8% (15.79-15.81)					

The 5-year relative survival after resection was 37.6% (95%CI: 33.5-41.7) for the younger patients versus 34.1% (95%CI: 32.5-35.7) for the older patients (p>0.05). Among patients who received palliative treatment; younger patients showed a worse 5-year relative survival compared to their older counterparts: 4.5% (95%CI: 2.8-6.1) and 6.7% (95%CI: 5.9-7.5) respectively. However, this difference was not significant (Figure 3).

Subgroup analysis for adenocarcinomas

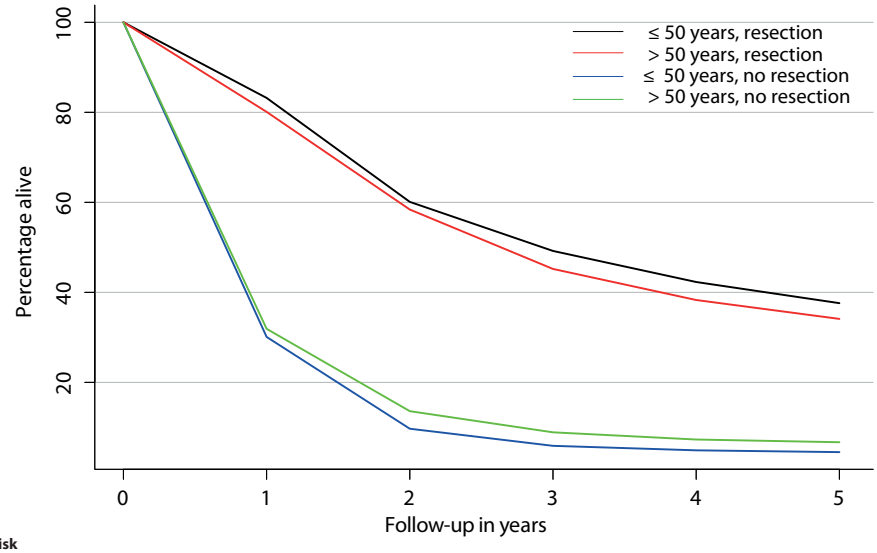
Some 9,807 (73.6%) patients were diagnosed with an adenocarcinoma. Younger patients presented more often with positive regional lymph nodes (N+) (71.9 vs. 67.1 %, p=0.001) and distant metastasis (M+) (53.0 vs. 46.3 %, p<0.001) and tumor grade was comparable between both age groups (p=0.434). Overall 5-year survival was significantly better for younger patients: 18.2% (95%CI: 2.9-6.5) as compared to 16.4% (95%CI: 5.1-6.7) for patients aged >50 years (p=0.021). The 5-year relative survival rates after surgery or palliative treatment did not differ significantly between both groups.

Table 3: Cox proportional hazard model

	Univariable analysis Hazard Ratio (95%CI)	Multivariable analysis Hazard Ratio (95% CI)	p
Gender			
Female	1	1	
male	1.10 (1.05-1.15)	1.10 (1.05-1.16)	<0.001
Age (yrs)			
≤ 50	1	1	
>50	1.08 (1.02-1.15)	1.12 (1.06-1.19)	<0.001
Tumor type			
Squamous cell carcinoma	1	1	
Adenocarcinoma	0.88 (0.84-0.92)	0.95 (0.91-0.99)	0.024
Tumor grade			
Good	1	1	
Moderate	1.82 (1.57-2.08)	1.77 (1.54-2.02)	<0.001
Poor	2.35 (2.06-2.68)	2.05 (1.79-2.35)	<0.001
Undifferentiated	3.20 (1.96-5.24)	4.11 (2.51-6.74)	<0.001
Unknown	2.33 (2.04-2.66)	1.69 (1.48-1.94)	<0.001
T			
1	1	1	
2	2.11 (1.85-2.40)	1.96 (1.72-2.25)	<0.001
3	2.39 (2.10-2.70)	1.94 (1.70-2.35)	<0.001
4	4.62 (4.06-5.27)	2.66 (2.32-3.03)	<0.001
Unknown	3.67 (3.24-4.15)	2.51 (2.20-2.85)	<0.001
HGD	0.25 (0.06-1.00)	0.31 (0.08-1.23)	0.095
N			
N0	1	1	
N+	1.93 (1.83-2.03)	1.46 (1.38-1.54)	<0.001
Unknown	3.32 (3.09-3.55)	1.73 (1.61-1.86)	<0.001
M			
M0	1	1	
M+	3.37 (3.23-3.51)	1.74 (1.66-1.83)	<0.001
Unknown	1.50 (1.39-1.61)	1.11 (1.03-1.20)	0.008
Neoadjuvant CRT*			
No	1	1	
Yes	0.39 (0.35-0.44)	0.93 (0.82-1.05)	0.234
Resection			
No	1	1	
Yes	0.29 (0.28-0.31)	0.52 (0.49-0.55)	<0.001
Resection margin			
R0 #	1	1	
R1 ##	2.12 (1.92-2.33)	1.87 (1.69-2.06)	<0.001
R2###	1.75 (1.36-2.25)	1.20 (0.93-1.55)	0.155
Unknown	3.56 (3.39-3.74)	1.61 (1.50-1.72)	<0.001

* CRT = Chemoradiation therapy, # R0 = Tumor free resection margin, ## R1= Vital tumor present within 1 mm from the proximal, distal, or circumferential resection margin, ###R2 = Residual macroscopic tumor

Figure 3: Relative survival accoring to age and resection



No at risk						
≤ 50 (resection)	**595**	**495**	**358**	**293**	**252**	**224**
> 50 (resection)	4,498	3,603	2,627	2,033	1,723	1,534
≤ 50 (no resection)	871	262	85	51	43	39
>50 (no resection)	7,367	2,350	1,002	656	538	420
5-year survival						
≤ 50 (resection)	37.6% (33.5-41.7)	≤ 50 (no resection)	4.5% (2.8-6.1)			
> 50 (resection)	34.1% (32.5-35.7)	> 50 (no resection)	6.7% (5.9-7.5)			

DISCUSSION

The incidence of esophageal cancer is rising among all age groups.[1-3] Hence, esophageal cancer is increasingly recognized in patients at a younger age. Controversy exists about the disease stage at initial presentation and prognosis of young patients with esophageal cancer. A previous study from our group based on an institutional database showed that clinicopathological characteristics and overall survival after surgery of patients aged ≤50 years did not differ from patients aged >50 years.[24] This is in agreement with previous studies,[25] and can be explained by the applied selection criteria and homogeneity of a cohort of surgical patients, since only patients without distant metastasis and/or local resectability plus an adequate performance status are eligible for surgery and included in the analysis.

The present study shows that in the Dutch population differences in tumor stage and treatment exist between younger and older patients with esophageal cancer. Patients aged ≤50 years presented more often with positive regional lymph nodes and distant metastasis. However, they attained a similar 5-year relative survival compared with their older counterparts. This can be partly explained by the higher percentage of young

patients who underwent multimodal treatment including surgery and neoadjuvant chemo- and/or radiotherapy. Also in the palliative setting, young patients were more likely to receive chemotherapy, whereas older patients were more frequently treated with less toxic radiotherapy schedules. Portale et al.[7] also reported that younger patients presented with more advanced disease, but with appropriate aggressive treatment, reached similar survival rates compared with their older counterparts.

Overall survival rates were significantly higher for younger patients, probably because older patients suffer from co-morbidities more frequently, hence might die of other causes than recurrent cancer. To adjust for survival in the general population with the same structure for age and gender, we calculated relative survival rates. Comparing of the relative survival rates between patients aged ≤50 years and patients aged >50 years showed no significant differences.

It is reasonable to assume that upper gastrointestinal symptoms in younger patients are more likely ascribed to a benign condition, hence diagnosis could be delayed. Whether non-reporting of symptoms by the patient, doctor's delay in referring youngsters for endoscopy or both are causative, is unknown. Several studies have reported that young patients indeed present with dysphagia that has been present for a longer period of time before diagnosis is made, and as such present with a more advanced disease stage as compared to older patients.[6-9] Clearly, there is room for improvement in recognition and timely detection of esophageal cancer in young patients.

The increased incidence of esophageal adenocarcinoma has occurred parallel to a rising prevalence of the Barrett's esophagus.[5] This implies an increase in the causal factor inducing Barrett's esophagus and/or esophageal adenocarcinoma, which is thought to be gastroesophageal reflux. Obesity and overweight are also independently associated with esophageal cancer and show an upward trend in prevalence among all ages.[26]

This would suggest that initiation and progression of cancer are uniform for both the young and the older patients. On the other hand, one could hypothesize that esophageal cancer in young patients has a different etiology than in their older counterparts. It is generally accepted that it takes several decades for a normal cell to turn into a cancer cell. Considering their young age, young patients have not had sufficient time to obtain enough mutations needed to turn a normal cell into a cancer cell. Therefore, it can be anticipated that young patients might develop esophageal cancer through another pathway than older patients. More insight into the molecular biology of the tumorigenic pathway is required to better understand the tumor initiation and progression of both the young and the old patients.

Young patients are generally treated according to protocols based on the average cohort of patients with esophageal cancer, with a peak incidence in age of 60-70 years.[4] Moreover, patients aged ≤50 years are likely underrepresented in clinical trials given the lower incidence in this age group. Koppert et al.[27] reported that the prevalence of co-morbidities among older cancer patients is high and older patients are treated less aggressively compared to younger cancer patients. Young patients suffer from co-morbidity less often compared to elderly patients,[14,18-20,28] and they generally have a better physical condition, which could lead to an improved tolerance for surgical and nonsurgical treatments. It can be expected that medical specialists are more eager to treat these young patients with more aggressive and potentially toxic therapies. Also an anticipated higher life expectancy could contribute to the higher rates of surgical treatment in younger patients as reported in the present study.

A limitation of our study is the lack of detailed information on duration of symptoms before diagnosis, co-morbidities, (non)surgical complications and causes of death, that are not available in the NCR database. Therefore, we were not able to calculate disease-specific survival or disease-free survival. Another limitation is the use of different editions of TNM-staging throughout the study period. For example, patients diagnosed with positive celiac axis lymph nodes were classified as stage M1a according to the 6th edition, however this stage changed into N1-3, depending on the number of positive lymph nodes, in the 7th edition.[29] However, it is unlikely, that this has had an impact on the distribution of tumor stages between the age groups. Improvements in overall survival in later-year cohorts could be attributed to improvements in multimodality treatment,[6,30] however these new treatments are considered to be equal managed in both age groups.

In conclusion, this population-based nationwide study revealed that esophageal cancer patients aged ≤50 years present with more advanced disease stage, wherefore they received more often treatment. This results in a marginal better overall survival rate but similar relative survival as compared to patients aged >50 years. Improvement should be made in the earlier recognition of alarm symptoms and the detection of esophageal cancer in young patients in order to prevent younger patients presenting with more advanced disease stages.

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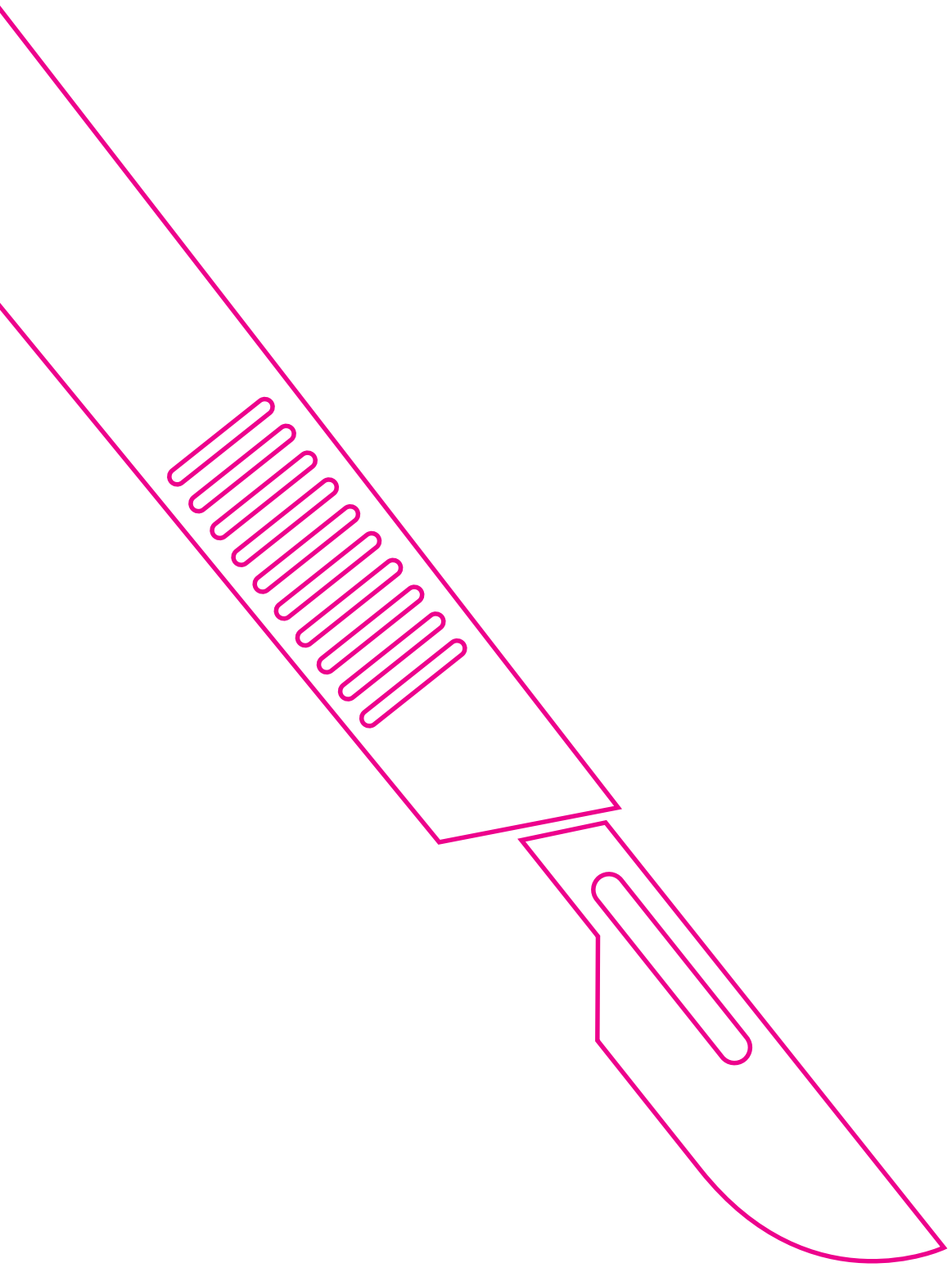

Chapter 9

Early onset esophageal adenocarcinoma: a distinct molecular entity?

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ABSTRACT

Background: Esophageal adenocarcinoma (EAC) is typically diagnosed in elderly with a median age of 68 years. The incidence of EAC has been rising over the last decades, also among young adults. The aim of the study was to investigate whether early onset EAC is a distinct molecular entity.

Methods: To identify early onset EACs, the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA) was searched. Twenty-eight tumors of patients aged ≤40 years were selected and matched with 27 tumors of patients aged ≥68 years. DNA was isolated from surgically resected specimen and sequenced on the Ion Torrent Personal Genome Machine with the Ion AmpliSeq Cancer Panel.

Results: No differences in mutational load between early onset and conventional EACs were observed (p=0.196). The most frequently mutated genes were *TP53* (73%) and *P16* (16%). Additional mutations in early onset EACs occurred exclusively in: *APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11*. In the conventional EACs additional mutations were exclusively identified in: *ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4*, and *VHL*.

Conclusion: Additional mutations besides *TP53* and *P16* seem to occur in different genes related to cell fate pathways for early onset EACs, while the additional mutations in conventional EACs are related to survival pathways.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is typically diagnosed in elderly adults with a median age of 68 years.[1] The incidence of EAC has been rising rapidly over the last decades, also among young adults.[2,3] The clinicopathological characteristics of these young adults with early onset EACs are different compared to the conventional EAC of the older patients: those with early onset EAC suffer from less co-morbidities, present with advanced disease stages more often, undergo more aggressive treatments, but ultimately obtain the same relative 5-year survival rates as their older counterparts.[4-6] Individuals can be predisposed to early onset EAC through heredity, since seven percent of the patients diagnosed with EAC can be considered familial Barrett's esophagus (FBE), of which the age at diagnosis is generally lower compared to sporadic cases,[7,8] yet no genetic defects related to FBE have been identified. Nevertheless, early onset EAC can be sporadic as well, lacking a genetic predisposition.

It has been accepted that cancer is generally a disease of the elderly population. In addition, evidence is obtained that the transformation of a normal cell into a malignant cell and subsequently the outgrowth to a clinically manifest lesion takes several decades.[9] This transformation process is driven by genomic instability leading to the accumulation of mutations. About three mutations in driver genes, which are causally involved in the tumorigenic process, have to accumulate to induce this malignant transformation.[10] As a result of the genomic instability also passenger mutations, which are not involved in the tumorigenic process, will accumulate. Hence, it can be anticipated that early onset EACs went through an accelerated transformation process and as a result could have a lower mutational load of (passenger) mutations, as has been reported for other tumors before.[11] In addition, it is possible that early onset EAC is a distinct molecular entity as has been demonstrated e.g. for colorectal carcinoma.[12]

The aim of the present study was to investigate whether early onset EAC is a distinct molecular entity. By next-generation sequencing with a standard cancer panel the mutational load and molecular profile of early onset EACs was determined and compared with the conventional EACs.

MATERIALS AND METHODS

Patient identification

PALGA, the nationwide network and registry of histopathology and cytopathology, contains pathology reports generated in the Netherlands since 1971 and has complete na-

tional coverage since 1991 encompassing the pathology laboratories from all academic and nonacademic hospitals in the Netherlands.[13] The PALGA database was searched, with approval of their Privacy Commission and Scientific Council, to identify all patients diagnosed with an adenocarcinoma of the esophagus or the gastroesophagealjunction (GEJ) and aged ≤40 years in the Netherlands. The following search terms were used: "primary carcinoma," "esophagus", "stomach," and "age ≤40 years". The search was performed from January 1990 to March 2013. Cases were further confirmed or excluded after careful evaluation of the individual pathology reports.

The selected early onset EACs were compared with a group of conventional EACs collected from the Pathology archive of the Erasmus MC Cancer Institute, University Medical Center, Rotterdam. Patients aged ≥68 years and diagnosed with an adenocarcinoma of the esophagus or GEJ for which an esophagectomy was performed were selected and matched with the patients with early onset EAC with regard to grade of tumor differentiation and TNM-stage (according to the classification of the American Joint Committee on Cancer (AJCC) Staging Manual 7th edition).[14]

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tumor tissues were provided by the participating laboratories of PALGA. The tissue blocks were assessed anonymously according to the Proper Secondary Use of Human Tissue code established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). In addition the study was approved by The Medical Ethical Committee of the Erasmus MC Cancer institute, University Medical Center, Rotterdam.

Tumor tissue areas composed of at least 50% neoplastic cells (indicated by a GI-pathologist) were manually microdissected from 10 to 15 hematoxylin-stained sections (4μm) of FFPE tissue blocks. DNA was extracted using proteinase K and 5% Chelex 100 resin. To determine the presence of microsatellite instability analysis were performed with the MSI Analysis System, Version 1.2 (Promega, Madison, WI, USA).

Next-generation sequencing

Ion semiconductor sequencing on the Ion Torrent Personal Genome Machine (PGM) was performed with the Ion AmpliSeq Cancer Hotspot Panel on tumor DNA according to the manufacturer's protocols. In short, libraries were made using the Ion AmpliSeq Library Preparation Kit. A template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion Sequencing Kit v2.0 on an Ion 316 chip. Data were analyzed with the Variant Caller v2.2.3-31149 (Life Technologies, Carlsbad, CA, USA). Variants were called when the position was covered at least 100 times. Variants

found in at least 25% of the called reads were considered reliable. Variants present in the ESP6500si or 1000genomes databases in ≥1% were excluded. Subsequently nonsynonymous somatic point mutations, insertions and deletions that change the protein amino acid sequence and splice site alterations were selected as driver mutations.

Data analysis

Early onset EAC was defined as patients diagnosed with EAC at the aged ≤40 years. This group was compared with the group of patients aged ≥68 years. Patient- and tumor characteristics were described using frequencies and percentages. Proportions were compared using χ^2 test for categorical variables. Differences in the mean number of mutations between the age groups were tested using independent samples t-test. Two-sided p-values <0.05 were considered statistically significant for all analysis. Data analysis was performed with SPSS version 20.0 (SPSS, Chicago, IL, USA).

Results

Thirty-seven patients diagnosed with EAC or adenocarcinoma of the GEJ and aged ≤40 years at time of diagnosis were identified in the PALGA database. Twenty-eight samples obtained from these patients passed quality controls and were included in the study (Mean age: 37.2 years, range 28-40 years, 89% male). Twenty-seven patients diagnosed with EAC or adenocarcinoma of the GEJ and aged ≥68 years at time of diagnosis were matched with patients aged ≤40 years based on TNM stage and tumor differentiation grade (Mean age: 74.6 years, range 68-83 years, 78% male). All tumors were tested microsatellite stable. Of the patients aged ≤40 years seven received some form of neoadjuvant therapy, while none of the patients aged ≥68 years did. Patients- and tumor characteristics are listed in Table 1 according to age groups.

Next-generation sequencing with the PGM revealed 83 mutations in 55 EAC samples before filtering: 36 mutations in the patients aged ≤40 years and 47 mutations in the patients aged ≥68 years (p=0.094). After filtering 78 mutations remained: 35 mutations were identified in the patients aged ≤40 years and 43 mutations in the patients aged ≥68 years. The mean number of mutations for the young adults and the older patients was, 1.25 (SD 0.844) and 1.59 (SD 1.083) respectively, and not significantly different (p=0.196).

The most frequently mutated genes were *TP53* (73%), *P16* (16%), *ATM* (7%), and *RB1* (7%). In the early onset EACs *TP53* was altered in 75% and *P16* in 11%, whereas in the conventional EACs a mutation in *TP53* was found in 70% and *P16* was mutated in 22%. Except for one, all *P16* mutations occurred simultaneously with a *TP53* mutation. In 43% of the early onset EACs and in 33% of the conventional EACs no additional mutations besides a *TP53* mutations or a *P16* mutations were identified. The genes *ATM*, *JAK3*, *PIK3CA*, and *RB1*

were mutated equally between both groups. Additional mutations in five individual early onset EACs occurred exclusively in the genes: *APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11*. In the conventional EACs additional mutations were exclusively identified in the genes: *ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4*, and *VHL* (Figure 1 and Table 2).

Table 1: Patient- and tumor characteristics according to age groups

	Early onset EAC n=28 (%)	**Conventional EAC n=27 (%)**	**p-value (χ^2)**
Mean Age (sd)	37.2 (3.023)	74.6 (4.395)	<0.001 (t-test)
Gender			0.249
Male	25 (89.3)	21 (77.8)	
Female	3 (10.7)	6 (22.2)	
Tumor type			0.718
EAC	16 (57.1)	17 (63.0)	
GEJAC	9 (32.1)	9 (33.3)	
Cardia	2 (7.1)	1 (3.7)	
Unknown	1 (3.6)	0 (0)	
TNM stage*			0.350
IA	5 (17.9)	3 (11.1)	
	3 (10.7)	0 (0)	
IB	0 (0)	1 (3.7)	
IIA	2 (7.1)	7 (25.9)	
IIB	9 (32.1)	7 (25.9)	
IIIA	3 (10.7)	2 (7.4)	
IIIB	5 (17.9)	6 (22.2)	
IIIC	1 (3.6)	1 (3.7)	
IV			
Differentiation grade			0.233
High-grade dysplasia	0 (3.6)	0 (0)	
Good	4 (14.3)	0 (0)	
Moderate	11 (39.3)	16 (59.3)	
Poor	9 (32.1)	9 (33.3)	
Unknown	4 (14.3)	2 (7.4)	

EAC= esophageal adenocarcinoma

GEJAC= gastroesophageal junction adenocarcinoma

*According to the classification of the American Joint Committee on Cancer (AJCC) Staging Manual 7th edition.

Figure 1: Mutations identified in early onset EACs and conventional EACs classified to signalling pathways

Table 2: Extensive information of classification of genes in signaling pathways and cellular processes according to age groups.

Gene	Gene Name	Early onset/ Conventional	Classification	Core pathway	Cellular process
ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	Conventional	Oncogene	Cell Cycle/Apoptosis	Cell Survival
APC	Adenomatous polyposis coli	Early onset	Tumor suppressor gene	APC	Cell Fate
ATM	Ataxia telangiectasia mutated	Early onset/ Conventional	Tumor suppressor gene	DNA Damage Control	Genome Maintenance
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Early onset	Tumor suppressor gene	APC	Cell Fate
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Early onset	Oncogene	APC	Cell Fate
FBXW7	F-box and WD repeat domain containing 7	Conventional	Tumor suppressor gene	NOTCH	Cell Fate
FGFR2	Fibroblast growth factor receptor 2	Early onset	Oncogene	PI3K; RAS ; STAT	Cell Survival
GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	Conventional	Oncogene	PI3K; RAS; MAPK	Cell Survival
GNAS	GNAS complex locus	Conventional	Oncogene	APC; PI3K; TGF-β, RAS	Cell Survival/Cell Fate
JAK3	Janus kinase 3	Early onset/ Conventional	Oncogene	STAT	Cell Survival
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Conventional	Oncogene	RAS	Cell Survival
MET	Met proto-oncogene (hepatocyte growth factor receptor)	Conventional	Oncogene	PI3K; RAS	Cell Survival
P16	Cyclin-dependent kinase inhibitor 2A	Early onset/ Conventional	Tumor suppressor gene	Cell Cycle/Apoptosis	Cell Survival
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, catalytic subunit alpha	Early onset/ Conventional	Oncogene	PI3K	Cell Survival
RB1	Retinoblastoma 1	Early onset/ Conventional	Tumor suppressor gene	Cell Cycle/Apoptosis	Cell Survival
SMAD4	SMAD family member 4	Conventional	Tumor suppressor gene	TGF-β	Cell Survival
STK11	Serine/threonine kinase 11	Early onset	Tumor suppressor gene	mTOR	Cell Survival
TP53	Tumor protein p53	Early onset/ Conventional	Tumor suppressor gene	Cell Cycle/Apoptosis; DNA Damage Control	Genome Maintenance
VHL	Von Hippel-Lindau tumor suppressor	Conventional	Tumor suppressor gene	PI3K; RAS; STAT	Cell Survival

Adapted from Vogelstein et al. 2013[9]

DISCUSSION

For the first time a molecular analysis was performed on an exclusive group of patients with early onset EACs, to determine whether this is a distinct entity based on molecular spectrum. In comparison with the conventional EACs no difference in the total mutational load, including common driver mutations, was observed in the early onset EACs. Although no evidently differences were observed between the two groups with regard to molecular profile, the additional mutations, besides mutations in *TP53* and *P16*, identified in some individual early onset EACs differed when compared to the additional mutations identified in the conventional EACs.

Presently, there is no accepted clear definition of early onset EACs. Recent publications demonstrate that five percent of the patients diagnosed with EAC are aged ≤40 years, [5] and 10% aged ≤50 years.[6] In these studies young adults with EAC were compared with the conventional EAC patients, with a median age of 68 years, based on clinicopathological characteristics, showing that these younger patients present with more advanced disease stages, receive more often aggressive treatment regimes, however, ultimately obtain relative survival rates comparable with their older counterparts.[6] In order to ensure a clear segregation of the two entities and to avoid any overlap, a more restrictive definition of both early onset EAC and conventional EAC was used and patients between 41 and 67 years were excluded in the present study.

EAC evolves from the premalignant condition Barrett's esophagus, following a multimorphological pathway, in which metaplasia evolves to low-grade dysplasia, high-grade dysplasia and ultimately into invasive adenocarcinoma, during this malignant transformation mutations accumulate over time.[9,15] The number of mutations in a tumor originating from self-renewing tissue, e.g. the esophagus, is directly correlated with age. The majority of these mutations are passenger mutations that have no effect on the neoplastic progression. Whereas, the minority are the driver mutations, which confer a selective growth advantage. The passenger mutations occur mostly during the pre-neoplastic phase, which is evidently longer for older patients than for the younger ones.[16] Based on this concept it can be hypothesized that the number of total mutations, i.e. mutational load, is lower in early onset EACs as compared to conventional EACs.

Although it did not reached the level of significance, probably due to the relatively small amount of patients, a higher amount of total mutations (i.e. passenger and driver mutations) was observed in the conventional EACs when compared to the early onset EACs, which is in line with concept as has been described previously. The current data did not revealed a significant difference in the load of driver mutations between the two age

groups. Since the use of the Cancer Hotspot panel in this study, by which only 207 gene "hot spot" regions are investigated that are frequently mutated in human cancers, not all genes were covered. Hence, a complete overview of the total mutation spectrum per patient could not be established. An alternative explanation for the comparable load of mutations between the early onset EACs and the conventional EACs can be the occurrence of an ultramutator phenotype in the young adults resulting in an accelerated accumulation of mutations. By this phenomenon, young adults with early onset EAC could bear a comparable mutational load as compared to their older counterparts despite their shorter time of tumorigenesis.[17]

At a first glance no evidently differences were observed between the early onset EACs and the conventional EACs, with regard to the molecular profiles: the tumor suppressor gene *TP53* was altered in approximately 72% of EACs (75% in early onset vs. 70% in conventional EACs), which is comparable with other studies.[18] Mutations of *TP53* have been suggested to be an early genetic event in the multimorphological pathway of esophageal adenocarcinogenesis and are facilitating the accumulation of mutations.[9,19] Alterations of tumor suppressor gene *P16* are additional early events in EAC, and present in approximately 12%.[18] Here, in 11% of the early onset EACs a *P16* mutation was identified, whereas in the conventional EACs a *P16* mutation was identified in 22%. A remarkable observation was made regarding the additional mutational spectrum; the genes *APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11* were exclusively mutated in five individual early onset EACs. Whereas these mutations were not identified in the conventional EACs, that instead, exclusively carried additional mutations in the genes *ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4*, and *VHL*. Since the additional mutations were identified in five individual early onset EACs it might be based on randomness. In addition, in a large whole exome sequencing study on EACs performed by Dulak et al.[18] mutations in *APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11* were identified in EAC patients, here categorized as conventional EAC of older patients (range: 51-85 years), although in very small amounts.

However, considering the classification of cancer cell signaling pathways i.e. cell fate, cell survival, and genome maintenance, it is striking that the additional mutations in the early onset EACs occurred mainly in genes classified in cell fate pathways (*APC*, *CDH1*, *CTNNB1*), while all additional mutations in conventional EACs were identified in genes classified in survival pathways (*ABL1*, *GNA11*, *KRAS*, *MET*, *SMAD4*, *VHL*, *GNAS*, *FBXW7*). In addition, the shared mutations occurred in genes classified in genome maintenance pathways (*TP53*, *ATM*) as well as in survival pathways (*JAK3*, *PIK3CA*, *P16*). Mutations classified in cell fate pathways disturb the balance between differentiation and division, favoring the latter, which causes a selective growth advantage. Mutations categorized in cell survival pathways allow cancer cells to proliferate under limiting nutrient concentrations, making

them survive in environments in which sister cells cannot.[9] Different biological pathways for patients with early onset cancers have been described earlier, for example in breast cancer, colorectal cancer, melanoma, and tongue cancer.[20]

Taken together, these findings indicate that the development of EAC requires, regardless of the age of onset, a *TP53* mutation mostly accompanied by a *P16* mutation. However, the additional mutations needed to probably induce the malignant transformation[10] in some early onset EACs seem to occur in different genes, related to different pathways, as compared to the additional mutated genes in conventional EACs. From a treatment perspective, different pathways could indicate different inhibitors in the means of targeting treatment as has been established for metastatic colorectal cancer.[21] The current study gives a clue for a distinct molecular biology for early onset EAC. More extensive sequencing of larger cohorts of young adults and older patients with EAC have to be performed to determine whether early onset EAC is truly a distinct molecular entity that needs probably a different targeting therapy in the future.

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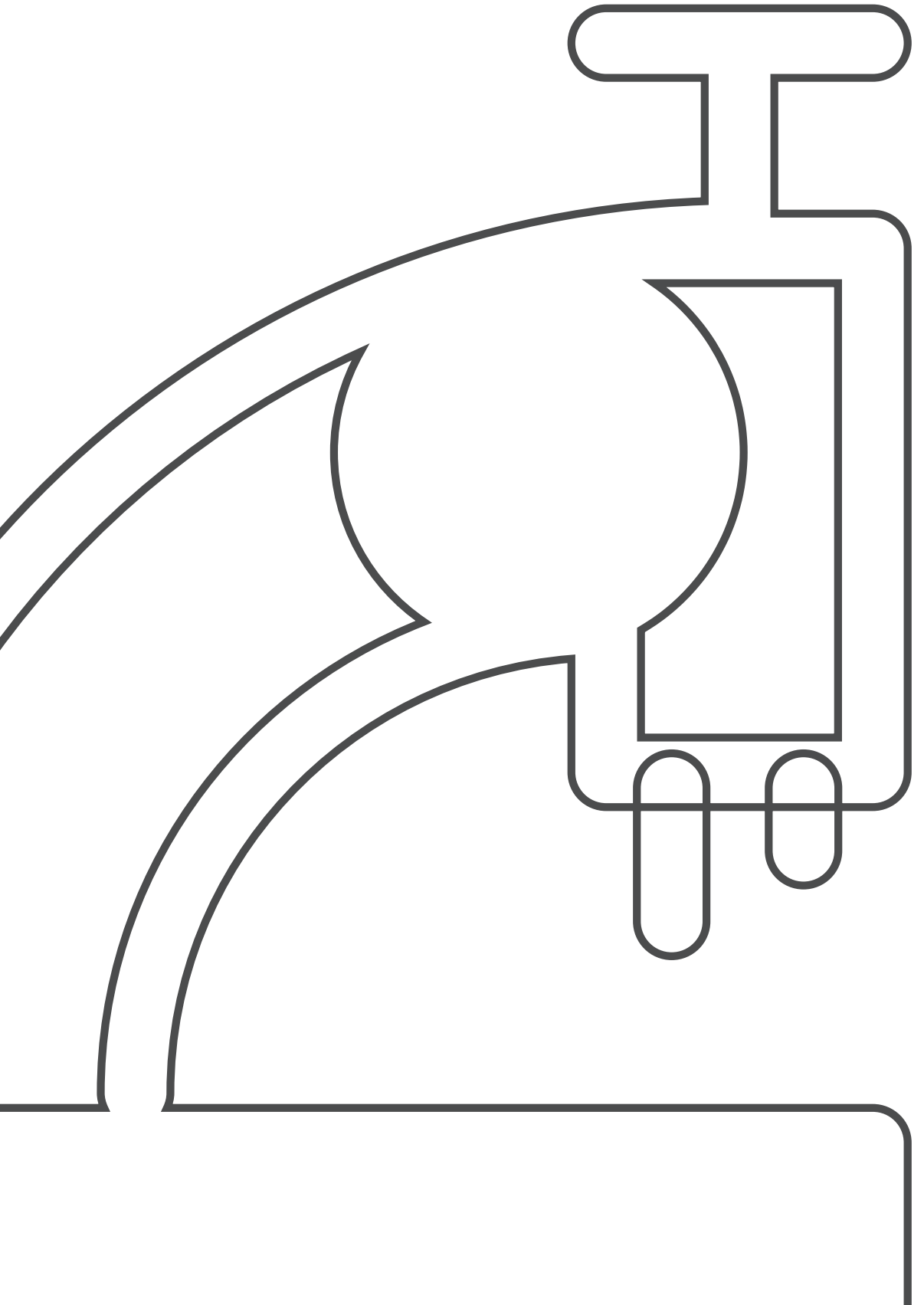
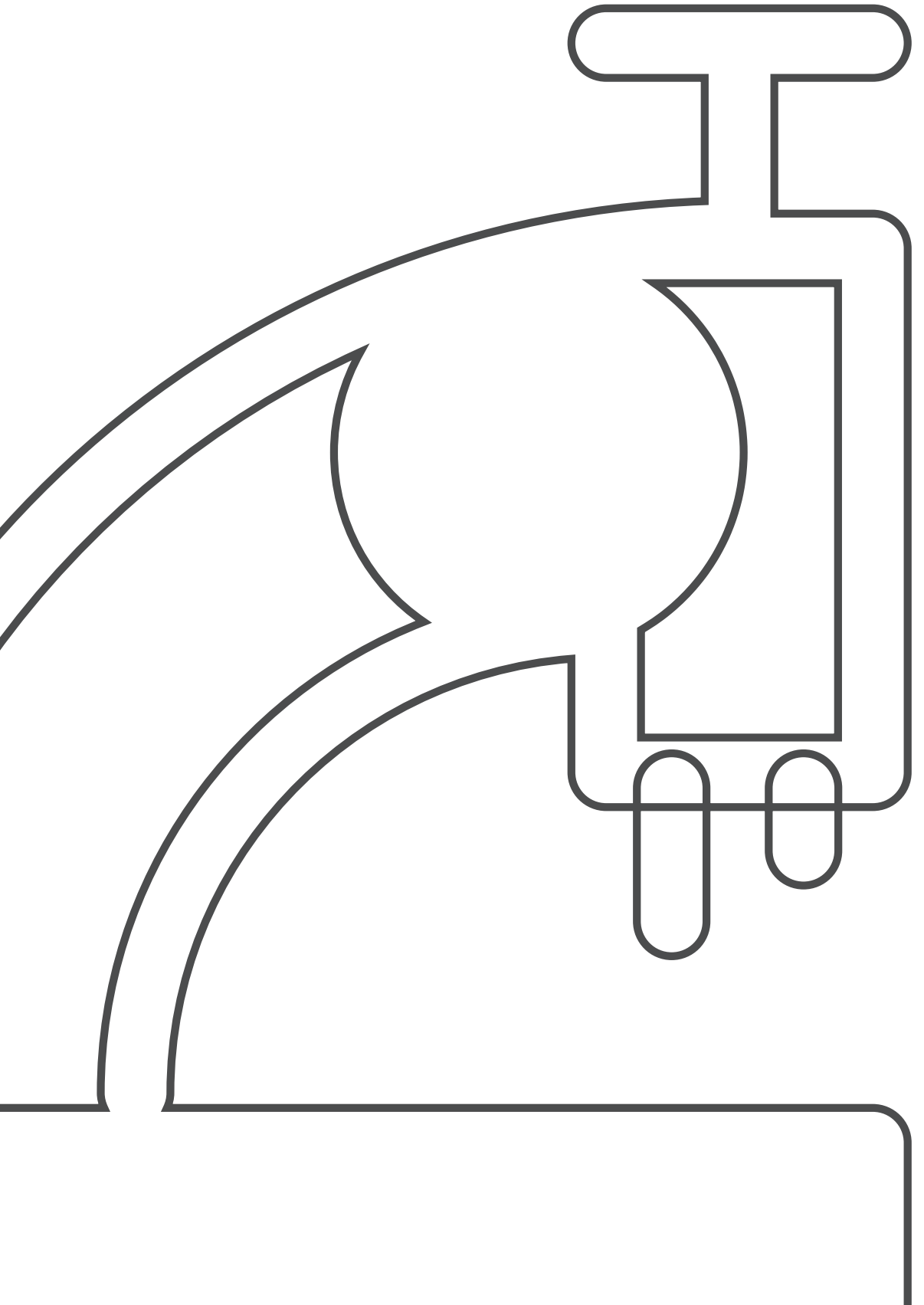


PART V

DISCUSSION AND SUMMARY



Chapter 10

General discussion and future perspectives

GENERAL DISCUSSION

Norman Barrett wrote in 1957: 'This paper concerns a condition whose existence is denied by some, misunderstood by others, and ignored by the majority of surgeons. It has been called a variety of names which have confused the story because they have suggested incorrect explanations.' Norman Barrett was an Australian-born British thoracic surgeon, after which the Barrett's esophagus (BE) was named to in 1950,[1] although it was Phillip Allison who proved the columnar-lined segment is esophagus and not stomach.[2] In 1953 the association between BE and gastroesophageal reflux (GER) was observed.[3] Subsequently in 1975, the relation between BE and esophageal adenocarcinoma (EAC) was suggested.[4] Over the past years endoscopic detection and surveillance of BE have gained insight in the morphological changes occurring during the transformation of BE towards EAC. Almost 60 years later however, there still is a real need to improve the understanding of this malignant transformation at a molecular level. In addition, although EAC has been considered a sporadic disease of the elderly, recently it has become clear that the population of EAC patients appeared to be relatively heterogeneous, including patients with sporadic cancers, patients with a positive family history for EAC and BE, and exceptionally young aged patients. This thesis aimed to elucidate the molecular biology of EAC by performing standard molecular analysis, i.e. whole-exome sequencing, targeted sequencing and Sanger sequencing on sporadic, familial and young patients with EAC. In addition, clinicopathological features of families with clustering of EAC and BE and young individuals with EAC were studied.

Molecular analysis of sporadic EAC

The assessment of oncological biomarkers in BE and EAC is based on the examination of small biopsy samples, which may not represent the entire molecular heterogeneity of a tumor. Genetic intratumor heterogeneity has been identified in several other cancer types e.g. clear cell renal cell carcinoma[5] and non-small cell lung cancer[6] and is thought to contribute to treatment failure and drug resistance. The model of branched tumor evolution leading to intratumor heterogeneity describes a tumor as a tree structure, with the trunk representing early molecular alterations, which clonally expand and therefore are homogeneously present throughout the entire tumor, reflecting a process involved before and during tumor initiation and early development. The branches of the tree represent later molecular alterations, which as a result are only present in different subclones of the tumor, contributing to intratumor heterogeneity and shaping the genome during tumor maintenance and progression.[7] In ***chapter 2*** of this thesis, the extent of intratumor heterogeneity was studied in EACs with the use of multiregional targeted sequencing. A panel of frequently mutated genes in EAC (*APC*, *P16*, *SMAD4*, *TP53*) was created based on the results of previous NGS studies.[8-10] This panel was sequenced on

multiple tumor regions obtained from single EACs. This revealed a homogeneous pattern of *TP53* mutations, as well as identical patterns of LOH of the *TP53* locus in all EACs, i.e. all separated tumor regions obtained from one EAC showed exactly the same *TP53* mutations and patterns of LOH of the *TP53* locus. In addition, in one paired EAC and BE sample the identical *TP53* mutation and pattern of LOH were detected. Although no mutations in *P16* were identified, a homogeneous pattern of LOH of the *P16* locus was identified in the majority of the EACs, as well as in the paired EAC en BE sample. While no mutations in *SMAD4* and *APC* were identified, LOH of the *SMAD4* and *APC* locus was observed in a heterogeneous pattern within the EACs, and thus not detectable in every sequenced region of the tumors. These findings indicate that alterations in both *TP53* and *P16* are located at the trunk of the so-called tree, representative for BE. *TP53* alterations precede LOH of *P16* in the malignant progression of EAC, however both are homogeneously present in the entire tumor. Alterations of *SMAD4* and *APC* seem to be located at the branches, representing intratumor heterogeneity of EACs.

The clonal evolution of BE has been studied before with interesting and conflicting interpretations of the data. Maley et al. proposed that a *P16* alteration clonally expands throughout the majority of cells of a Barrett segment, and subsequently *TP53* alterations occur on top of these and also expand.[11] Leedham et al. suggested a genetically heterogeneous BE, however with a strong selective advantage of cells with the inactivation of *TP53*.[12] The data presented in this thesis suggests, although based on the tumor biopsies and not BE, a full clonal expansion of *TP53* inactivation, followed by the inactivation of *P16*. This is in line with a recent study on the clonal architecture of BE and EAC obtained by paired exome sequencing. The data suggests that *TP53* mutations are present early in BE progression relative to other alterations including inactivation of *P16*, followed by a genome doubling, resulting in a heterogeneous tumor.[13] EACs mirror spatial and temporal heterogeneity, although the study in this thesis revealed homogeneous alterations of *TP53* and *P16*, which are the most frequently altered genes in EACs, on top of these heterogeneous alterations (*APC*, *SMAD4*) in the original EACs differ among tumor regions and distinct disease site (BE and lymph node metastasis). This mandates taking biopsies at multiple time points from the premalignant lesion, the primary tumor and, although not studied in this thesis, the metastatic sites. Reconstructing clonal architectures of EAC in more detail is necessary to obtain key insight into the etiology of BE and EAC. Our attempt of a representation of the mutational landscape of EAC, although in a limited number of cases and a relatively small number of genes, has revealed a considerable level of heterogeneity that likely affects the identification of oncological biomarkers that could serve as markers for risk stratification or as targets for therapy.

Somatic hotspot mutations in the promoter of the gene coding for telomerase reverse transcriptase (*TERT*) catalytic subunit have recently been described in bladder cancers[14], melanomas,[15] gliomas,[16] and thyroid cancers,[17] but not in breast, colorectal and prostate cancer. In addition, *TERT* promoter mutations could not be detected in esophageal SCC.[18] In ***chapter 3*** of this thesis, for the first time, the occurrence of *TERT* promoter mutations in EAC was evaluated with the use of a multiplex SNaPShot assay. Telomerase increases telomere length at chromosome ends. It is active in stem cells to prevent chromosome shortening, replicative senescence and genomic instability. In differentiated cells of somatic tissues, telomerase becomes downregulated, however in cancer cells it can become reactivated.[19] However, since *TERT* promoter mutations were not detected in EACs, these tumors might have alternative mechanisms to maintain telomere length, and therefore would be less likely to benefit from activating mutations in *TERT*.

Many studies have focussed on the molecular biology of EACs using the tumor as a starting point, by which tumor-specific (somatic) mutations in the genes *TP53*, *P16*, *SMAD4*, *PIK3CA*, *ARID1A* were identified.[8-10] However, research into the genetic alterations of BE developing into EAC is important as well. Assessing an individual's genetic susceptibility, based on common genetic variants in the population, may identify patients with BE, who have a high risk for malignant progression. Single nucleotide polymorphisms (SNPs) are the most common type of genetic variations and represent differences of single nucleotides at specific positions in the DNA sequence. Each variation is present to some appreciable degree in a population. SNPs within the coding sequences of genes, i.e. exons, can change the amino-acid sequences of a protein and therefore the activity of the protein. SNPs located upstream or downstream from a gene may affect gene expression, when located in the promotor region. SNPs located in the non-coding regions of a gene, i.e. introns, do not affect the amino-acid sequence but, can still be related to variants located in an exon. However, the vast majority of the SNPs are located outside the genes and functional consequences of these genomic variations are mostly unknown (www.nature.com). Genome-wide association studies (GWAS) screen thousands of unrelated cases (diagnosed with the disease of interest) and controls to identify common genetic variants that have an association with a specific disease. The first GWAS on BE and EAC revealed several associated SNPs: rs10419226 (*CRTC1*), rs11789015 (*BARX1*), rs2687201 (*FOXP1*), rs9936833 (*FOXF1*), rs2178146 (*FOXF1*) and rs3111601 (*FOXF1*).[20]

In ***chapter 4*** of this thesis the association between these six SNPs and the risk of EAC was validated in an independent and large case–control study. The SNPs rs10419226 (*CRTC1*) and rs11789015 (*BARX1*), were associated with EAC, based on a significantly increased risk of EAC for the minor allele T of rs10419226 (*CRTC1*) and a significantly decreased risk of EAC for the minor allele G of rs11789015 (*BARX1*). In addition, both SNPs showed a

dose-effect in the genotypic analysis: homozygosity for two minor alleles gave a stronger effect than heterozygous genotypes. Rs10419226 as well as rs11789015 are located in the first intron of the genes *CRTC1* and *BARX1*, respectively. Both variants have no obvious effect on the coding sequence of the gene but could be linked to an associated variant located in an exon. Alternatively, since the SNPs are both located in the first intron of the gene, they might affect the promoter and cause up- or downregulation of the expression of the gene.

A more recent GWAS[17] supported the association of rs11789015 (*BARX1*) and rs2687201 (*FOXP1*) with the risk BE (not EAC), as previously reported by Levine et al.,[20] but not replicated by our study. In addition, two new SNPs were identified: rs3072 (*GDF7*) and rs2701108 (*TBX5*).[21] The impact of SNPs on the function of the genes must be interpreted with caution. Functional studies are required to determine whether the SNPs cause up- or downregulation of gene expression and impact on cell behaviour. In addition, the effect of the SNPs can be cell type specific or stage specific or can be influenced by environmental factors. These results provide evidence that the risks of BE and EAC are influenced by genetic variants that are common in the population, and therefore the susceptibility to BE and EAC is not equally distributed among individuals in the population. Clearly, some people have a higher susceptibility than others because of their genetic load, which in the context of changing environmental conditions may increase their absolute risk of cancer. Although the individual effect size of any given SNP is small, collectively these SNPs could still account for a substantial proportion of variation in risk.

Familial clustering of EAC

Chapter 5 of this thesis consist of a review about familial EAC. Since 1978 several families have been described with clustering of BE and EAC.[22-27] Families consisting of two or more first- or second-degree family members diagnosed with BE and/or EAC are termed familial Barrett's esophagus (FBE). This group comprises approximately seven percent of all patients diagnosed with BE or EAC.[28-30] The majority of the patients meeting the criteria of FBE are diagnosed at a younger age compared to sporadic cases.[29-31] In addition, a segregation analysis of 881 pedigrees of FBE supports an incompletely dominant inheritance model with a polygenic component,[32] i.e. a trait influenced by many genes. Moreover the presence of BE and EAC cases among multiple generations of one family also suggests a phenotypic autosomal dominant pattern of inheritance. [24,33] Orloff et al. identified germline mutations in the genes *MSR1*, *ASCC1*, and *CTHRC1* with the use of a linkage analysis on affected siblings diagnosed with BE or EAC.[34] However, information about the presence of identical germline mutations in affected siblings is lacking and the role of these genes in the development of FBE is unknown. The presence of several cases of BE and EAC among one family can be based on a coincidence, can be explained by

shared environmental factors between family members or is caused by the presence of a genetic defect concerning a rare variant, of which the importance is not elucidate yet. Extensive candidate gene and linkage research has been unsuccessful to date in identifying genetic variants that are associated with FBE.

In ***chapter 6*** of this thesis a family fulfilling the criteria of FBE was investigated by whole -exome sequencing on germline DNA of the proband diagnosed with BE (LGD). Germline and tumor DNA of affected relatives diagnosed with EAC was investigated successively. A rare heterozygous germline missense variant in the *MSX1* gene was identified in all but one family member. In addition somatic loss of the wild type allele of the *MSX1* gene was identified in the tumor DNA, indicating a potential role of this germline variant in the development of BE and EAC in this particular family.

MSX1 encodes a homeobox protein and interacts with bone morphogenetic proteins (BMPs) 2 and 4. Recent studies established BMP4 signalling as an important interconnected regulatory pathway that contributes to transformation of the epithelial cells from the normal stratified squamous mucosa to an intestinal columnar cell type. BMP4 was found to be present in inflamed squamous epithelium but not in normal squamous mucosa.[35,36] In addition, *MSX1* has been proposed as a gene in which mutations may contribute to tooth agenesis as well as non-syndromic forms of cleft lip and/or cleft palate.[37-40]

The presence of this genetic variant in this family with clustering of BE and EAC can be specific for this particular family. However, other families fulfilling the criteria of FBE should be screened for the presence of *MSX1* variants, while it might also be common a mutation in families with clustering of BE and EAC. Although this *MSX1* variant can be exclusive for this particular family, when demonstrated to be pathogenic it potentially elucidates a more common pathogenic pathway in EAC.

Young patients with EAC

Treatment for cancers has substantially improved over the past few decades, resulting in increased survival. Unfortunately, the outcome of young adults diagnosed with cancer including melanoma, colorectal cancer, breast cancer, and thyroid cancer remains poor.[41] EAC is most commonly diagnosed at the age of 70 years.[42] However, the incidence of EAC in young adults (aged ≤50 years) has been rising (www.cijfersoverkanker.nl).

Chapter 7 and ***chapter 8*** of this thesis show that more than 10% of patients diagnozed with EAC in the Netherlands are aged ≤50 years. This is in accordance with a study from the USA.[43] Patients with advanced EAC often present with dysphagia. Young patients

report a longer period of dysphagia prior to diagnosis, as has been found by others.[43-46] Upper gastrointestinal symptoms in younger patients might often be ascribed to benign conditions instead of being recognized as cancer symptoms. In addition, current guidelines do not indicate an endoscopy for younger patients (age ≤50 years) presenting with dysphagia without other alarming signs or symptoms. [47] This may cause a delay in diagnosis.

In ***chapter 7*** some 1,314 patients with EAC, who underwent esophagectomy were studied. There were no differences in tumor grade, TNM-stage, and survival between patients aged ≤50 years and their older counterparts. This is in agreement with the study of Markar et al.[43] The similarity in pathology and outcome after surgery of the two age groups can be explained by the applied selection criteria resulting in a homogeneous cohort of patients eligible for surgery based on TNM-stage and adequate performance status. In ***chapter 8,*** 13,331 patients diagnosed with EAC were selected, including patient with locally advanced tumors, patients with distant metastasis, and patients who were not fit for surgery. This revealed that younger patients presented with positive lymph nodes and distant metastasis more often. However, despite a more advanced tumor stage, younger patients underwent surgery more often (with or without neoadjuvant therapy) as compared to older patients. In patients that did not undergo surgery the younger group received more often chemotherapy, while radiotherapy, which is less toxic, was applied more often in older patients. A possible explanation might be the higher prevalence of co-morbidities in older patients,[48-51] whilst younger patients have a better physical condition. Hence, more aggressive treatments are offered to the younger patients. Nonsurgical complications were seen less often and in-hospital mortality was lower in younger age groups. This could explain the finding of a better overall survival in the younger patients. It can be expected that medical specialists treat younger patients with more aggressive and potentially toxic therapies to obtain improved survival rates. As expected, considering the higher prevalence of co-morbidities and incidence of complications among older patients, the overall survival for the younger patients was higher as compared to their older counterparts. This was true for overall survival after surgical and nonsurgical treatments. After adjustment for the survival in the general population with the same structure for age and gender, the relative survival rates were similar between both age groups. Despite the more advanced disease stage in younger patients present with at the time of diagnosis, they obtain relative survival rates similar to that of the elderly patients, most likely due to application of more aggressive treatment strategies.

There is a relative lack of clinical trials including young adults with cancer. If biological differences exist between younger and older patients with EAC optimal treatment may also be different for the two age groups. It has been described that the oncogenic pathways

in colorectal cancer, breast cancer and melanoma are different between younger and old patients.[52,53] From a molecular point of view individuals can be predisposed to early onset cancer through hereditary factors, including EAC as discussed in chapter 5 and chapter 6. However, the majority of early onset cancers are probably sporadic without a genetic predisposition.

In ***chapter 9*** of this thesis the molecular spectrum, i.e. mutational load and mutational profile, of patients diagnosed with EAC aged ≤40 years (early onset EACs) was determined and compared with patient diagnosed with EAC at the average age of ≥68 years. In order to ensure a clear segregation of the two entities and to avoid any overlap, patients between 41 and 67 years were excluded. Conceptually, in tumors originating from self-renewing tissues, the number of mutations is correlated with age. More than half of the mutations in tumors occur during the pre-neoplastic phase, which is evidently longer for older patients.[54] In ***chapter 9*** no difference in mutational load was observed between the EAC patients aged ≤40 years and the patients aged ≥68 years, which can probably be explained by the presence of an increased mutator phenotype in the young adults, i.e. cancer progression in which mutations develop in a rapid burst resulting in an accelerated accumulation of mutations. Hence, young adults with early onset EAC have a comparable mutational load as their older counterparts.[55]

With regard to the molecular profile, others have found as previously, *TP53* is the most commonly mutated gene in EACs,[8-10,56] followed by *P16*.[9] This mutation pattern was observed in chapter 9 as well: *TP53* was altered in 75% and *P16* in 16% of the patients with EAC. Although the percentage of *TP53* and *P16* mutations was comparable between the patients aged ≤40 years and the patients aged ≥68 years, the profile of additional mutations found in early onset EACs includes different genes compared to the older patients with EACs. Moreover, the genes mutated in the young EAC patients were mostly classified to cell fate pathways (*APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11*), while the genes mutated in the conventional EACs were classified to cell survival pathways (*ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4*, and *VHL*).[57] These findings indicate that the development of EAC requires, regardless of the age of onset, a *TP53* mutation mostly accompanied by a *P16* mutation. However, the additional mutations needed to probably induce the malignant transformation in young patients with early onset EACs seem to occur in different genes, related to different pathways, as compared to the additional mutated genes in older EACs patients. Different pathways underlining the possible different genetic make up of EAC between young and older patients could indicate that (targeted) treatment may have to be adapted.

Observations from the clinical and molecular studies suggest that there might be a distinct biology of EAC affecting young patients. With molecular profiling and NGS technology including whole genome and exome sequencing, becoming widely available, this may improve the molecular classification of EAC and help to identify specific biological entities, and refine our understanding of the impact of age on cancer biology, and impact on patient management.

FUTURE PERSPECTIVES

Cell free tumor DNA

Molecular testing on tissue samples derived from surgical resection specimens or small tumor biopsies remains the standard of care. However, intratumor heterogeneity in EAC would mandate multiple biopsies from a tumor, it surrounding premalignant BE and the metastatic sites at multiple time points. This is neither feasible nor ethical given the invasiveness of the procedure, risk of complications, and economic and logistic consequences. Molecular analysis of circulating cell-free tumor DNA (ctDNA) derived from blood samples is a novel method, which can be performed at multiple time-points and possibly better represents the dominant molecular profile of EAC than a single site biopsy, since the fragments of ctDNA are released in the blood from diverse tumor sites. This technique is also known as "liquid biopsy" and could also be used for monitoring the molecular profile of EACs over time. Possible clinical applications might be the analysis of ctDNA to evaluate the response to nCRT, to identify the complete responders prior to surgery to prevent them of having an esophagectomy. In addition it may be used as a biomarker to monitor disease progression or recurrence.

Familial EAC

It is been estimated that five percent of all human cancers are caused by an inherited factor. The patterns of inheritance in most familial cancer syndromes are consistent with an autosomal dominant inheritance pattern with a high penetrance. This means that offspring of patients have a 50% risk to inherit the disease and most of the individuals, who are susceptible will develop symptoms. Cancers diagnosed before the age of 50 years have relatively high chance to have been caused by an inherited factor.

Seven percent of patients diagnosed with BE and EAC is considered familial, and the pattern of inheritance of families with clustering of BE and EAC is consistent with a phenotypic autosomal dominant inheritance. The definition of FBE suggests familial BE and familial EAC to be part of the same genetic trait. However, BE is much more prevalent among the common population and familial BE does not necessarily have to be the

underlying condition of familial EAC. Although familial EAC might be the result of accelerated malignant progression from familial BE, it might also be distinct from most familial BE. The possibility of familial EAC as a distinct entity should be considered. A (inter)national consortium for familial EAC should be established to enlarge our knowledge about familial EAC, to set up and coordinate research, and improve the care for patients with familial EAC. Most important is to find consensus about an accepted definition of familial EAC. Criteria that warrant further testing could be based on the criteria as defined for hereditary diffuse gastric cancer: [58]

- Gastric cancer diagnosed in one family member before the age of 40 year or
- Gastric cancer diagnosed in two first- or second-degree relatives, of whom one was diagnosed before the age of 50 years or
- Gastric cancer diagnosed in three first- or second-degree relatives, regardless of the ages.

A clear and scientifically valid definition of familial EAC creates the opportunity to start a wide search for the identification of germline defects in these families, with the aid of new techniques e.g. genome/exome sequencing, enables testing of unaffected family members for the presence of the specific germline defect in order to counsel predisposed individuals at an early stage in their life.

Young patients with EAC

Over the last decades it has become apparent that patients diagnosed with cancer aged ≤40 years have not obtained the same improvements in survival as their older counterparts. In addition, it is striking that in terms of long-term survival expectations the most favourable ages are >40 years. Reasons for this deficit in survival improvements are the lack of awareness of cancer in younger patients and the lack of clinical trial activity and participation among younger patients with cancer.[59] When focussing on EAC, although survival rates are comparable between the younger and the older patients, clinical trials barely include younger patients. It should be recommended to include younger patients into clinical trials. In addition, more biological studies should be performed in order to obtain more knowledge about how tumors from younger patients differ from those of older patients on a molecular level to produce new targets for intervention, so that survival rates for younger patient can be improved.

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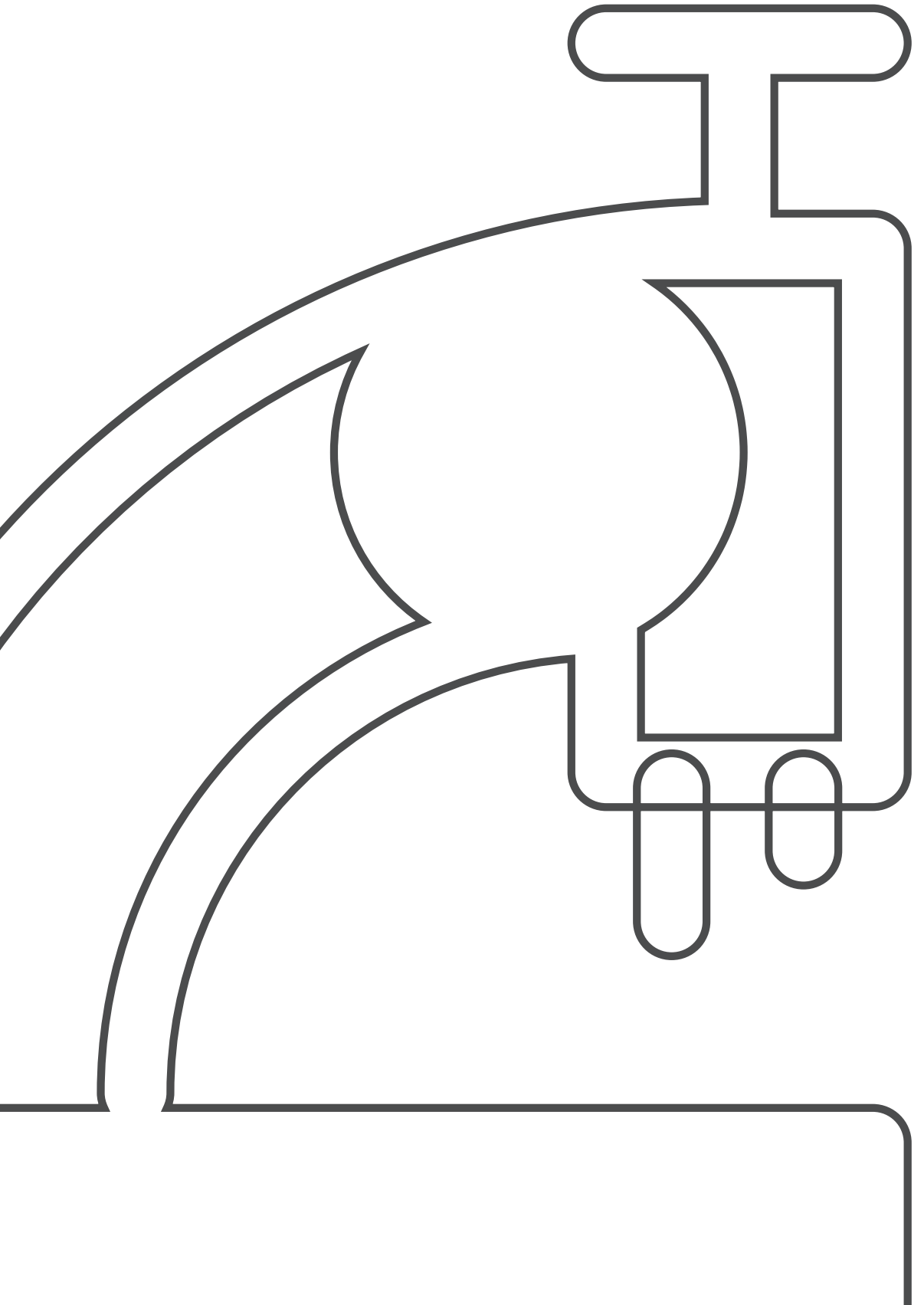


Chapter 11

Summary/Samenvatting

SUMMARY

This thesis highlighted that the population of EAC patients appears to be relatively heterogeneous, including patients with sporadic cancers, patients with a positive family history for EAC and BE, and young aged patients. The aim of this thesis was to elucidate the molecular biology of EAC by performing standard molecular analysis, i.e. whole-exome sequencing, targeted sequencing and Sanger sequencing on sporadic, familial and young EAC cases. In addition, clinicopathological features of families with clustering of EAC and BE and young individuals with EAC were studied.

The introduction to this thesis (***chapter 1)*** highlights the growing concerns about EAC. The incidence of both EAC and the premalignant lesion BE has been rising over the last decades. Despite the improvements in multimodality treatment (nCRT followed by surgery) the prognosis of EAC remains relatively poor. In addition, there is a lack of discriminative oncological biomarkers that might support the rationale for a BE surveillance program, in order to identify BE patients with a high risk of neoplastic progression.

In ***chapter 2***, a considerable level of genetic intratumor heterogeneity was revealed with the aid of multiregional targeted sequencing of a panel of frequently mutated genes in EAC. *TP53* alterations were found to be present early in the BE progression, followed by *P16* inactivation, and therefore both these alterations were homogenously present in the entire tumors. However, alterations of *SMAD4* and *APC* differed among tumor regions and distinct disease sites (BE and lymph node metastasis), representing intratumor heterogeneity of EACs.

Chapter 3 comprises a short-report, which revealed, with the aid of a multiplex SNaPShot essay, that somatic shotspot mutations in the promoter of the gene coding for telomerase reverse transcriptase (*TERT*) catalytic subunit do not occur in EAC. This suggests that EACs might have alternative mechanisms to maintain telomere length, and may therefore be less likely to benefit from activating mutations in *TERT*.

In ***chapter 4***, SNPs as patient specific biomarkers were considered to avoid the possible consequence of inter- and intratumor heterogeneity in the search for oncological biomarkers. The first GWAS on BE and EAC identified, among several others, the SNPs rs10419226 (*CRTC1*) and rs11789015 (*BARX1*) to be associated with the risk of EAC. This association was validated in a case-control study. These findings suggest that the risk of EAC is influenced by genetic variants that are common in the population, and therefore the susceptibility to EAC is not equally distributed among the population.

In ***chapter 5*** the appearance of families with clustering of BE and EAC was described. Families consisting of two or more first- or second-degree family members diagnosed with BE and/or EAC are termed "familial Barrett's esophagus" (FBE) and comprise approximately seven percent of all patients diagnosed with BE or EAC. Most patients meeting the criteria for FBE are diagnosed at a younger age than sporadic cases and a (phenotypic)

autosomal dominant pattern of inheritance has been suggested. Until today, extensive candidate gene and linkage analysis has been unsuccessful in identifying genetic variants that are associated with FBE.

In ***chapter 6*** a family meeting the criteria for FBE was investigated by whole-exome sequencing. A rare variant in the *MSX1* gene was identified heterogeneously in the germline DNAs of all affected family members, and in addition somatic loss of the wild type allele of the *MSX1* gene was identified in the tumor DNAs. This suggests a prominent role of this germline defect in the development of BE and EAC in this particular family. Although this *MSX1* variant may be unique for this particular family, it may also indicate a more common pathogenic pathway in EAC, when it is demonstrated to be pathogenic.

Chapter 7 and ***chapter 8*** demonstrated that currently in the Netherlands about ten percent of the patients diagnosed with EAC age ≤50 years. Young patients presented with positive lymph nodes and distant metastasis more often when compared to their older counterparts. However, despite the more advanced tumor stage the younger patients underwent surgery more often. With regard to palliative treatment, younger patients received chemotherapy more often while in the older patients radiotherapy was applied more frequently. This was probably due to the better physical condition and the absence of co-morbidities in young patients with EAC. Despite the more advanced disease stage younger patients present with at the time of diagnosis, they obtain comparable relative survival rates with elderly patients, probably due to application of more aggressive treatment strategies.

In ***chapter 9*** the molecular spectrum, i.e. mutational load and mutational profile, of patients diagnosed with EAC aged ≤40 years was determined and compared with patients diagnosed with EAC at the average age of ≥68 years. No difference in mutational load was observed between the EAC patients aged ≤40 years and the patients aged ≥68 years. The amount of *TP53* and *P16* mutations in the young and older patients was comparable, while the profile of additional mutations was different between the two age groups. The genes mutated in the young EAC patients were mostly classified to cell fate pathways (*APC*, *CDH1*, *CTNNB1*, *FGFR2*, and *STK11*), while the genes mutated in the older EACs patients were classified to cell survival pathways (*ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4*, and *VHL*). From a treatment perspective, different pathways could indicate different inhibitors for targeted therapy.

Chapter 10, the general discussion of this thesis, comprises brief summaries of all previous chapters, the main conclusions and points of discussion. Overall it is concluded that the search for oncological biomarkers is hampered by the extent of intratumor heterogeneity of EACs. To avoid the consequences of intratumor heterogeneity, common genetic variants in the population (SNPs) can be used to asses individual genetic susceptibility for BE and EAC.

Seven percent of all patients diagnosed with BE or EAC has at least one other first- or second-degree family member diagnosed with BE or EAC, defined as FBE. In the search for genetic variants associated with FBE, a mutation in the *MSX1* gene was identified by whole-exome sequencing in a family consistent with the criteria for FBE. Approximately ten percent of the EAC patients aged ≤50 years. They present with more advanced disease stage, however obtain relative survival rates similar to the elderly patients, most likely due to application of more aggressive treatment strategies. In addition, although the mutational load between tumors from young and older patients is comparable, the mutational spectrum is different besides the frequent mutations in *TP53* and *P16*.

Future recommendations arising from this thesis are:

- Molecular analysis of circulating cell-free tumor DNA (ctDNA) derived from blood samples is a novel method, which can be performed at multiple time-points and possibly represents the dominant molecular profile of EAC better than a single site biopsy. Possible clinical applications might be the analysis of ctDNA to evaluate the response to nCRT, there by identifying the complete responders prior to surgery to prevent them from undergoing an esophagectomy
- Familial EAC should be considered a distinct entity . A (inter)national consortium for familial EAC should be established to enlarge our knowledge about familial EAC, to set up and coordinate research, and toimprove the care for patients with familial EAC. It is highly important is to reach consensus about an accepted definition of familial EAC.
- It should be recommended to include younger patients into clinical trials. In addition, more biological studies should be performed in order to obtain more knowledge about how tumors from younger patients differ from those of older patients on a molecular level to produce new targets for intervention, so that survival rates for younger patient can be improved.

NEDERLANDSE SAMENVATTING

De incidentie van slokdarmkanker is de laatste decennia sterk toegenomen in Nederland en andere Westerse landen. Deze stijging is met name te wijten aan een toename van het adenocarcinoom. Barrett epitheel is het premaligne voorstadium van het adenocarcinoom. De kans om vanuit Barrett epitheel een adenocarcinoom te ontwikkelen wordt geschat op minder dan één procent per jaar.

Doordat de diagnose slokdarmkanker veelal in een laat stadium van de ziekte wordt gesteld, komt slechts minder dan de helft van de patiënten in aanmerking voor een in opzet curatieve behandeling. Dit resulteert in een 5-jaars overleving van ongeveer 50%. De overige patiënten worden palliatief behandeld, waarmee een 2-jaars overleving van negen procent wordt behaald.

Gezien de slechte prognose van slokdarmkanker worden patiënten met Barrett epitheel onderworpen aan een endoscopisch onderzoek. Dit onderzoek wordt, afhankelijk van de aanwezigheid van laaggradige dysplasie, om de 6 maanden, 12 maanden, 3 jaar of 5 jaar verricht. De beoordeling van laaggradige dysplasie is sterk afhankelijk van de hoeveelheid en de plaats van de biopten en van de patholoog. Echter, momenteel is de diagnose laaggradige dysplasie de enige voorspeller voor het ontwikkelen van een adenocarcinoom. Het vinden van een moleculaire marker die kan voorspellen welke patiënten met Barrett epitheel een hoog risico hebben op het ontwikkelen van een adenocarcinoom is noodzakelijk. Daarnaast zou een moleculaire marker, als target voor aanvullende therapieën, kunnen bijdragen aan het verbeteren van de prognose van slokdarmkanker.

Tijdens de zoektocht naar moleculaire markers voor slokdarmkanker wordt er vanuit gegaan dat het een niet-erfelijke ziekte betreft van de oudere mens. Echter, in dit proefschrift wordt de nadruk gelegd op verschillende groepen patiënten met slokdarmkanker, zoals patiënten met een positieve familiegeschiedenis voor Barrett epitheel en/of het adenocarcinoom en jonge patiënten met slokdarmkanker, naast de grootste groep patienten met een niet-erfelijke tumor. Het doel van dit proefschrift is om door middel van standaard moleculaire analyses de moleculaire biologie van het adenocarcinoom van de slokdarm te ontrafelen met inachtneming van de verschillende subpopulaties.

Geen twee tumoren, ook al van hetzelfde type, zijn hetzelfde. Zelfs binnen één tumor zijn grote verschillen aanwezig, hetgeen intratumor-heterogeniteit wordt genoemd. Verschillende delen van een tumor op verschillende tijdstippen laten variatie zien op moleculair niveau. Intratumor-heterogeniteit draagt bij aan de tumorgroei via een vertakt patroon (Branched evolution), zoals Marco Gerlinger voor het eerst beschreef in niertumoren. Als gevolg van intratumor-heterogeniteit worden niet alle mutaties van een tumor gedetecteerd in alle biopten, waardoor het aantal mutaties van de gehele tumor wordt onderschat. In ***hoofdstuk 2*** werd door middel van het sequensen

van meerdere biopten van vijf adenocarcinomen van de slokdarm een zekere mate van intratumor-heterogeniteit vastgesteld. Mutaties in *TP53* en *P16* werden in nagenoeg alle tumorbiopten aangetroffen en tevens in het bijbehorende Barrett epitheel. Dit geeft aan dat zowel mutaties in *TP53* en *P16* vroeg in de ontwikkeling van een adenocarcinoom van de slokdarm ontstaan, zich klonaal verspreiden en daardoor in alle biopten aanwezig zijn. Echter, mutaties in *APC* en *SMAD4* bleken niet aanwezig in alle tumorbiopten en ook niet in het Barrett epitheel. Dit betekent dat deze mutaties in een later stadium van de ziekte ontstaan en een zekere mate van intratumor-heterogeniteit veroorzaken in het adenocarcinoom van de slokdarm.

Hoofdstuk 3 betreft een zogeheten 'short-report' waarin met behulp van een SNaPshot analyse werd aangetoond dat veel voorkomende mutaties in de promotor van het gen dat codeert voor telomerase reverse transcriptase (*TERT*) niet voorkomen in het adenocarcinoom van de slokdarm. Dit suggereert dat deze tumoren een alternatief mechanisme gebruiken om lengte van de telomeren te behouden.

Om de consequenties van intratumor-heterogeniteit te vermijden in de zoektocht naar moleculaire markers kan gebruik gemaakt worden van patiënt specifieke markers in plaats van tumor specifieke markers. Dit kan bijvoorbeeld in de vorm van een SNP (een plaatst op het chromosoom bestaande uit een enkel basenpaar (C en G of A en T), waarop in minstens één procent van de populatie een variatie in de nucleotide wordt gevonden). Recent zijn door middel van een genoomwijde associatiestudie (GWAS) enkele SNPs geïdentificeerd die geassocieerd zijn met het risico op Barrett epitheel en het adenocarcinoom van de slokdarm.

In ***hoofdstuk 4*** werd de associatie met SNP rs10419226 in het gen *CRTC1* en met SNP rs11789015 in het gen *BARX1* en het risico op een adenocarcinoom van de slokdarm bevestigd door middel van een patiënt-controle studie. Dit zou kunnen betekenen dat kwetsbaarheid voor het ontwikkelen van een adenocarcinoom van de slokdarm niet evenredig is verdeeld over de populatie en dat bepaalde individuen een hoger risico hebben op basis van hun genetische profiel.

Het adenocarcinoom van de slokdarm is in het algemeen een niet-erfelijke vorm van kanker. Echter, de laatste jaren zijn er steeds meer aanwijzingen voor een erfelijke variant van het adenocarcinoom van de slokdarm. ***Hoofdstuk 5*** geeft een overzicht van de literatuur die tot op heden bekend is over de erfelijke vorm van het adenocarcinoom van de slokdarm. Er wordt gesproken van "familiaire Barrett's esophagus" (FBE) indien er twee of meer eerste- of tweedegraads familieleden zijn gediagnosticeerd met Barrett epitheel of een adenocarcinoom van de slokdarm. De prevalentie hiervan wordt geschat op ongeveer zeven procent. Het merendeel van de patiënten die voldoen aan de criteria van FBE krijgen de diagnose op een lagere leeftijd dan patiënten met de niet-erfelijke variant. De overerving in deze families lijkt via een (fenotypisch) autosomaal dominant patroon te verlopen. Vooralsnog is er geen eenduidig oorzakelijk gendefect bekend voor

FBE. In ***hoofdstuk 6*** werd een familie beschreven die voldoet aan de criteria van FBE. Deze familie werd tevens onderworpen aan kiembaan DNA onderzoek, waarmee zowel gezond weefsel als tumorweefsel van de aangedane familieleden werd onderzocht. Een zeldzame mutatie in het gen *MSX1* werd gevonden in de gezonde weefsels van de aangedane familieleden en tevens werd er verlies van het wild type allel gevonden in het tumorweefsel van twee familieleden. Dit zou kunnen betekenen dat de mutatie in *MSX1* een prominente rol speelt in de ontwikkeling van Barrett epitheel en adenocarcinoom van de slokdarm in deze specifieke familie.

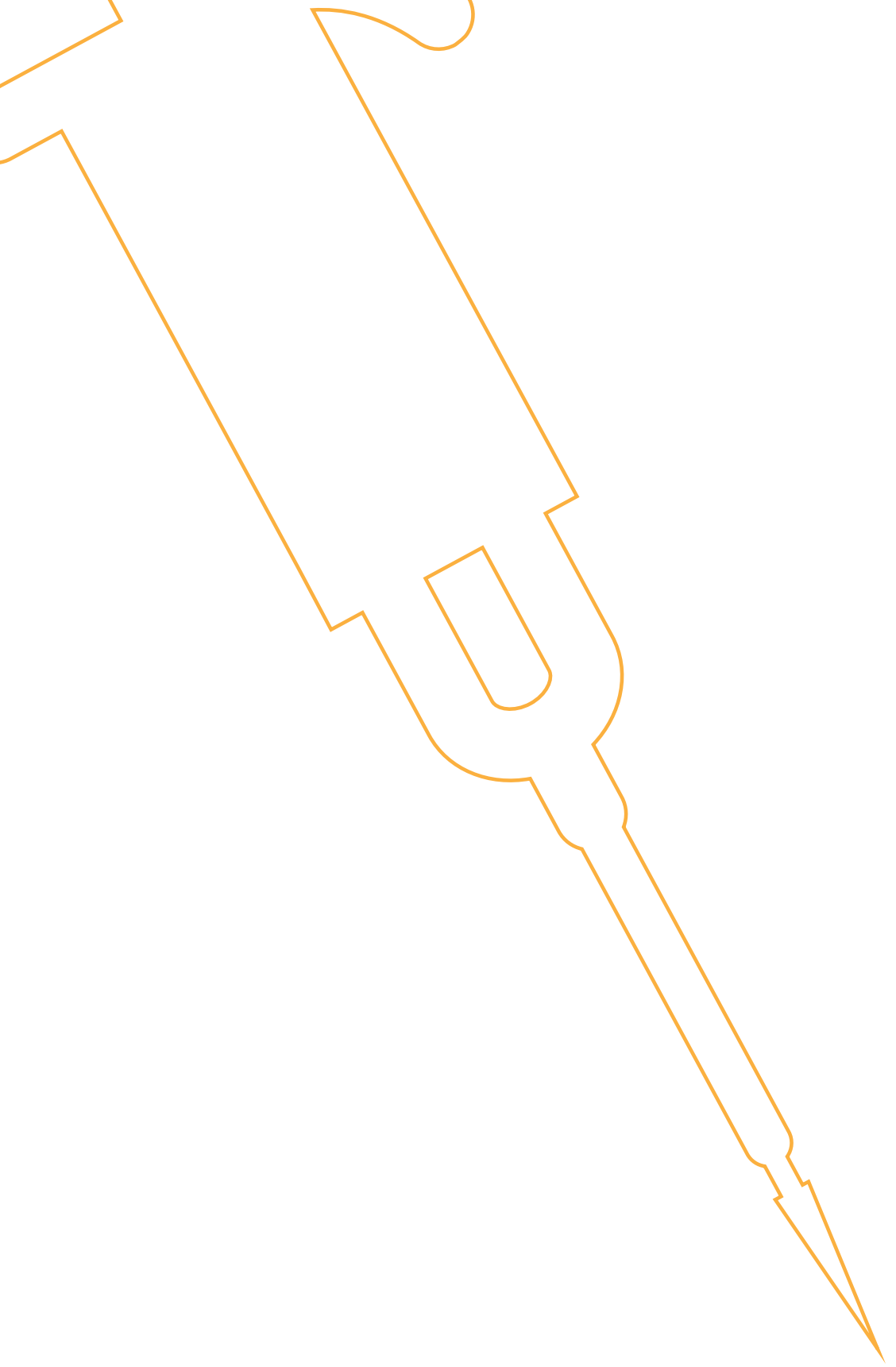
In ***hoofdstuk 7*** en ***hoofdstuk 8*** werd beschreven dat in Nederland ongeveer tien procent van de patiënten met een adenocarcinoom van de slokdarm ≤50 jaar is. Deze jonge patiënten presenteren zich vaker met uitzaaiingen in de lymfeklieren en andere organen als ze vergeleken worden met oudere patiënten met een adenocarcinoom van de slokdarm. Ondanks dit verder gevorderde ziektestadium ondergingen jonge patiënten vaker een slokdarmresectie en werd vaker chemotherapie gegeven in het kader van een palliatieve behandeling, terwijl de oudere patiënten vaker radiotherapie kregen. Het krijgen van meer agressieve vormen van therapie voor jonge patiënten kon mogelijk verklaard worden door de betere fysieke conditie en de afwezigheid van co-morbiditeiten, waardoor ze de therapieën beter tolereerden. Dit resulteerde uiteindelijk in een overeenkomstige relatieve overleving tussen de jonge en de oudere patiënten met een adenocarcinoom van de slokdarm.

Over het algemeen wordt aangenomen dat kanker een ziekte van de oudere mens is. Deze gedachte wordt ondersteund door de aanwijzingen dat de verandering van een normale cel in tumor cel en vervolgens een manifeste tumor enkele tientallen jaren duurt, en minstens drie mutaties in 'driver' genen vereist (genen die de celgroei regelen). In ***hoofdstuk 9*** werd onderzocht of adenocarcinomen van de slokdarm afkomstig van jonge patiënten (≤40 jaar) op moleculair niveau verschillend zijn van de tumoren afkomstig van oudere patiënten (≥68 jaar). Een panel bestaande uit de meest voorkomende kankergerelateerde genen werd onderzocht in het tumorweefsel van een groep jonge patiënten en vergeleken met het tumorweefsel van een groep oudere patiënten. De hoeveelheid mutaties bleek niet verschillend tussen beide leeftijdsgroepen. De genen *TP53* en *P16* bleken evenredig gemuteerd in zowel de jonge als de oudere patiënten. Echter, naast de *TP53* en de *P16* mutatie bleek het palet aan andere mutaties te verschillen tussen de jonge patiënten en de oudere patiënten. In de jonge patiënten bleken de genen *APC*, *CDH1*, *CTNNB1*, *FGFR2*, en *STK11* gemuteerd en in de oudere patiënten de genen *ABL1*, *FBXW7*, *GNA11*, *GNAS*, *KRAS*, *MET*, *SMAD4* en *VHL*. Deze bevindingen suggereren dat adenocarcinomen van de slokdarm in jonge patiënten, naast de veel voorkomend mutaties in *TP53* en *P16*, andere mutaties hebben dan oudere patiënten.

Hoofdstuk 10 is de algemene conclusie van dit proefschrift en bevat een korte samenvatting van alle voorgaande hoofdstukken met tevens de conclusies en de punten van discussie. Geconcludeerd kan worden dat de zoektocht naar moleculaire markers verstoord wordt door een zekere mate van intratumor-heterogeniteit, waardoor biopten van een adenocarcinoom van de slokdarm een onderschatting geven van de hoeveelheid mutaties. Om de gevolgen van intratumor-heterogeniteit te omzeilen kan gebruik worden gemaakt van zogeheten SNPs, waarmee de kwetsbaarheid van een individu voor het krijgen van een adenocarcinoom van de slokdarm ingeschat kan worden. Zeven procent van de patiënten met Barrett epitheel of een adenocarcinoom van de slokdarm hebben tenminste nog één eerste- of tweedegraads familielid met een van deze beide aandoeningen. De term 'familiaire Barrett's esophagus' wordt hiervoor gebruikt. In een familie met familiaire Barrett's esophagus werd een zeldzame mutatie in het *MSX1* gen gevonden. De relevantie van het *MSX1* gen in andere families die aan de criteria voldoen moet nog onderzocht worden. Tien procent van de patiënten met een adenocarcinoom van de slokdarm zijn ≤50 jaar en presenteren zich met een verder gevorderd ziekte stadium. Echter, als gevolg van agressievere therapieën behalen zij een overeenkomstige relatieve overleving met de oudere patiënten. Naast de veel voorkomende mutaties in de genen *TP53* en *P16*, bezitten de tumoren van jonge patiënten andere mutaties dan die van de oudere patiënten.

Aanbevelingen voor de toekomst:

- Moleculaire analyse van circulerend celvrij tumor DNA afkomstig uit bloed bevat het volledige moleculaire profiel van een tumor en kan tevens op een minimaal invasieve manier op multipele tijdsintervallen worden afgenomen. Het analyseren van circulerend celvrij tumor DNA kan klinisch relevant zijn voor het evalueren van de respons op neoadjuvante chemoradiotherapie, om te voorkomen dat patiënten die volledig responderen geopereerd worden.
- Familiair adenocarcinoom van de slokdarm zou als een op zichzelf staande entiteit moeten worden beschouwd. Men neemt aan dat het familiair voorkomen van zowel Barrett epitheel als het adenocarcinoom het gevolg is van eenzelfde erfelijke aanlegfactor. Echter, gezien de hoge prevalentie van Barrett epitheel in de algemene populatie, hoeft dit premaligne voorstadium niet per definitie ook de onderliggende conditie te zijn voor het veel zeldzamere familiaire adenocarcinoom.
- Om de overleving van jonge patiënten met een adenocarcinoom van de slokdarm te verbeteren, moeten meer jonge patiënten geïncludeerd worden in klinische trials. Daarnaast zouden er meer moleculair biologische studies uitgevoerd moeten worden om te achterhalen of tumoren van jonge patiënten op moleculaire niveau verschillen van die van oudere patiënten om zodoende nieuwe aangrijpingspunten voor therapieën te ontwikkelen.



Part VI

APPENDICES

LIST OF PUBLICATIONS

van Nistelrooij AM, Andrinopoulou ER, van Lanschot JJ, Tilanus HW, Wijnhoven BP. Influence of young age on outcome after esophagectomy for cancer. *World J Surg. 2012;36:2612-21.*

van Nistelrooij AM, Zwarthoff EC, Post E, Lurkin I, van Marion R, Korpershoek E, Biermann K, Wijnhoven BP, Dinjens WN. Absence of *TERT* promoter mutations in esophageal adenocarcinoma. *Letters to the editors. Int J Cancer. 2014;134:2014-5.*

van Nistelrooij AM, Dinjens WN, Spaander VM, van Lanschot JJ, Wijnhoven BP. Hereditary factors in esophageal adenocarcinoma. *Mini-review*. *Gastrointest tumors.2014;1:93-8.*

van Nistelrooij AM, van Steenbergen LN, Spaander MC, Tilanus HW, van Lanschot JJ, Lemmens VE, Wijnhoven BP. Treatment and outcome of young patients with esophageal cancer in the Netherlands. *J Surg Oncol.2014;109:561-6.*

van Nistelrooij AM, van der Korput JA, Broer L, van Marion R, van Berge Henegouwen MI, van Noesel CJ, Biermann K, Spaander VM, Tilanus HW, van Lanschot JJ, Hofman A, Uitterlinden AG, Wijnhoven BP, Dinjens WN. Single Nucleotide Polymorphisms (SNPs) in *CRTC1* and *BARX1* are associated with esophageal adenocarcinoma. *J Carcinog. 2015;21:5.*

van Nistelrooij AM, van Marion R, Biermann K, PALGA-group, van Lanschot JJ, Wijnhoven BP, Dinjens WN. Early onset esophageal adenocarcinoma: a distinct molecular entity? *Oncoscience.2016;3:42-8.*

van Nistelrooij AM, van Marion R, Koppert LB, Biermann K, Spaander MC, Tilanus HW, van Lanschot JJ, Wijnhoven BP, Dinjens WN. Molecular clonality analysis of esophageal adenocarcinoma by multiregion sequencing of tumor samples. *Submitted.*

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PHD PORTFOLIO

Summary of PhD training and teaching

Name of PhD student:	Anna Maria Josina van Nistelrooij
Erasmus MC department:	Surgery and Pathology, Erasmus MC Cancer Institute
PhD period:	March 2013 – October 2016
Promotores:	Prof. dr. J.J.B van Lanschot
	Prof. dr. F. van Kemenade
Supervisors:	Dr. B.P.L. Wijnhoven
	Dr. W.N.M. Dinjens

1. PHD TRAINING		**Year**	**Workload**
Courses			
- Research Master Clinical Research	NIHES	2011-2014	120 ECTS
- Research integrity	Erasmus MC	2013	2 ECTS
Presentations			
Congres van de Nederlandse Vereniging voor Gastro-Enterologie (NVGE) – voorjaarsconferentie The influence of young age on outcome after esophagectomy for cancer. (oral)	Veldhoven	2012	1 ECTS
13th World Congress of the International Society for Diseases of the Esophagus The influence of young age on outcome after esophagectomy for cancer. (poster)	Venice, Italy	2012	1 ECTS
'OESO World congress' – cancers of the esophagus Next-generation DNA Sequencing" of patients with confirmed Familial Barrett's Esophagus. (oral)	Paris, France	2013	1 ECTS
Congres van de Nederlandse Vereniging voor Gastro-Enterologie (NVGE) – najaarsconferentie Treatment and prognosis for young patients with esophageal cancer. (oral)	Veldhoven	2013	1 ECTS
11th Congress of the European Society for Diseases of the Esophagus Next-generation DNA Sequencing of familial esophageal adenocarcinoma. (oral)	Rotterdam	2013	1 ECTS

26ste Symposium van Experimenteel Onderzoek Heelkundige Specialismen (SEOHS) Early genomic aberrations in esophageal adenocarcinoma as potential markers for malignant progression. (oral)	Maastricht	2013	1 ECTS
Keystone Symposia Conference - Stem Cells and Cancer Early genomic aberrations in esophageal adenocarcinoma as potential markers for malignant progression. (poster)	Banff, Canada	2014	1 ECTS
12th Congress of the European Society for Diseases of the Esophagus Single Nucleotide Polymorphisms (SNPs) in *CRTC1* and *BARX1* are associated with esophageal adenocarcinoma", congres van de 'European Society for Diseases of the Esophagus. (***awarded for best oral presentation***)	Bologna, Italy	2014	1 ECTS
Congres van de Nederlandse Vereniging voor Gastro-enterologie (NVGE) voorjaarsconferentie SNPs associated with esophageal adenocarcinoma. (oral)	Veldhoven	2015	1 ECTS
Congres van de Nederlandse Vereniging voor Gastro-enterologie (NVGE) voorjaarsconferentie Molecular profile of young esophageal adenocarcinoma patients. (oral)	Veldhoven	2015	1 ECTS

2. Teaching

2e jaars keuzeonderwijs – Kanker: de arts/onderzoeker in relatie tot de patient		2013-2015	3 ECTS
Erasmus anatomy and research project (EARP) – abdomen. (assistant)		2013	3 ECTS
Erasmus anatomy and research project (EARP) – abdomen. (tutoring)		2014	3 ECTS

CURRICULUM VITAE

Annemarie was born on November 7th 1988 in Gilze en Rijen, where she grew up as well. She attended secondary school (Cambreur College) in Dongen, from which she graduated in 2007. The first year after graduation she studied Health Policy & Management at the Erasmus University Rotterdam. In 2008 she started her medical training at the Erasmus Medical Center Rotterdam. Parallel to her medical study she started a Research Master in Clinical research at the Netherlands Institute for Health Sciences, for which she attended Summer school at Johns Hopkins University School of Public Health in Baltimore, United States. As part of the Research Master program she started her research project at the laboratories of Molecular Diagnostics under the supervision of dr. Dinjens in collaboration with the Department of Surgery under supervision of dr. Wijnhoven. After she obtained her Master of Science Degree in Clinical Research and finished the theoretical part of her medical school, she got the opportunity to proceed with her research project as a PhD candidate at the department of Surgery and Pathology (prof. dr. van Lanschot, prof. dr. van Kemenade). In order to finish her medical school, she started her clinical rotations in March of 2014. In February of 2016 she obtained her medical degree, after which she started as a resident (not in training) at the department of Surgery at the Ikazia Hospital Rotterdam under the supervision of dr. den Hoed.

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