Genetic alterations during neoplastic progression in Barrett's esophagus

(Genetische veranderingen bij neoplastische progressie van Barrett's oesophagus)

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Yan-song Bian

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Promotoren:

Prof.dr.Th.G.van der Kwast
Prof.dr. F.T. Bosman

Overige leden:

Dr.H.van Dekken
Dr.L.H.J. Looijenga
Prof.dr.H.W. Tilanus

CONTENTS

LIST OF ABBREVIATIONS

APC	adenomatous polyposis coli
CDK	cyclin-dependent kinases
COBRA	combined bisulfite restriction analysis
CRC	colorectal cancer
DCC	deleted in colorectal carcinomas
DSH	dishevelled
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelio-mesenchymal transition
Fz	frizzle
GSK	glycogen synthase kinase
HG	high grade dysplasia
IHC	immunohistochemistry
LEF	lymphocyte enhancing factor
LG	low grade dysplasia
LOH	loss of heterozygosity
MSI	microsatellite instability
MSP	methylation specific PCR
Ms-SNuPE	methylation sensitive, single nucleotide primer extension
MS-SSCA	methylation sensitive, single strand conformation analysis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
SSCP	single strand conformation polymorphism
Tcf	T cell factor
TRAP	telomeric repeat amplification protocol

CHAPTER I

General introduction and outline of the study

1.1 Genetic alterations during neoplastic progression in Barrett's esophagus

1.1.1 Barrett's esophagus and esophageal adenocarcinoma

1.1.1.1 Barrett's esophagus

Barrett's esophagus is an acquired condition in which the normal squamous epithelium of the distal esophagus is replaced by a metaplastic columnar epithelium. Although Barrett's esophagus was originally hypothesized to result from a congenital abnormality, most cases probably arise as a complication of chronic gastro-esophageal reflux disease (Hamilton 1985). The most important clinical implication of this condition is the associated predisposition to adenocarcinoma of the esophagus and gastric cardia: there is a 30 to 125 fold increased risk for patients with Barrett's esophagus compared with the general population (Riddell et al, 1983; Hameeteman et al, 1989; Williamson et al 1991). The average time-interval from the development of Barrett's esophagus (median age: 40) to the clinical presentation of Barrett's adenocarcinoma (mean age: 64) has been estimated at 20-30 years (Cameron et al, 1992).

There is controversy regarding diagnostic criteria for Barrett's esophagus. Some authors have defined the condition according to an arbitrary extent of esophageal columnar lining whereas others have felt that the presence of specialized intestinal metaplasia in the esophagus establishes the diagnosis. Studies have clarified that intestinal metaplasia was not only the most common and distinctive type of epithelium detected within the columnar metaplastic mucosa, but this is associated with greatest malignant potential. Cancer risk in Barrett's esophagus is restricted to patients with specialized (intestinal) epithelium, which can be identified in more than 90% of adult patients presenting segments more than 3 cm of esophageal columnar lining (Haggitt 1994). Therefore, Barrett's esophagus has come to be defined by the histological presence of intestinal metaplasia containing goblet cells (Sharma et al, 1998, 1999).

1.1.1.2 Histological sequence from Barrett's esophagus to adenocarcinoma

Esophageal adenocarcinoma is the second most rapidly increasing carcinoma in Western countries, with an annual incidence of approximately 0.8% (Bit et al, 1991; Pera et al, 1993). Unfortunately, esophageal adenocarcinoma is rarely discovered in time for cure, and 93% of patients will eventually die of their disease (Silverberg et al, 1990). As

the prognosis of invasive carcinoma is poor, with a 5 year survival rate less than 10%, early diagnosis of malignancy is essential for optimal treatment and better prospects for the patient (Fléjou et al, 1994).

Virtually all esophageal adenocarcinomas arise from precursor lesions (dysplasia). Dysplasia is characterized by architectural and cytological abnormalities and can be classified in low-grade (LG) and high-grade (HG) using the same criteria as for dysplasia in gastric mucosa and in inflammatory bowel disease (Haggitt 1994). Histological evaluation of neoplastic progression in Barrett's esophagus has shown that Barrett's adenocarcinoma usually arise within regions of HG dysplasia, which in turn are associated with regions in which the mucosa has abnormalities in the LG dysplasia range (Haggitt et al, 1978; Hamilton et al 1987; Reid et al, 1988; Thompson et al, 1983). Barrett's specialized metaplasia is typically found in association with all of these abnormalities. The recognition of Barrett's esophagus as a condition that predisposes to malignancy has resulted in recommendations for endoscopic surveillance of Barrett's patients for esophageal adenocarcinoma and columnar epithelial dysplasia (Sjogren et al, 1983; Sarr et al, 1985; Sanfey et al, 1985). Thus, patients appear to progress from Barrett's specialized metaplasia to abnormalities in the LG dysplasia range to HG dysplasia, and eventually to carcinoma (Hameeteman et al, 1989; Reid et al, 1990).

Although dysplasia has been proposed as a surveillance tool for developing adenocarcinoma, there are significant problems involved (Reid et al, 1988; Petras et al, 1991; Sampliner et al, 1993; Saganet al, 1994; Cameron et al, 1995; Paraf et al, 1995), such as:

-Sampling errors: dysplastic mucosa is not always detectable macroscopically, it is often multicentric, and may involve the entire esophagus, or may be limited in extent.

-Distinction between reactive or regenerative changes and dysplasia (Indeterminate for dysplasia).

-Regression of the dysplasia

-Intra- and interobserver variation in diagnosis and in dysplasia grading.

-Differentiation of HG dysplasia from invasive cancer.

Consequently, efforts are required to further understand the pathogenesis of columnar metaplasia and its progression to cancer. Molecular genetic analysis together

with image analysis of histological spectrum of Barrett's esophagus enable better understanding of the mechanism of malignant degeneration and this might ultimately lead to more adequate cancer prevention and/or therapeutic interventions.

1.1.1.3 Barrett's esophagus: A unique model system of human neoplastic progression

Barrett's esophagus is a unique model system for investigations of intermediate events of human neoplasia since the metaplastic epithelium sequentially develops into LG dysplasia, HG dysplasia, early adenocarcinoma and invasive cancer. It is generally accepted that in the pathogenesis of Barrett's adenocarcinoma stepwise accumulation of genetic alterations, including specific mutations and a generalized increasing genetic instability play a key role. In patients with Barrett's esophagus, genetic events in neoplastic progression can be evaluated by serial biopsies of the same patient over time and correlated with histological evidence of progression (Reid et al, 1992). Furthermore, in addition to cancer, esophagectomy specimens often show the surrounding epithelium in which the cancer arose premalignant changes, permitting the genetic study of multiple steps of neoplastic progression from metaplasia to adenocarcinoma in a single esophagectomy specimen (Rabinovitch et al, 1989; Reid et al, 1988).

1.1.2 Genetic events in Barrett's esophageal adenocarcinoma development

Cancer is a genetic disease, caused by an accumulation of genome alterations partly through genetic instability and characterized by a dysregulation of cell proliferation. In general, there are four genetic routes which contribute to genetic instability and uncontrolled cell proliferation: 1) activation of oncogenes which stimulates cell growth; 2) inactivation of tumor suppressor genes, which regulate cell growth by inhibition of proliferation; and 3) inactivation of DNA (mismatch) repair genes which normally limit the mutation rate of growth controlling genes. Recently, a fourth class of genes, mitotic checkpoint genes, was described (Cahill et al, 1998). These latter genes are dominant and lead to chromosomal instability (aneuploidy).

Neoplastic transformation occurring in Barrett's esophagus is apparently a consequence of multiple genetic events, which include changes in gene structure, gene expression and protein structure (Jankowski et al, 1992, 1993, 1999; Rustgi et al, 1997;

Souza et al, 1997; Fitzgerald et al, 1998). These events tend not to accumulate stochastically but in a preferred order, certain molecular alterations appearing early in the sequence from metaplasia to carcinoma, whereas others seem to be late events (Hamilton et al, 1987; Krishnadath et al, 1995).

1.1.2.1 Oncogenes

Proto-oncogenes are regulatory genes found in normal human cells that perform critical cell functions including signal tranduction and gene transcription. A mutated proto-oncogene (an oncogene) may therefore have dramatic and deleterious effect on cell function which may ultimately result in carcinogenesis. Mechanisms that activate proto-oncogenes include point mutation, gene amplification, or chromosomal translocation, all of which deregulate gene expression or create an abnormal protein with an abnormal function. In the process of neoplasia, many oncogenes may be involved, the same oncogene may be involved in early or late stage of neoplastic development, and there may be cooperative effects of two distinct oncogenes. The net result is that the protein products of oncogenes drive the cell to persistent proliferation. Few oncogenes have been shown to be involved in Barrett's adenocarcinoma, and only a small percentage of Barrett's adenocarcinoma display mutations in oncogenes.

1.1.2.1.1 Oncoproteins involved in signal transduction

The ras family of oncogenes (H, K and N) encode specific proteins, designated p21, which appear to be essential components of normal cell division and differentiation. Ras oncogenes are thought to function analogously to the G proteins, acting as signal-transducting molecules in the plasma membrane. Point mutations of ras genes (especially base substitutions in codon 12, 13 or 61) commonly occur after exposure to chemical carcinogens and constitute an early step in cancer development (Cooper, 1990). Activated ras oncogenes have been detected with a frequency of 40% in colon cancer (Bos et al, 1987; Forrester et al, 1987) and 95% in pancreatic cancer (Almoguera et al 1988). In Barrett's esophagus, K-ras mutations were detected in 4 of 55 (7.2%) adenocarcinomas, 4 of 105 (3.8%) cases of HG dysplasia, 1 of 105 (1%) cases of LG dysplasia and 1of 252 (0.4%) metaplastic lesions by one group (Trautmann et al, 1996) while other investigators

found no evidence for point mutations of the K-ras gene in human esophageal cancers (Victor et al, 1990; Meltzer et al, 1990). Using monoclonal antibodies to the K-ras associated proteins, however, expression was found in 20-40% esophageal adenocarcinomas and 14% of premalignant lesions (Jankowski et al, 1992; Galiana et al, 1995). It seems that the ras oncogene is less important in Barrett's esophagus and thus, carcinogenesis in Barrett's esophagus may proceed via different pathways than in other digestive tract cancer such as sporadic colon cancer.

c-src and its viral homologue v-src are cellular oncogenes which encode a nonreceptor tyrosine kinase involved in a number of signal transduction pathways (Erpel et al, 1995 ; Wasilenko et al, 1990). Many studies have shown that alterations in c-src may deregulate cell adhesion and anchorage-dependent growth control, thereby maintaining cells in a proliferative state (Takekula et al, 1990). In Barrett's esophagus, src-specific activity has been demonstrated to be 6 fold higher in adenocarcinomas than in adjacent premalignant lesions, and 3 to 4 fold higher in metaplasia than in normal esophageal mucosa suggesting an important role for c-src early in the malignant transformation of Barret's esophagus. Further studies showed that the mechanism for activation of src in Barrett's esophagus may depend on dephosphorylation of src at tyrosine 527 (Kumble et al, 1997).

1.1.2.1.2 Nuclear regulatory oncoproteins

The products of the oncogenes jun, fos and myc function in the nucleus as transcriptional regulatory proteins. The rapid induction of nuclear oncogenes in response to transient growth factor stimulation may lead to a long-term alteration in the program of gene expression, and thus result in sustained cell proliferation (Coop, 1990). The c-jun protein has been shown to be expressed in Barrett's esophagus and esophageal adenocarcinoma. In contrast, neither c-myc nor c-fos was expressed in premalignant lesions or Barrett's adenocarcinoma (Jankowski et al, 1991). It remains unclear whether amplification of nuclear oncogenes is a primary or a secondary event in the malignant progression of Barrett's esophagus.

1.1.2.1.3 Growth factors and their receptors

Transforming Growth Factor Beta (TGF-β) is a potent and ubiquitous mediator of cell growth. TGF-β plays various roles in the process of malignant progression. It is a potent inhibitor of normal stromal, hematopoietic, and epithelial cell growth. However, at some point during cancer development the majority of transformed cells become either partly or completely resistant to TGF-β growth inhibition. There is also evidence that in the later stages of cancer TGF-β is secreted by tumor cells and contributes to cell growth, invasion, and metastasis and decreases host-tumor immune responses. Epidemiological data indicates that hypomorphic TGF-β signaling contributes to the development of a variety of cancers including esophageal adenocarcinomas. Studies have demonstrated that TGF-β expression is enhanced in endoscopic biopsies of esophagitis, nondysplastic Barrett's esophagus as well as esophageal adenocarcinomas (Triadafilopoulo et al, 1996). Loss of expression of the functional receptor for TGF-β (TGF-β type II receptor) appears to be associated with Barrett's esophagus and esophageal adenocarcinomas (Souza et al, 1996; Garrigue-Antar et al, 1996). However, further works are required to understand the exact role of TGF-β and its receptor during the development of Barrett's esophageal adenocarcinomas.

The oncogene c-erbB-2 encodes a truncated version of the cell surface receptor for epidermal growth factor (EGF) in which the EGF domain is deleted. As a result, in contrast to the normal epidermal growth factor receptor (EGFR) in which the kinase activity is upregulated following EGF binding, the receptor is a constitutively active protein-tyrosine kinase. Evidence has shown that esophageal adenocarcinoma is associated with aberrant expression of EGF (Gullick 1991; Jankowski et al, 1991). Overexpression of membranous c-erbB-2 has been demonstrated in both malignant and premalignant Barrett's esophageal tissues (Jankowski et al, 1992). Increased expression of the c-erbB-2 product p185 has been observed in Barrett's mucosa and adenocarcinoma, and tumors of cardia, but there was no significant association between levels of expression and the grade, histology or prognosis of the tumor (al Kasspooles et al, 1993). By immunohistochemistry, expression of c-erbB-2 was only detected in dysplastic lesions, suggesting that it may be a late event in the development of Barrett's adenocarcinomas (Hardwick et al, 1995). However, these results conflict with other reports which have shown a significantly lower expression of the c-erbB-2 protein in Barrett's

adenocarcinoma (Houldsworth et al, 1990; Sauter et al, 1993). Whether the expression level of c-erbB-2 protein is of independent prognostic importance in the malignant progression of Barrett's esophagus remains unclear.

1.1.2.2 Tumor-suppressor genes

Tumor suppressor genes are a class of cancer-related recessive genes, both copies of which must be inactivated for their tumor suppressive effect to be lost. One allele of these genes is frequently inactivated by Loss of heterozygosity (LOH), and the remaining copy is often inactivated by mutation or promoter hypermethylation.

1.1.2.2.1 Loss of heterozygosity

LOH involving a chromosomal arm or locus suggests the presence, at or near that locus, of a tumor suppressor gene. Several groups have evaluated chromosomal regions for LOH in Barrett's esophagus. Allelic loss of 17p (locus of the p53 gene) has been found to be an early event that occurs in diploid cells and typically precedes the development of aneuploidy and other allelic losses during neoplastic progression in Barrett's esophagus (Blount et al, 1991, 1994 ; Huang et al, 1992 ; Meltzer et al, 1991). LOH of 5q (locus of the APC gene) was demonstrated in Barrett's epithelium adjacent to dysplasia as well as in dysplastic epithelium and carcinoma, suggesting that LOH of 5q may be an early event in Barrett's metaplasia (Zhuang et al, 1996). In addition, LOH of 13q (locus of the Rb gene) was observed in 50% of esophageal adenocarcinomas. LOH of 18q21.1 (locus of DCC and MADH4 genes) in aneuploid cell populations has been found in 45% of Barrett's adenocarcinoma and 46% of premalignant lesions (Barrett et al, 1996). In 75% of the samples containing only diploid cells, the same LOH was present as was detected in the corresponding carcinoma, suggesting that allelic loss of 18q21.1 occurs as an early event in Barrett's epithelium and precedes the development of aneuploidy and adenocarcinoma.

LOH in Barrett's esophagus appears to follow a preferred sequence in association with neoplastic progression. Allelic loss of 17p typically occurs earlier than that at 5q (Blount PL et al, 1993). More studies are needed to assess the significance of these observations.

1.1.2.2.2 p53 gene

The p53 tumor suppressor gene encodes a nuclear phosphoprotein which functions as a transcription factor that controls the expression of many genes important in the regulation of the cell cycle and in triggering apoptosis after certain type of genome damage (Kastan et al, 1991; Figure 1). Deletion of one allele in the short arm of chromosome 17 and a functionally inactivating mutation of the other allele is among the most common combination of genetic abnormalities documented in human cancers (Levine, 1992). p53 was the first such gene to be investigated in Barrett's-associated neoplasm (Casson et al, 1991; Ferec et al, 1994; Krishnadath et al, 1995a; Younes et al, 1997). Mutations in p53 occur frequently in esophageal adenocarcinoma and dysplastic lesions (50-60%). However, histological lesions with which p53 alterations are associated are a matter of debate: several research groups have suggested that p53 mutation usually is damaged early during the progression from metaplasia to adenocarcinoma (Blount et al, 1994; Hamelin et al, 1994). However, a p53 mutation early on in the sequence is not sufficient to cause adenocarcinoma (Jankowski et al, 1997). Others found p53 abnormalities to be a late event and some abnormalities have been detected in HG lesions but not in the synchronous invasive carcinoma, indicating genetic divergence even during progression from HG dysplasia to adenocarcinoma (Hamelin et al, 1994). Recently, Barrett's adenocarcinoma with synchronous HG dysplasia were found to present genetic alterations in the carcinoma as well as in the HG dysplastic epithelium supporting the hypothesis of a clonal progression of the disease (Neshat et al, 1997). Although most research in Barrett's esophagus has focused on mutations occurring in exons 5-8 of the p53 gene, it is conceivable that mutations in other exons or in related proteins could affect biological function. Additional evidence for the involvement of p53 mutations in the neoplastic progression towards Barrett's adenocarcinoma comes from studies of p53 protein overexpression by means of multiparameter flow cytometry and immunohistochemistry (IHC) (Ramel et al, 1992; Hamelin et al, 1994; Krishnadath et al, 1994). Alterations of the p53 gene result in the production of an aberrant protein, which is deprived of its normal cell turnover regulatory function. The aberrant protein is characterized by a prolonged half-life due to increased post-translational stability, and therefore it can be visualized by IHC. In contrast, wild type protein is normally not

detectable by IHC due to a very short half-life (Rodrigues et al, 1990). Although the sensitivity of IHC for detection of p53 mutations is relatively low, as critically reviewed previously (Ireland et al, 1997), the obvious advantage of this rapid technique is its wide availability in most laboratories. Moreover, it may well be that IHC which explores p53 at the protein level is a better test in some cases because mechanisms other than mutation may lead to dysfunction of the p53 protein. Several studies using IHC showed increasing p53 accumulation in the dysplasia-adenocarcinoma progression of Barrett's esophagus (Younes et al, 1993; Hamelin et al, 1994; Polkowski et al, 1995). However, neither p53 mutation nor protein accumulation was observed in metaplastic lesions. It appears that in Barrett's esophagus p53 is not affected in the nondysplastic mucosa. These results indicate that p53 staining may have potential value for confirming a suspected diagnosis of LG dysplasia, as suggested by some investigators (Khan et al, 1998).

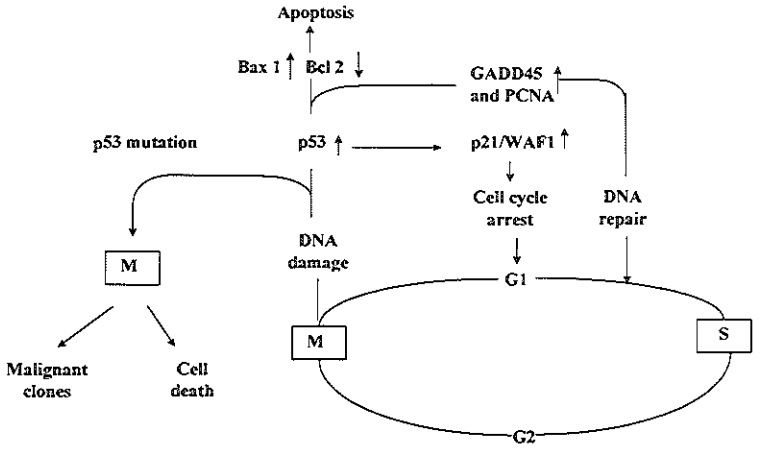

Figure 1. *p53 and cell cycle regulation. p53 is the guardian of the genome. When DNA is damaged, p53 is turned on. p53 then stops the cell cycle at a G1/S checkpoint and stimulates DNA repair. If damage is severe, p53 induces apoptosis. This prevents the cell from cycling in the presence of DNA damage. When p53 function is lost by mutation, the cell continues to cycle with increasing genomic instability, genetic accumulates and tumorigenesis progresses.*

1.1.2.2.3 APC gene

Frequent LOH involving other chromosomal loci has supported the potential role of additional tumor suppressor genes in Barrett's esophagus. One locus at which LOH is common in esophageal adenocarcinomas is on chromosome 5q21, a locus which harbors the APC (adenomatous polyposis coli) gene. The APC protein is located in the cytoplasm, where it interacts with several other intercellular proteins, including β-catenin, a protein that can enter the nucleus and activate transcription of growth-promoting genes. An important function of the APC protein is to cause degradation of β-catenin, thus maintaining low level of the latter in the cytoplasm. Inactivation of the APC gene, and the consequent loss of APC protein, increases the cellular level of β-catenin, which in turn translocates to the nucleus and activates nuclear transcription factor such as lymphocyte enhancing factor (LEF) and T cell factor (Tcf-4), which leads to increased cell proliferation (Fearon, 1997). In Barrett's esophagus, allelic loss of APC is frequently found in adenocarcinoma, but there are conflicting results concerning the presence of APC LOH in premalignant lesions. Using microdissection techniques, Zhuang et al. (1996) were able to demonstrate allelic loss at the APC locus in the same metaplastic monoclonal populations which had apparently progressed to dysplasia and adenocarcinoma. However, Gonzalez et al. (1997) failed to detect LOH of APC in dysplasia, but microdissection to enrich the cells of interest was not used. APC mutation was found to be a rare event and seemed to develop late in the progression to adenocarcinoma (Gonzalez et al, 1997).

1.1.2.2.4 APC/β-catenin/Tcf signal pathway

β-catenin is known to act as a key regulator in the cadherin mediated cell adhesion system (McCrea et al, 1991). Alterations of β-catenin can induce disorders in this system and seem to be associated with tumor invasion and metastasis in many cancers, including esophageal carcinoma (Takayama et al, 1996; Bailey et al, 1998 ; Kimura et al, 1999). In addition to simply supporting cell-cell adhesion, a role for β-catenin in the Wnt signal transduction pathway has been discovered (Morin et al, 1997). More detailed investigation of β-catenin in this pathway might provide valuable insights into the mechanisms of cellular transformation and tumor progression in Barrett's esophagus.

In normal non-transformed cells, not exposed to the Wnt-1 ligand, glycogen synthase kinase-3β (GSK3β) is active and together with wild-type APC binds free β-catenin. In this multiprotein complex GSK-3β phosphorylates β-catenin, leading to its dissociation from the complex and to its degradation in the cytoplasm (Rubinfeld et al, 1993, 1996; Papkoff et al, 1996). Residual β-catenin binds tightly both to E-cadherin at adherence junctions and to the actin cytoskeleton, providing polarised non-motile cells (Peifer, 1997). In non transformed cells exposed to the Wnt-1 ligand, or in transformed cells, the activity of GSK-3β is inhibited, or the multiprotein complex is not correctly formed (i.e. through mutation of APC or β-catenin). As a result, β-catenin can no longer be degraded, resulting in an excess of free (monomeric) β-catenin. When the cytoplasmic concentration rises, monomeric β-catenin binds to other newly synthesised proteins, especially transcription factors LEF and Tcf-4, and is transferred to nucleus (Behrens et al, 1996; Morin et al, 1997). Nuclear proteins in combination with β-catenin can activate or suppress individual gene promoters, resulting in down regulation of E-cadherin and up regulation of expression of genes implicated in epithelio-mesenchymal transition (EMT), which appears to be closely associated with invasion (Behrens et al, 1994; Figure 2).

Several studies have examined the role of the E-cadherin/β-catenin complex in maintenance of cell-cell adhesion and the disturbance of β-catenin in the Wnt pathway in various types of tumor (Morin et al, 1997; Inomata et al, 1996; Takayama et al, 1996; Bailey et al, 1998; Kimura et al, 1999). A significant percentage of gastric adenocarcinomas and squamous cell carcinomas of the esophagus have shown abnormal expression of the E-cadherin/β-catenin complex. In a recent report, a strong reduction of the expression of β-catenin was observed in dysplasia and adenocarcinoma in Barrett's esophagus (Bailey, 1998). Patterns of catenin expression seem to convey prognostic information but this has not been sufficiently explored (Nakanishi et al., 1997; Krishnadath et al., 1997).

In colorectal cancer the nuclear accumulation of β-catenin has been found frequently, predominantly at the invasion front (Brablertz et al, 1998). It is tempting to speculate that the nuclear expression of β-catenin is the result of disorders in the APC/β-catenin/Tcf signal pathway. Mutations of APC gene have been found in 60% to 80% of

colorectal cancers and they occur relatively early during colorectal tumorigenesis (Powell et al, 1992). Mutations or deletions of the β-catenin gene have been found in 10% of colorectal cancers without APC gene mutations (Sparks et al, 1998; Iwao et al, 1998). β-catenin accumulation in the cytoplasm and nuclei has been found associated with APC mutation both in colorectal and desmoid tumors (Inomata et al, 1996 ; Alman et al, 1997). Nuclear accumulation of β-catenin have also been reported in Barrett's adenocarcinoma, however, this was not a result of mutations in either APC or β-catenin gene (Seery et al., 1999; Washington et al., 1998; Wijnhoven et al., 2000; Ninomiya et al., 2000).

It has been recently established from a series of genetic, cellular, and biochemical investigations that the frizzle (Fz) gene family of seven transmembrane proteins serves as receptor for Wnt signaling (Yang-Synder et al, 1996; Bhanot et al, 1996). Recently a novel member of the human Fz gene family has been cloned from human esophageal carcinoma. Expression of the FzE3 cDNA in esophageal carcinoma cells appeared to stimulate complex formation between APC and β-catenin, and was followed by nuclear translocation of β-catenin (Tanaka et al, 1998). Therefore, in Barrett's adenocarcinomas mechanisms other than APC or β-catenin mutations might explain the disruption of the APC/β-catenin pathway. These will include Fz gene transcription, deletion of exon 3 of the β-catenin gene and GSK3β or Axin gene mutations.

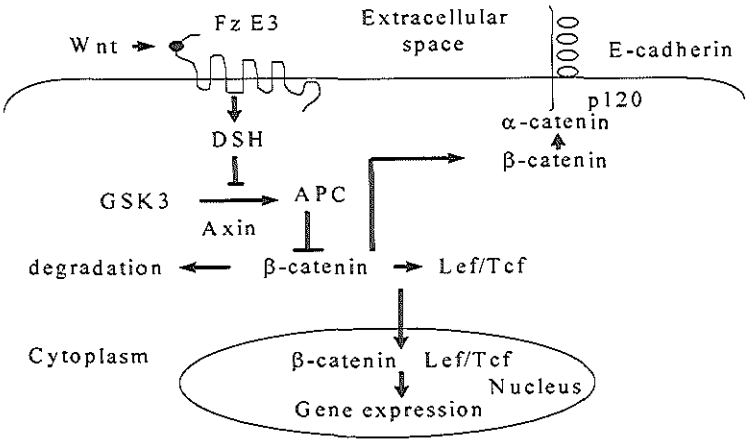

Figure 2. *Components of the APC/β-catenin/Tcf pathway. Wnt signal acts through cell-surface receptors of the Frizzed group, which transduce the signal into the cytoplasm via a protein called Dishevelled (DSH). A complex of proteins, including glycogen synthase kinase-3 (GSK3), Axin, the APC tumor suppressor protein, and β-catenin regulate the signal activity of β-catenin. In the most popular model, this complex targets β-catenin for degradation, and residual β-catenin holds cells together by binding tightly both to E-cadherin at adherent junctions and to the actin cytoskeleton, providing polarised non-motile cells. In transformed cells the presented Wnt-1 signal inhibits the activity of GSK-3β or the multiprotein complex is not correctly formed (i.e. mutation of APC or β-catenin) leading to β-catenin accumulation in the cytoplasm. β-catenin is then transported into the nucleus with newly synthesised proteins, especially transcription factors such as lymphocyte enhancing factor (LEF) and T cell factor (Tcf-4).*

1.1.2.2.5 p16 gene

Inactivation of the p16 tumor suppresser gene is one of the most frequent genetic abnormalities in human neoplasia (Sherr CJ, 1996). The p16 gene, located on 9p21, encodes a cell cycle regulatory protein which inhibits cyclin-dependent kinases (CDK) 4 and 6, preventing the phosphorylation of pRb protein and the release of transcription factor E2F, inhibiting cell cycle progression from G1 to S phase (Serrano et al, 1993; Kamb et al, 1994; Lukas et al, 1995). Genetic alterations of the p16 gene lead to its inactivation, resulting in deregulation of cell proliferation and the occurrence of genomic instability (Zhou et al, 1996; Figure 3). In various tumor types, the p16 gene has been shown to be inactivated by different mechanisms: homozygous deletion, point mutation, and hypermethylation of the promoter. In Barrett's esophagus, 9p21 LOH was found to be a frequent abnormality, occurring relatively early in the progression from Barrett's esophagus to adenocarcinoma. However, inactivation of the p16 gene by mutation of the remaining allele was reported in only 23% of the patients and no homozygous deletions of p16 were detected in esophageal adenocarcinomas (Barrett et al, 1996). Recently the p16 promoter was found to be hypermethylated at a high frequency in esophageal adenocarcinoma (Wong et al, 1997; Klump et al, 1998). Promoter hypermethylation of p16 and LOH at 9p21 were detected in 8 of 21 patients with adenocarcinoma and in 3 patients with only premalignant lesions (Wong et al, 1997). In a recent study, p16 CpG

island methylation, 9p21 LOH, and /or p16 mutations were found in biopsies from more than 85% of patients at all of the histological grades of progression in Barrett's esophagus. The results indicated that p16 genotype was strongly correlated with Barrett's length, and both p16-/- and p16+/- clonal cell populations had the ability to expend over extensive regions of Barrett's epithelium, creating a field of abnormal epithelial cells (Wong et al, 2001). However, the correlation of 9p loss and p16 gene promoter hypermethylation has not yet been analyzed in a significant number of samples, and the relationship between methylation/LOH and expression of p16 protein in esophageal adenocarcinoma has not been fully clarified either. Furthermore, it has remained unclear at which step p16 inactivation occurs during neoplastic progression of Barrett's esophagus and whether or not only p16 promoter hypermethylation is sufficient for p16 gene inactivation.

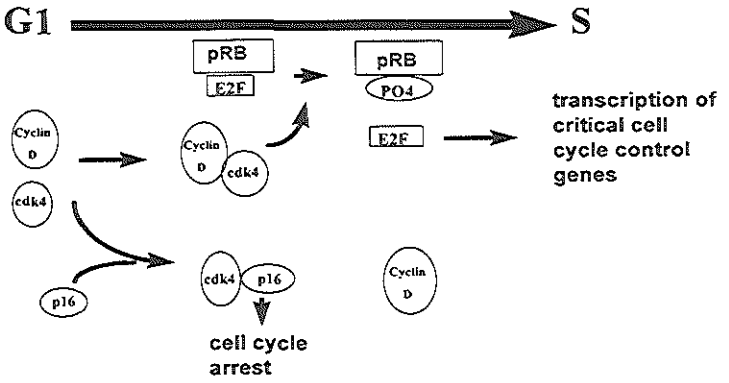

Figure 3. *The cell cycle and the G1/S controlling elements. p16 and cyclin D1 regulate the function of CDK4 and/or CDK6, which phosphorylates pRB. This effectively inactivates pRB, causing the release of E_2F and transcription of critical cell-cycle proteins that allow the cycle to progress through the restriction point.*

1.1.2.3 Altered proliferation and cell cycle abnormalities

Dividing normal cell populations maintain the balance between cell proliferation and cell loss. Uncontrolled cell growth due to an increased proliferation and/or a decreased apoptosis may result in tumor formation.

In Barrett's esophagus with dysplasia or adenocarcinoma, increased proliferative activity has been observed (Reid et al, 1993). Immunohistochemical studies of Barrett's esophagus using monoclonal antibodies that recognize the proliferation associated antigen Ki-67 and proliferating cell nuclear antigen (PCNA) showed increased proliferative fractions (Gray et al, 1992; Jankovski et al, 1992). The Ki-67 labeling index was found to be low in junctional or gastric type of Barrett's epithelium, moderate in intestinal type epithelium and high in severe dysplastic epithelium or adenocarcinoma. Staining for PCNA was mainly found in basal cells of the neck and foveolar regions of Barrett's intestinal type epithelium. Although proliferative activity, which correlates with the degree of dysplasia, seems an objective measure for malignant transformation in Barrett's epithelium, its diagnostic value remains to be proven.

An alterative to measuring the number of proliferating cells is to analyze the cell cycle. The cell cycle is governed by cyclin-dependent kinases (CDKs) that integrate mitogenic and growth inhibitory signals. Loss of cell cycle check points may be the result of altered expression of cytokines and growth factors, although genomic alterations of cell cycle-associated genes also occur.

Cyclins are from a family of proteins that form complex with CDKs. The cyclin D1-CDK4/6 can phosphorylate the Rb protein resulting in the release of transcription factor E2F, which in turn led to the cell transition across the G1 check point. Increased nuclear expression of cyclin D1 is observed in 20-60% of esophageal adeenocarcinomas and the cyclin D1 overexpression presents as early as in metaplasia. Amplification of cyclin D1 gene was found in 16-26% of esophageal adeenocarcinomas (Arbert et al, 1996; Morgan et al, 1999). However, cyclin D1 immunoreactivity was not always associated with gene amplification (Roncalli et al, 1998).

1.1.2.4 Genes that regulate apoptosis

Apoptosis, or programmed cell death, is one of the mechanisms responsible for cell loss. It also provides a protective mechanism by removing DNA damaged cells that could led to tumor formation. Many genes have been found to be involved in apoptosis such as Fas and bcl-2 genes.

The Fas/APO-1 (CD95) gene encodes a transmembrane protein that plays an important role in apoptosis. Reduced or absent expression of Fas protein on the cell surface has been observed in esophageal adenocarcinoma while wild-type Fas protein is retained in the cytoplasm. The result raised the possibility that retention of wild-type Fas protein in the cytoplasm may represent the mechanism by which malignant cells evade Fas-mediated apoptosis (Hughes et al, 1997).

Increased expression of the bcl-2 oncogene is one mechanism by which apoptosis may be blocked in malignant cells. Bcl-2 overexpression has been found as an early event in the dysplasia-carcinoma sequence of both ulcerative colitis-related and gastric neoplasia (Williams et al, 1991). However, in one study evaluating the role of bcl-2 in Barrett's esophagus, no immunoreactivity was found in any case of Barrett's mucosa, regardless of the presence of dysplasia or adenocarcinoma (Goldblum et al, 1995). In contrast to these roles in gastric neoplasia, bcl-2 alterations in apoptosis do not seem to be important in the neoplastic progression of Barrett's esophagus.

1.1.2.5 Telomerase and Barrett's adenocarcinoma

Telomerase is a ribonnucleoprotein reverse transcriptase that utilizes its own RNA template for the addition of telomeric sequences to chromosome ends, thereby maintaining telomeric length. Most normal human cells do not have detectable telomerase activity; thus, progressive telomere shortening occurs throughout life. By using a sensitive polymerase chain reaction (PCR)-based assay, called telomeric repeat amplification protocol (TRAP), telomerase activity has been demonstrated in male germ cells and in proliferative cells of rapidly renewing tissues such as small intestine (Hiyama et al, 1995). Regarding some tumor types, telomerase has been detected already in preneoplastic lesions (e.g., lung, breast), whereas, for other tumor types, the alteration of telomerase occurs when the preneoplastic lesions progresses (e.g., thyroid, pancreas) (Shay et al,

1997). The increase in telomerase activity that accompanies most neoplastic and many preneoplastic conditions may permit the emergence of a population of immortalized cells, thereby facilitating the subsequent accumulation of genetic mutations. Using the TRAP assay, telomerase activity was detected in four of eight biopsy specimens from patients with Barrett's esophagus (Ueda et al, 1997). The author concluded that Barrett's epithelium may contain a population of immortal cells that are at risk for malignant progression. However, as for the TRAP assay tissue extracts are used, the precise cellular source of telomerase activity in these samples could not be determined. By in situ hybridization, another study demonstrated that the vast majority of esophageal adenocarcinomas and HG dysplasia cases contain high levels of telomerase RNA (hTR) and that the greatest increase occurs during the transition from LG dysplasia to HG dysplasia. These results suggest that the presence of telomerase hTR RNA in Barrett's epithelium may indicate the emergence of a population of immortalized metaplastic cells with an increased proliferative capacity. The increased proliferation could facilitate the accumulation of genetic mutations, resulting in the progression to esophageal adenocarcinoma (Morales et al, 1998)

1.1.2.6 DNA content and cytogenetics

1.1.2.6.1 Aneuploidy

With the exception of germ cells, all normal cells are diploid (2N). Evolution from a normal to a malignant cell may be associated with changes in total DNA content or ploidy. Aneuploidy is an alteration in the cellular DNA content which results from uncontrolled cell division (Krishnadath et al, 1995b). By flow cytometry aneuploidy was detected in a majority of Barrett's adenocarcinomas (Giaretti et al, 1997). Aneuploidy is also observed in premalignant lesions in Barrett's esophagus (Reid et al, 1992). Furthermore, patients with aneuploidy or increased G2/tetraploid cell populations in the initial flow-cytometric analysis more frequently progressed to HG dysplasia or adenocarcinoma (Reid et al, 1992). In investigation of the contribution of DNA abnormalities in Barrett's epithelium, multiple aneuploid clones have been noted in dysplasia and its associated adenocarcinoma, suggesting that the genome of the metaplastic mucosa is unstable (Rabinovitch et al, 1989). It has been postulated that

aneuploidy cell clones may spread to involve large regions of the esophagus. Once an aneuploid clone quires the capacity for invasion, carcinoma may develop; while with continued genomic instability tumor cell heterogeneity may result (Reid, 1991).

1.1.2.6.2 Chromosome abnormalities

In addition to aneuploidy, specific chromosomal abnormalities have been studied by in situ hybridization using chromosome specific repetitive DNA probes. It has been found that aneuploidy and loss of the Y chromosome correlates with the development of neoplasia. However, no abnormalities of the X chromosome were observed (Krishnadath et al, 1995b). Trisomy of chromosomes 5 and 7 has also been reported in Barrett's esophagus (Garewall et al, 1989, 1990). It has been suggested that these karyotypically abnormal cells may result from an increased rate of cell proliferation and subsequently may undergo clonal expansion (Krishnadath et al, 1995b; Garewall et al, 1990).

Using comparative genomic hybridization (CGH), some studies have been reported recently on adenocarcinomas arising at and around the gastroesophageal junction, including Barrett's esophageal adenocarcinoma. Frequent losses were detected on 4pq, 5q, 9p, 14q, 16q, 17p, 18q, 21q, and Y, whereas frequent gains were found on 1q, 3q, 5p, 6p, 7pq, 8q, 12q, 13q, 15q, 17q, 18p, 20q, and Xpq (Moskaluk et al, 1998; van Dekken et al, 1999a; Walch et al, 2000). Loss of 14q31-q32.1 was observed in a significantly higher frequency in Barrett's esophageal adenocarcinoma than in gastric cardia cancers (van Dekken et al, 1999a). In a case of multifocal Barrett's adenocarcinoma and adjacent HG dysplasia, a subset of shared alteration has been described suggesting these different tumor sites were due to clonal expansion of the same malignancy (van Dekken et al, 1999b). Furthermore, in a study of a genome-wild overview, which was based on CGH, the frequency of losses and gains were found significantly increased in the subsequent stages of malignant transformation. Losses of 5q21-q23, 9p21, 17p12-13.1, 18q21, and Y were revealed in LG dysplasia. This was followed by loss of 7q33-q35 and gain of 7p12-p15, 7q21-q22, and 17q21 in HG dysplasia along with high level amplification (HLA) of 7q21 and 17q21. In the invasive cancers, additional losses of 3p14-p21, 4p, 4q, 8p21, 13q14-q31, 14q21.3-q31, 16q21-q22, and 22q as well as gains of 3q25-q27, 8q23-24.1, 12p11.2-12, 15q22-q24, and 20q11.2-q13.1 were distinguished along with HLAs of 8p12-p22 and

20q11.2-q13.1. Loss of 7q33-q35 was found to represent a significant distinction between LG and HG dysplasia, whereas loss of 16q21-q22 and gain of 20q11.2-q13.1 were disclosed to significantly discriminate between HG dysplasia and adenocarcinoma (Riegman et al, 2001).

1.1.2.6.3 Microsatellite instability

A form of genetic instability that has come to light recently is microsatellite instability (MSI). This type of molecular abnormality has been observed in a variety of human cancers. DNA sequences termed microsatellites are 1 to 6 nucleotide motifs randomly repeated numerous times in the human genome. In malignant epithelia, as a result of rapid cell proliferation, defects occur in mismatch repair genes such as hMSH2, hMLH1, hPMS1 and hPMS2 resulting in uncorrected DNA replication errors. As a consequence, the repetitive microsatellite sequences seen in malignant tissues vary in size compared with normal, nonmalignant tissues. This is called microsatellite instability. This variation in the length of microsatellite repeats represents a mutational process of insertions or deletions within the tumor DNA. As microsatellites are scattered through the genome, such mutations must be widespread. Thus, a mutator phenotype appears to be associated with and may possibly cause some cancers. In Barrett's adenocarcinoma, MSI was found to be an early event in metaplasia before aneuploidy occurs (Meltzer et al, 1994; Keller et al 1995; Gleeson et al, 1996). However, it is not yet known which mismatch repair genes are responsible for the MSI observed in Barrett's adenocarcinoma, and whether environmental factors play a role in MSI genesis (Brentall, 1995).

1.1.3 Perspectives

1.1.3.1 Prognostic molecular markers in Barrett's esophageal adenocarcinoma

Current strategies to detect early cancers of the esophagus rely on periodical endoscopic biopsy surveillance of Barrett's patients for the histological detection of dysplastic lesions. However, as a result of our limited understanding of the pathophysiology of this disease, at the present time, it is not possible to predict which Barrett's patients will progress to dysplasia or adenocarcinoma. An improved understanding of biological and molecular events by dissecting the metaplasia-dysplasia-

carcinoma sequence is essential to develop new strategies for the optimal management of cancer risk in patients with Barrett's esophagus. Consequently there has been an ongoing search for molecular factors determining the malignant potential of Barrett's esophagus. In order for molecular markers to be clinically useful, they must be simple, noninvasive, cost-effective, and highly sensitive and specific.

Genome instability is a hallmark of neoplastic progression in Barrett's esophagus, and the progression to cancer has been shown to involve an accumulation of genetic and cell cycle abnormalities (Neshat et al, 1994; Reid et al, 1996). LOH involving five chromosome arms (5q, 9p, 13q, 17p, and 18q) has been detected in the pathogenesis or progression of Barrett's adenocarcinomas (Barrett et al, 1996a). In particular, 17p and 9p21 LOH occur as early events during the progression to cancer in Barrett's esophagus and are associated with inactivation of the cell cycle-regulatory protein, p53 and the cyclin-dependent kinase inhibitor p16 (Reid et al, 1996 ; Neshat et al, 1994 ; Blount et al, 1994 ; Barrett et al, 1996b). Inactivation of p53 by 17p LOH and mutation of the remaining allele develop in diploid cells of dysplastic Barrett's epithelium and are strongly predictive for the subsequent development of aneuploidy (Neshat et al, 1994; Blount et al, 1994). Mutations of p53 seem to accumulate mainly in the transition from low to high grade dysplasia. Inactivation of other tumor suppressor genes by mutation (APC, p16) or hypermethylation (p16) as well as amplification of oncogenes such as c-erbB2 are relatively late events in the development of adenocarcinoma. Among the phenotypic changes is the Ki67 positive proliferative compartment, of which the occurrence is correlated with the degree of dysplasia. Moreover, reduced expression of the cadherin/catenin complex develops primarily in invasive carcinomas. The results in large number of biological markers investigated indicate that single marker can not replace the current practice of histological assessment of dysplasia in routine clinical practice. Therefore, the ongoing search for biological markers not only continues to aid our understanding of the behavior and nature history of Barrett's esophagus on a cellular and molecular level, but combination of these markers might essentially assess in more clearly defining which patients are at risk for the development of carcinoma in Barrett's esophagus.

1.1.3.2 Molecular Model of esophageal adenocarcinoma carcinogenesis

Epidemiological studies have shown that development of a detectable tumor is the result of a series of events (at least 2) that occur over a period of many years. Correspondingly, observations in experimental animals and in humans show that cancer may develop along a variety of molecular pathways characterized by stepwise acquisition and accumulation of aberrant cell morphology and behavior. In 1990, Fearon and Vogelstein proposed a model of successive genetic changes leading to colorectal cancer (CRC), in which a number of genes were involved, including APC, K-ras, DCC, and p53 (Fearon et al, 1990). The original proposal stressed that accumulation of mutations in these genes was essential for the development of CRC, rather than the exact sequence of changes. In Barrett's adenocarcinoma however, the metaplasia-dysplasia-adenocarcinoma sequence (MCS) differs from the colorectal adenoma-carcinoma sequence (ACS) in several important aspects. First, colorectal adenomas arise in native epithelium, whereas in Barrett's esophagus premalignant lesions arise in metaplastic mucosa. Secondly, Barrett's metaplasia arises in a background of reflux-induced chronic inflammation and ulceration, whereas this is not the cases in ACS, although in Barrett's esophagus also neoplasm associated with important molecular genetic defects. Third, K-ras and APC gene mutations occur frequently in the colorectal cancer, but are very uncommon in Barrett's dysplasia or adenocarcinoma.

In MCS, perhaps one of the earliest cellular events is the selection and propagation of the metaplastic cell clones, resulting specialized intestinal metaplasia. Allelic loss of several loci in chromosomes 5, 9, 17 and 18, on which tumor suppressor genes reside (APC, p16, p53 and DCC), have been identified in nondysplastic Barrett's epithelium, and these may contribute to the initiation and/or progression from Barrett's metaplasia to adenocarcinoma (Huang et al, 1992). Subsequently, loss of cell cycle check points by p53 mutation or p16 promoter hypermethylation and genomic instability (including anueploidy and microsatellite instability) may contribute to slow clonal expansion perhaps by increasing proliferation. Inhibition of apoptosis, in which p53 and c-erbB-2 may be involved, occurs late and then only in a select proportion of cells with HG dysplasia. Loss of 7q33-35 was also found to represent a significant distinction between LG and HG dysplasia (Riegman et al, 2001). Invasive cancer may be preceded by loss of 16q21-q22,

gain of 20q11.2-q13.1 and alterations of cell adhesion especially involving cadherin/catenin complexes. Subsequent cumulative genetic errors may result in the generation of multiple clones of transformed cells, thereby expanding the population of altered cells with an angiogenic or metastatic potential (Figure 4).

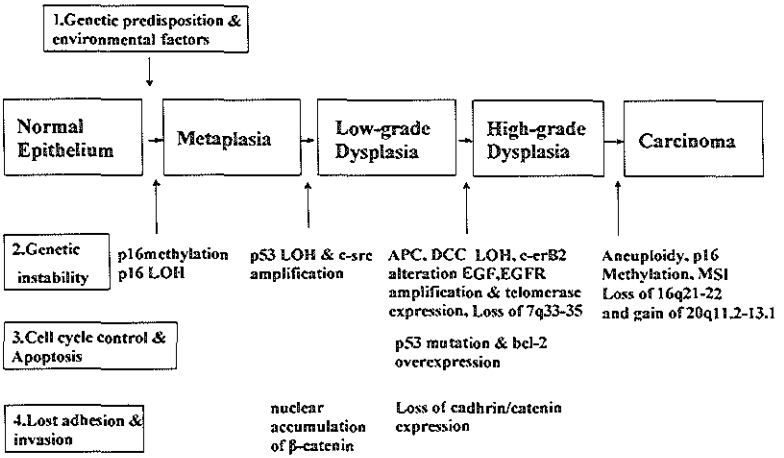

Figure 4. *Schematic representation of the key molecular events that may lead to carcinogenesis in the metaplsia-dysplasia-adenocarcinoma sequence of Barrett's esophagus.*

Several issues are incompletely elucidated at present. 1) The origin of stem cells that give rise to Barrett's metaplasia are unknown. 2) The nature history of dysplastic glands, especially the low-grade dysplasia is unclear. 3) We do not know which biological processes are essential determinants of early invasion.

In conclusion, more studies are needed to improve our understanding of the biology of Barrett's esophagus. Although no common and simple molecular pathway of progression is evident, a combined analysis of the histological spectrum of Barrett's esophagus and accompanying specific molecular alterations will increase our knowledge concerning the pathogenesis of the metaplasia-dysplasia-adenocarcinoma sequence.

1.2 Outline of the present study

The aim of our study was to better understand the molecular mechanisms during neoplastic progression in Barrett's esophagus, mainly focusing on p16 gene transcriptional silencing and the role of disruption of the APC/β-catenin/Tcf pathway. In addition, expression and mutation of p53 during the neoplastic progression from Barrett's esophagus to adenocarcinoma was characterized and an evaluation of the correlation between gene mutation and/or protein accumulation with clinicopathological findings and survival was performed.

In **chapter 2**, a methodological validation of microdissection and DNA extraction from archival tissue sections is reported. This procedure allows 15 PCR reactions to be routinely performed on lesions as small as 1 mm in diameter. Its usefulness was demonstrated in the study of loss of heterozygosity (LOH) on chromosome 18q.

In **chapter 3**, we established a new method for promoter hypermethylation analysis based on bisufite modification followed by PCR single-strand conformation analysis (MS-SSCA). The application for p16 gene promoter hypermethylation analysis showed that this approach is rapid, specific, semi-quantitative and works well with DNA extracted from microdissected fixed tissue sections.

In **chapter 4**, we characterized expression and mutation of p53 during the neoplastic progression from Barrett's esophagus to adenocarcinoma, and test the reliability of immunohistochemistry for p53 overexpression as an indicator of p53 mutation in this context. A evaluation of the association of both gene mutation and protein accumulation with clinicopathological findings and survival were also studied.

In **chapter 5**, we determined whether there is a correlation between β-catenin nuclear accumulation and exon 3 mutaion of this gene. Expression and mutation of β-catenin in the progression of Barrett's esophagus to adenocarcinoma were characterized.

In **chapter 6**, to investigate the potential role of p16 gene inactivation during neoplastic progression in Barrett's esophagus, we analyzed p16 gene promoter hypermethylation, mutation, LOH of 9p21 as well as expression of p16 protein. The relationship among these features was also determined.

In **chapter 7**, our findings are discussed in the context of the current literature.

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CHAPTER II

Microdissection by exclusion followed by an appropriate DNA extraction for multiple PCR analyses from archival tissue sections

Bénédicte Baisse, Yan-Song Bian, and Jean Benhattar.

Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

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ABSTRACT

An inverse microdissection technique is described which has been developed to allow analysis of invasive lesions as small as 1 mm^2 on formalin-fixed, paraffin-embedded histological tissue sections. Unwanted cells are completely removed before sampling of target cells; an optimized DNA extraction and subsequent polymerase chain reaction (PCR) complete the procedure. Using this approach, 15 PCR reactions can be performed on each microdissected-area and, therefore, the study of several genetic alterations can be carried out on the same small microdissected-area without contamination by normal cellular DNA. Detection of LOH on the long arm of chromosome 18 was performed on archival formalin-fixed tissues to demonstrate the advantages of this method: simplicity, rapidity, sensitivity and reasonable price.

The study of genetic alterations is a crucial facet of human cancer research. However, molecular analysis of genetic changes has been limited by the diversity of cell types present in specimens. The genomic DNA present in non-neoplastic cells may complicate the results of molecular analysis depending on the ratio of neoplastic to non-neoplastic genomic DNA and the sensitivity of the assays. With the development of polymerase chain reaction (PCR) technology which can amplify small quantities of DNA, several kinds of microdissection-based methods have been established. A first category uses ablation with ultraviolet radiation to destroy or remove the unwanted regions, the remaining target tissue is then collected mechanically (7). The main disadvantage of this method is the possibility of damage to the target DNA. A second category involves use of a tool under manual guidance to separate cells of interest from the histologic section. The tool can be a pipette (11), micropipette tip (10), needle (4), blade (5) or adhesive gum (8) either hand-held or connected to a micromanipulator arm (6). These techniques are not sufficiently precise to allow microdissection of diffuse tumors because internal stromal cells are generally not discarded and thus, the ratio of tumoral cells to normal cells is not optimized. A third category uses a laser-based technique: laser capture microdissection (3)

or UV laser microbeam (1), but this sophisticated method is unfortunately very costly and not accessible to all laboratories.

We present here a protocol for microdissection of archival paraffin-embedded tissue using a manual approach based on exclusion of unwanted normal cells and subsequent DNA extraction. This procedure allows 15 PCR reactions to be routinely performed on lesions as small as 1mm in diameter, even in cases of diffuse tumor infiltration. Its application will be demonstrated in the study of loss of heterozygosity (LOH) on chromosome 18q.

Sections of formalin-fixed, paraffin-embedded tissues were cut at 7 µm, collected on clean untreated glass slides, dewaxed twice with xylene and rinsed twice with methanol. The first section was stained with hematoxylin and eosin and used for histological control. Following sections were stained with 0.01% toluidin blue for 30 s, washed, air dried and either directly microdissected or stored in a closed box at room temperature.

Areas of specific histological interest were selected on the stained section by a pathologist and, in order to recognize the selected area, the slide was inverted and the area of interest surrounded with a felt pen. Microdissection was performed using wide field microscopy (for example an inverted microscope for cell culture) with a surgical scalpel blade, and consisted of removal of unwanted (non-tumorous) cells by gentle scraping. When the area of interest was entirely microdissected, the slide was washed with pure water under light pressure until unwanted cells were completely eliminated. If necessary, scraping, and washing were repeated until only the selected cells remained. A glass capillary was saturated with TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) to slightly moisten the selected cells which were detached from the slide by gentle scraping, without risk of contamination by neighboring normal cells. The collection of tissue fragments adhering to the tip of the glass capillary could be transferred to a microcentrifuge tube. In order to minimize loss of material, the capillary tip was broken off and left in the tube during the DNA extraction. The cells were immediately resuspended in 27 µl of TE buffer, 3 µl of digestion buffer (500 mM KCl, 150 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$ and 1.5% Tween-20) and 0.5 mg/ml of proteinase K, incubated for at least 24 hours at 55°C. An equal volume of a 10% chelex-100 resin solution (Bio Rad Laboratories,

Hercules, California, USA) was added and the mixture was incubated 1 hour at 55°C, then boiled for 10 minutes. After quick centrifugation, the supernatant containing the DNA was subsequently extracted with phenol followed by phenol/chloroform and co-precipitated in ethanol with 10 µg of glycogen either at -20°C, overnight or, more rapidly, 1 hour at -20°C followed by 1 hour at -80°C. Extracted DNA was resuspended in 30 µl of 10 mM Tris-HCl pH 8.0 solution, and kept at -20°C until use. Two microsatellite markers, D18S69 and D18S58 from chromosome 18q were chosen for LOH analysis (9). PCR amplifications were carried out, for 40 cycles, with 2 µl of extracted DNA in a total volume of 20 µl. An aliquot of 5 µl of each PCR product was mixed with 2 µl of formamide loading buffer, denatured 10 min at 90°C, and loaded on a 6% denaturing polyacrylamide gel containing 8 M urea. After electrophoresis in 0.5X TBE buffer, at 500 V for 90 min, gels were stained for 20 min in the dark with a SYBR Gold gel stain (Molecular Probes, Eugene, OR, USA) diluted 1:10'000 in 1X TBE buffer and visualized to UV light using a CCD camera (2).

In order to optimize the PCR amplifications we adapted the DNA extraction to the microdissected material. PCR amplification of material from simple scraping resulted in amplification of about 40% of samples and allows only one PCR reaction on each microdissected area. Thus, with this easy and rapid method only one genetic alteration can be studied for each area interest on a given slide. When a digestion step with proteinase K was performed, about 60% of DNA samples were successfully amplified. Use of chelex, a product known to be a chelator of metals which are potential inhibitors of Taq polymerase, after the proteinase K digestion step allowed virtually all samples to be amplified. However, PCR amplification of the extracted DNA was not reproducible after few rounds of freezing and thawing, probably due to instability in the digestion and chelex mixtures. Phenol extraction and ethanol precipitation after the chelex step were added to circumvent this problem. Using this complete extraction procedure, DNA amplifications have been successful from DNA extracted from tissue fixed in sublimed formaldehyde, in Bouin's solution and buffered formalin. From paraffin-embedded tissue, the extracted DNA can be preserved at -20°C for several months and was sufficient to perform 15 PCR reactions. Thus, using our DNA extraction protocol, we were able to study several genes in the same

area on the same slide and apply different techniques for gene analysis such as Single-Strand Conformational Polymorphism (SSCP) or LOH analysis.

The microdissection by exclusion technique that we developed allows the isolation of tumor areas as small as $1mm^2$, surrounded by stromal cells and muscular cells. The case shown in Figure 1 has been chosen specially because of the fact that the tumor infiltrates the stroma and thus makes the sampling of the tumor difficult by currently described microdissection approaches. The Figure 1 illustrates two of the major advantages of the microdissection by exclusion. First, during the scraping procedure, the tumoral cells remain adherent, whereas the unwanted tissues, represented by black points, were detached from the slide (Fig. 1B). The scraping was performed in 10 to 20 minutes. The second and more important advantage is that it allows the microdissection procedure to be controlled. Indeed, after washing of scraped sections (Fig. 1C), independent monitoring by a pathologist of the complete removal of normal tissue is possible prior to collection of tumor tissue, and continued removal of normal tissue remains possible. Depending on the size of the area selected and the histology of the tumor, we succeeded to collect between 95 to 100% of the tumor tissue. We recommend that surrounding tissue to be generously discarded, guaranteeing a larger working area for cells, and thus reducing contamination.

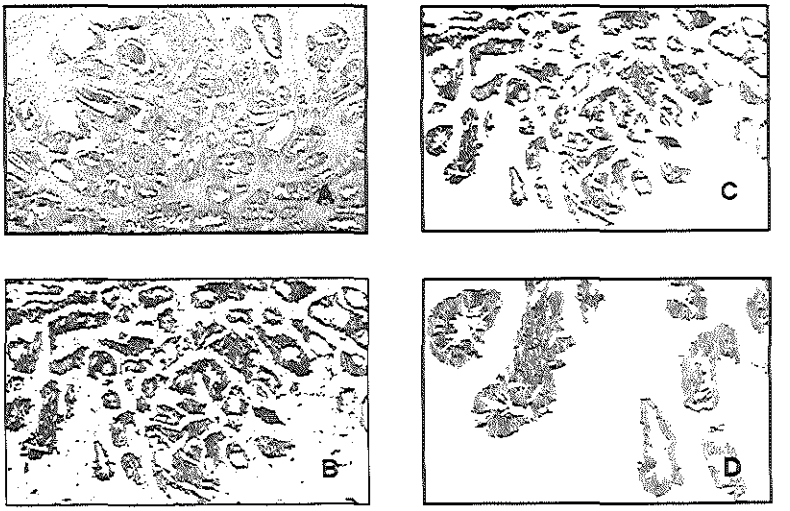

Figure 1. *Colorectal carcinoma from paraffin-embedded tumor sections.* ***A:*** *Hematoxylin and eosin stained section corresponding to the control (original magnification. x 20).* ***B:*** *consecutive section stained with toluidin blue after scraping. Arrows show some of the scraped unwanted tissues.* ***C:*** *same section as B, after washing of both scraped material and toluidin blue present on the slide.* ***D :*** *magnification (x 40) of framed part from C.*

We have tested our protocol of microdissection by exclusion on several cases in different tissues (colon-rectum, esophagus, endometrium and lymph node) and applied LOH analysis, where the presence of normal DNA is a limiting factor for interpretation of results. Results obtained following analysis of allelic loss of chromosome 18q in three informative cases of colorectal carcinoma are shown in Fig.2. Analysis of cases 1 and 2 was achieved with the microsatellite marker D18S69 and case 3 with D18S58. In comparison with the pattern of a normal sample, the lesion in the first case presented a sample without LOH, a sample with LOH in the second and a sample with microsatellite instability in the last one. To highlight the improvement achieved by the microdissection by exclusion and its application to genetic alteration studies, a diffuse lesion (see Fig. 1) was analyzed by standard microdissection (4) and by microdissection by exclusion (Fig. 2, case 2). The LOH was not detectable by the standard method (Fig. 2Ta), due to the presence of non neoplastic cells conferring a normal pattern to the sample, whereas, use of the exclusion method, rendered the LOH obvious (Fig. 2Tb).

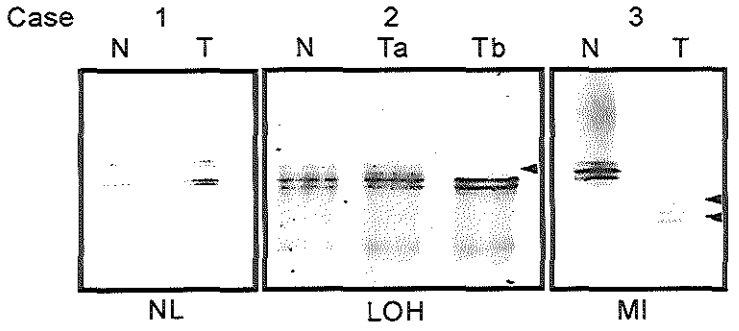

Figure 2. *Examples of no loss (NL), loss of heterozygosity (LOH), and microsatellite instability (MI) on chromosome 18q in paired normal (N) and colorectal tumor (T. Ta and Tb) tissues. Case 2, a diffuse tumor sample. LOH detection was performed after either direct microdissection (Ta) or microdissection by exclusion (Tb). Arrows indicate regions of either allelic loss or microsatellite instability.*

In conclusion, the experiment described above among many others performed routinely during the last year in our laboratory highlights the advantages of the microdissection by exclusion followed by an adapted DNA extraction for all kind of fixed archival tissues. Optimization of all the steps of the DNA extraction and purification allowed us to perform multiple PCR reactions on the same microdissected sample. This permits PCR-based studies of clonal diversity of various carcinomas even when diffuse. The microdissection by exclusion described here appears to be more precise than other current approaches, especially for diffuse lesions. Indeed, contamination by normal cells can be completely avoided, control and repeated scraping remain possible, and photography prior to sampling can be performed to document the tumoral cells studied. The principle of the microdissection by exclusion is, in a way, similar to UV laser microdissection (1); however, our methodology avoids the use of a sophisticated and costly apparatus which is not possible for all laboratories.

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CHAPTER III

Promoter Methylation Analysis on Microdissected Paraffin-Embedded Tissues Using Bisulfite Treatment and PCR-SSCP

Yan-Song Bian, Pu Yan, Maria-Chiara Osterheld, Charlotte Fontolliet, and Jean Benhattar

Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

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ABSTRACT

Methylation sensitive single-strand conformation analysis (MS-SSCA) is a new method of screening for DNA methylation changes. The combination of bisulfite modification and PCR, results in the conversion of unmethylated cytosines to thymines whereas methylated cytosines remain unchanged. This sequence conversion can lead to methylation-dependent alterations of single strand conformation, which can be detected by SSCA. An analysis of mixtures of methylated and unmethylated DNA at known ratios revealed that the relative intensities of the corresponding bands following MS-SSCA were maintained. MS-SSCA was applied for methylation analysis of human p16 promoter region, using genomic DNA obtained from either frozen, fixed or microdissected fixed tissue sections. MS-SSCA is a rapid, specific, and semi-quantitative approach which allows the detection of methylation of the p16 gene promoter. In reconstruction experiments, the method permits the detection of 10% or less of cells harboring a methylated p16 promoter. We have been successful in analyzing by MS-SSCA almost all (96%) tumor samples microdissected from archival paraffin-embedded fixed tissue sections and obtaining reproducible results. In addition, when microdissection was performed, the clonality of this genetic alteration could be identified.

INTRODUCTION

Hypermethylation of the 5'CpG Island in the promoter region is one of the mechanisms for inactivation of tumor suppressor genes in human neoplasia (7, 8). Detection of DNA methylation has recently attracted considerable attention. Although the exact sequence location of methylated cytosines can be determined by bisulfite genomic sequencing (3, 4), this approach is technically difficult and time-consuming. Methylation specific PCR (MSP) is an alternative qualitative method for methylation analysis, easy to perform and highly sensitive (6). However, we obtained false positive results using DNA from fixed tissues due to PCR overamplification. Southern blot analysis is a quantitative method to analyze DNA methylation levels at specific gene loci, but this requires much more DNA (>5 μg) and can not be performed on DNA isolated from paraffin sections. Combined bisulfite restriction analysis (COBRA) provides an excellent methylation analysis with both high sensitivity and quantitative accuracy (10). However, this method also potentially generates false positive results when enzymatic digestion and/or bisulfite modification are incomplete (9). These limitations hinder routine analysis of samples especially from paraffin-embedded fixed tissues. Recently, a new method of screening for methylation changes has been developed based on bisulfite treatment of DNA followed by PCR and single strand conformation analysis (2). In this study, we applied this procedure to the methylation analysis of p16 promoter region in human cancers. We specially investigated the use of this semi-quantitative method on bisulfite treated genomic DNA from frozen, fixed and microdissected archival paraffin-embedded fixed tissue sections.

MATERIALS AND METHODS

Tissues, Cell lines, and DNA extraction

Frozen tumor samples from cases of bladder, colon, esophagus and soft tissue tumors, as well as their associated normal tissues were obtained from the Tissue Bank of the Institute of Pathology, University of Lausanne. Archival formalin-fixed and paraffin embedded tissues of each case were also studied. All tissue samples, frozen and fixed, were histologically characterized. A colon cancer-derived cell line (SW620), and placenta tissue were also used. Genomic DNA from frozen and archival fixed tissues and cell lines were extracted by using QIAGEN DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer.

Microdissection and DNA extraction from fixed tissue sections

After deparaffinization in xylene and rinsing in methanol, sections were stained with 0.01% toluidin blue and selected areas were microdissected and extracted as previously described (1). In brief, nontumorous tissues were completely eliminated before tumor cells collection. By this procedure, microdissected areas were determined to contain at least 95% cancer cells. The microdissected cells were immediately resuspended in 27 µl of TE buffer, 3 µl of digestion buffer (500 mM KCl, 150 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$ and 1.5% Tween-20) and 0.5 mg/ml of proteinase K, incubated for at least 24 hours at 55°C. An equal volume of a 10% chelex-100 resin solution (Bio Rad Laboratories, Hercules, California, USA) was added and the mixture was incubated 1 hour at 55°C, then boiled for 10 minutes. After quick centrifugation, the supernatant containing the DNA was subsequently purified with phenol followed by phenol/chloroform and co-precipitated in ethanol with 10 µg of glycogen at -20°C. Extracted DNA was resuspended in 20 µl of 10 mM Tris-HCl pH 8.0 solution and kept at -20°C until use.

Bisulfite Modification

One µg of genomic DNA from cell lines, frozen or fixed tissue and all the DNA obtained in the case of microdissected tissues, in a volume of 40 µl water, was first heated at 100°C for 10 min and then denatured for 10 min at 37° by addition of 2.7 µl of freshly

prepared 3N NaOH. Twenty eight microliters of 10 mM hydroquinone (Sigma) and 500 µl of 4.8M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and the reagents were well mixed. The mixture was incubated at 55°C for 4 hr (6). After adding 80 µl H_2O and 365 µl 100% EtOH, the modified DNA was purified by passing through a column from the QIAGEN DNeasy Tissue Kit. After washing with the wash buffer contained in the kit, DNA modification was completed on the column by addition of 500 µl of 0.2 M NaOH/90% EtOH. The reaction was performed during 10 min at room temperature, washing was done twice, and DNA was eluted into 50 µl of 10 mM Tris-HCl solution and stored at -20°C.

PCR-SSCP

A 194 bp fragment of the p16 gene promoter containing 11 CG dinucleotides was amplified by PCR using the following primers specific to the upper modified strand: 5'-GGGGGAGATTTAATTTGG-3' (sense) and 5'-CAACCCCTCCTCTTTCTT (anti-sense). After an initial denaturation for 5 min at 95°C, the PCR reaction was carried out, in presence of 5% DMSO, for 35 (with DNA from frozen tissues) or 40 (with DNA from fixed tissues) cycles using *Taq* DNA polymerase and the following amplification profile: denaturation at 94°C for 30 sec, annealing at 54°C for 45 sec, and extension at 72°C for 75 sec. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. Five microliters of PCR product was denatured with 5 µl of denaturing buffer (100 mM NaOH and 2 mM EDTA) at 50°C for 10 min. After addition of 1.5 µl of formamide dye, the samples were immediately analyzed in a 30% MDE gel (AT Biochem, Malvern, PA) in 0.5X TBE (45 mM Tris-HCl, 1 mM EDTA, 40 mM boric acid), with a constant voltage of 20 V/cm for 5 h at 20°C. After electrophoresis, gels were released from plates, stained for 20 min in the dark with a SYBR Gold gel stain (Molecular Probes, Eugene, OR, USA), diluted 1:10'000 in 1xTBE buffer, and visualized with UV light using a CCD camera. For each sample, the final results were confirmed by analysis of two PCR products which have been done at the same time.

RESULTS AND DISCUSSION

Semi-quantitative MS-SSCA analysis

The differences of methylation-dependent sequence are introduced into the genomic DNA by sodium bisulfite modification and then the modified DNA is amplified by PCR using primers without CpG repeats and complementary to the deaminated DNA strand. This combination of bisulfite modification and PCR results in the conversion of unmethylated cytosine residues to thymine whereas methylated cytosine residues, present at CpG sites, are retained as cytosine. This sequence conversion can lead to the methylation-dependent alteration of single strand conformation which can be detected by SSCP. The outline of the MS-SSCA, bisulfite modification followed by PCR-SSCP analysis, is shown in Fig 1. Three patterns of band mobility were detected by MS-SSCA: no methylation (0%), full methylation (100%) and a mixture of both. The percentage of DNA methylation therefore can be directly reflected by the ratio of intensity between the methylated and unmethylated bands. To check the reliability of this approach, this analysis was performed on genomic DNAs where the p16 promoter region was found to be either fully methylated (SW620 colorectal cell line) or fully unmethylated (placenta) at all CpG sites. To determine the dynamic range of semi-quantitative methylation analysis by PCR-SSCP, we prepared mixtures of SW620 and placenta genomic DNAs in different ratios (0%, 10%, 20%, 50%, 80%, 90%, 100%) prior to the bisulfite modification and processed as independent samples throughout a complete bisulfite modification followed by a PCR-SSCP analysis. The results of this experiment are shown in Figure 2. Linear regression analysis of the data shows that MS-SSCA yields reliable semi-quantitative results across a wild range of DNA methylation levels. This technique allows the detection of at least 10% fully methylated p16 gene promoter in the presence of unmethylated one (Figure 2, lane 2).

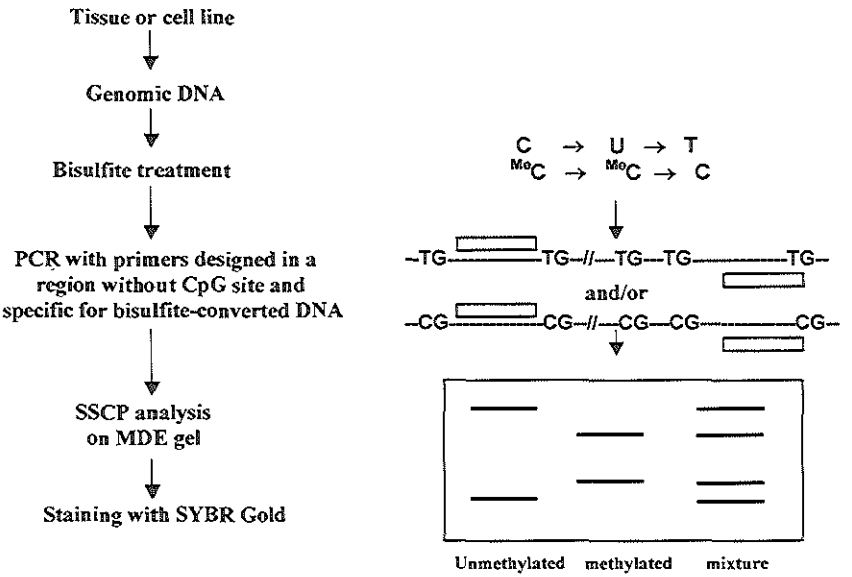

Figure I. *Outline of the MS-SSCA procedure. This method based on bisulfite treatment of genomic DNA followed by PCR using primers specific to the modified strand and without CpG sites (indicated by large bands), and then semi-quantitatively analyzed by nonradioactive single strand conformation polymorphism (SSCP). According to the sequence differences created by bisulfite-PCR, three patterns are distinguishable by SSCP: unmethylated, methylated and mixture between unmethylated and methylated alleles*

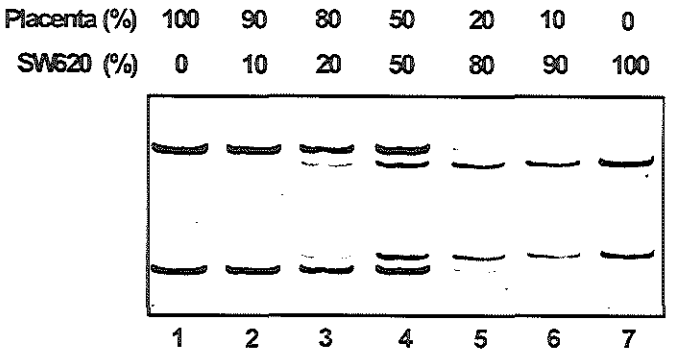

Figure 2. Determination of semi-quantitative methylation analysis by MS-SSCA. DNAs from a human colorectal cancer cell line (SW620) and from normal human tissue (placenta) were mixed at various ratios, as indicated above the gel, to determine the dynamic range of semi-quantitative methylation analysis by MS-SSCA. The p16 promoter was fully methylated for SW620 (100%) and unmethylated for placenta (0%). Linear regression analysis of the data shows that MS-SSCA method yields reliable semi-quantitative results across a wild range of DNA methylation levels and it is sensitive to 10% methylated alleles of a given CpG island locus.

Application of MS-SSCA in frozen and fixed human cancer tissues

To test if this method could be applied to the routine analysis of clinical tumor specimens of frozen or archival fixed tissues, we analyzed human cancers (from bladder, colon, esophagus and soft tissue) and their associated normal tissues for p16 methylation status. All the genomic DNAs tested were found to be amplifiable by PCR after bisulfite treatment. Only three patterns of mobility were detected by MS-SSCA: no methylation, full methylation and mixture between no and full methylation. These results suggested that partially methylated alleles (methylation only occurs in some of the CpG sites within the analyzed region) were not present in these tissues or cell lines. Analysis of p16 promoter methylation in three cases of esophageal squamous cell carcinoma by using frozen and fixed tissues are shown in figure 3. In comparison with the pattern of unmethylated normal esophageal squamous epithelium, case 1 had no methylation (Figure 3, in case 1 compare lane 5,7 to lanes 4 and 6) while a mixture between no and full methylation was observed for cases 2 and 3 (Figure 3, in case 2 and 3 compare lanes 5,7 to lanes 4 and 6). Since the

tumor tissues are constituted of tumor and nontumorour cells (e.g. inflammatory cells, stroma) in variable proportions, it remains unclear whether it is the tumor cells which harbor a methylated p16 promoter. In addition, it could be necessary to determine the level of tumor cells which contain one or both methylated p16 alleles. This crucial problem can not be resolved by the analysis using piece of tissues, fixed or frozen and only microdissection can answer this question.

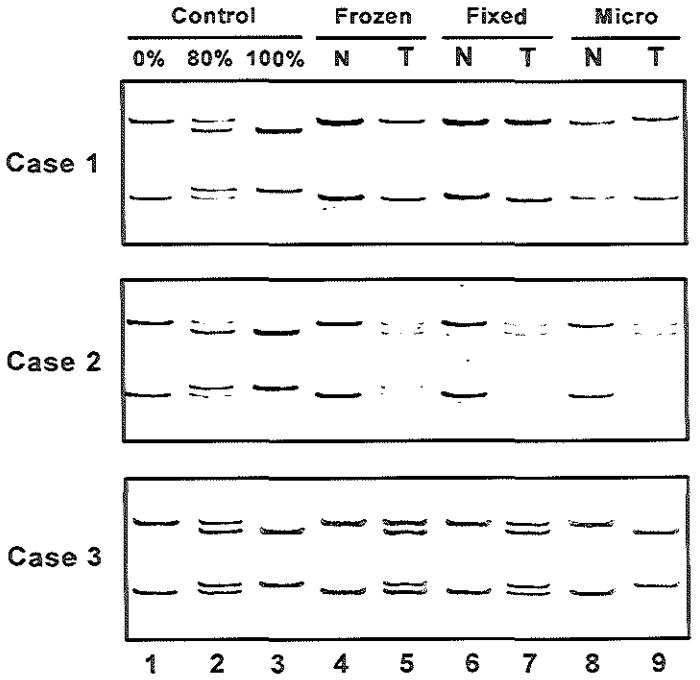

Figure 3. *MS-SSCA analysis of human esophageal squamous cell carcinomas and their associated normal tissues. To test whether this method could be applied to the routine analysis of any kind of clinical material : Frozen (Lanes 4-5), Fixed (Lanes 6-7) and Microdissected fixed (Lanes 8-9) tissues were used. Mixtures of SW620 and placenta genomic DNAs in different ratios (0%, 80% and 100%) were used as control (Lanes 1-3). T indicates tumor, N indicates normal tissue.*

Application of MS-SSCA in microdissected formalin-fixed and paraffin embedded tissues

Methylation analysis has not been extensively applied to microdissected archival fixed tissues. Gonzalgo et al. (5) developed a rapid quantitative method (Ms-SNuPE) for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA, followed by single nucleotide extension, and claimed that this technique could be applied to DNA obtained from microdissected pathological sections. However, this approach is more laborious than other techniques and also uses radioactive tracer. We tested two other alternative methods, including MSP (6) and COBRA (10), and found that both resulted in a relatively high number of false positive cases, probably due to an incomplete bisulfite modification of the genomic DNA (9), and false negative cases, essentially due to poor PCR amplification when MSP approach was used (data not shown).

There are some points which are important for successful analysis of methylation status by MS-SSCA using microdissected formalin-fixed and paraffin embedded tissue sections. Since a significant amount of DNA lost during bisulfite modification, three to five fold more tissue is necessary compare with PCR-SSCP analysis of unmodified genomic DNA, for which 1 mm^2 of tissue generally suffices (1). In addition, DNA extraction from microdissected sections need to be optimal. Addition of chelex, a product known to be a chelator of metals which are potential inhibitors of *Taq* polymerase, during DNA extraction after the proteinase K digestion step but before phenol/chloroform and ethanol precipitation steps, allowed most of samples to be amplified (1). Bisulfite modification is then performed essentially as described for genomic DNA obtained from frozen or fixed tissues. Part of this modification, mainly the washing steps and the final alkali treatment, was done on the QIAGEN DNeasy Tissue Kit column. This procedure is easy to perform, limits the risk of contamination, and yields a high amount of relatively pure modified DNA, which is essential for the next PCR amplification step. Nevertheless, to avoid false results due to poor quality of extracted DNA in some microdissected samples, two PCR reactions need to be performed. The same mobility of the PCR products analyzed by SSCP assures a correct result. In only few cases (about 4%),

different mobility was observed. In these cases, the whole procedure has to be repeated from the beginning.

This approach has been applied to the analysis of clinical tumor specimens. As an example, the analysis of p16 promoter methylation in esophageal squamous carcinoma is shown in figure 3. Normal esophageal mucosal cells were also microdissected but MS-SSCA never showed p16 promoter methylation. For the tumor tissues, similar results were obtained from frozen, fixed and microdissected formalin-fixed and paraffin embedded tissues (Figure 3). In case 2, even after microdissection, MS-SSCA showed a mixture of fully methylated and unmethylated p16 promoter suggesting that half of the DNA molecules were fully methylated and the remainder unmethylated. (Figure 3, in case 2 compare lane 9 to lanes 5 and 7). Conceivably, either the p16 promoter was methylated at all CpG sites in one allele with the other allele totally unmethylated, or part of the tumor cells is fully methylated. In case 3, the p16 promoter region was fully methylated at all the CpG sites and in all microdissected tumor cells (Figure 3, in case 3 compare lane 9 to lanes 5 and 7). As shown in cases 2 and 3, microdissection was necessary to confirm that the p16 promoter was methylated in tumor cells. Analysis after microdissection also revealed that the methylation status of the p16 promoter is complex. Indeed, during our experiments, together with LOH analysis of chromosome 9p21 where p16 is located (data not shown), we found that 1) complete methylation represents either methylation of one allele and deletion of the other allele or methylation of both alleles; 2) a mixture of complete methylation and no methylation at a ratio 1:1 represents methylation of only one allele; and 3) a mixture with other ratios is probably due to intratumor heterogeneity (only part of tumor cell population harbors a methylated p16 promoter). From this point of view, microdissection is a very useful tool to determine the methylation status of the p16 promoter within tumor cells in a tissue.

In conclusion, our improved MS-SSCA has several advantages over existing methods: it is rapid, specific, semi-quantitative and works very well with DNA extracted from microdissected fixed tissues. Indeed, MS-SSCA can detect methylation changes in as little as 1 ng of DNA. This technique is also sensitive and allows the detection of 10% or even less of fully methylated p16 gene promoter in the presence of unmethylated one. The advantage of this method not only allows the analysis of methylation status within a lesion

but, when microdissection is performed, also gives further information about the clonality of this genetic alteration.

ACKNOWLEDGEMENT

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CHAPTER IV

p53 gene mutation and protein accumulation during neoplastic progression in Barrett's esophagus

Yan-Song Bian, M.D., Maria-Chiara Osterheld, M.D., Fred T. Bosman, M.D. Ph.D., Jean Benhattar, Ph.D., Charlotte Fontolliet, M.D.

Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

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ABSTRACT

The aim of the present study was to characterize expression and mutation of p53 during the neoplastic progression from Barrett's esophagus to adenocarcinoma, and to test the reliability of immunohistochemistry for p53 overexpression as an indicator of p53 mutation in this context. A evaluation of the association of both gene mutation and protein accumulation with clinicopathological findings and survival were also studied. A total of 77 samples from 30 esophagectomy specimens with Barrett's esophagus and adenocarcinoma of patients in longitudinal clinical follow-up were analyzed. Different lesions (intestinal metaplasia, dysplasia and adenocarcinoma) as well as normal squamous cell esophageal epithelia were sampled from formalin-fixed paraffin-embedded tissues by microdissection. Mutations in p53 exons 5 to 9 were detected by PCR-SSCP and confirmed by direct DNA sequencing. Nuclear accumulation of p53 protein was analyzed immunohistochemically from tissue sections adjacent to those used for microdissection. p53 gene mutations were found in 17 and p53 protein accumulation were found in 20 tumor samples. Of the 17 adenocarcinomas with a p53 mutation, 16 stained positive for p53 protein. p53 mutations were detected significantly more frequent in high-grade dysplastic than in low-grade dysplastic lesions (77% vs. 29%, $p<0.01$). In contrast, nuclear accumulation of p53 was detected in 85% of high-grade and 71% of low-grade dysplastic lesions. In 8 cases with p53 mutation, the mutation identified in the tumors was also detected in premalignant lesions, mainly in high-grade dysplasia. In 4 cases of p53 mutated tumors, clones with different p53 mutations were detected in premalignant lesions. Neither p53 mutations nor p53 protein accumulation were found in metaplastic lesions. In summary, we found p53 mutations occurred mainly during the transition from low-grade to high-grade dysplasia in the neoplastic progression of Barrett's esophagus but not in the nondysplastic Barrett's mucosa. Mutational analysis of p53 by PCR-SSCP and p53 accumulation by immunohistochemistry were mostly concordant in adenocarcinoma and high-grade dysplastic lesions, but frequently discordant in low-grade dysplastic lesions. No correlation between p53 gene mutation or p53 accumulation and clinicopathological findings was observed in this study.

INTRODUCTION

Barrett's esophagus represents a peculiar form of healing in the lining of the distal esophagus which occurs in response to chronic gastro-esophageal reflux (1, 2). The most important clinical significance of this disease is the associated predisposition to adenocarcinoma of the esophagus and gastric cardia (3, 4). It is hypothesized that cancer arises in Barrett's esophagus not de novo but as a result of progression from a metaplastic intestinal epithelium through increasing degrees of dysplasia and finally to invasive carcinoma (5-8). An increased understanding of molecular alterations during this neoplastic progression might allow to improve tumor control and prevention and finally lead to better management of the disease.

The p53 tumor suppressor gene encodes a nuclear phosphoprotein which functions as a transcription factor that controls the expression of many genes important in the regulation of the cell cycle and in triggering apoptosis after certain type of genomic damage (9). Deletion of one allele in the short arm of chromosome 17 and a functionally inactivating mutation of the other allele at the specific location are among the most common genetic abnormalities documented in human cancers (10). Numerous studies have found evidence to suggest that p53 inactivation contributes to the development of Barrett's esophageal adenocarcinoma. Casson et al (11) first demonstrated p53 mutations in Barrett's adenocarcinoma and premalignant lesions. Later, p53 gene mutations were found to be common and develop as relatively early events in the progression to cancer in Barrett's esophagus (12). These results were reconfirmed by many other investigators (13-15). A close association between 17p allelic loss and p53 gene mutation was also found (16). Additional evidence for the involvement of p53 mutations in the neoplastic progression towards Barrett's adenocarcinoma comes from studies of p53 protein overexpression by means of multiparameter flow cytometry and immunohistochemistry (17-19). Some studies correlated p53 alterations with clinicopathologic findings and outcome (20-22). However, despite the increasing amount of information, the precise role of p53 alterations in the natural history from benign to malignant lesions in Barrett's and the potential clinical significance of these observation remain unclear (23).

Wild-type p53 protein has a short half-life and the level present in the nucleus of normal cells is below the sensitivity of immunohistochemical detection. Missense

mutations in p53 gene result in a protein with a prolonged half-life which then accumulates in the nucleus (24). The increased levels of p53 are due to metabolic stabilization rather than overexpression (25). p53 protein overexpression detected by immunohistochemistry (IHC) has been proposed as an indirect method of detecting a p53 mutation. It is relatively cheap, quick and easy compared with other techniques, such as DNA sequencing or PCR-SSCP, and in addition, the technique is available in most Pathology laboratories.

Studies on colorectal and ovarian cancer showed that mutation of p53 gene and overexpression of p53 protein correlated significantly (26, 27). In contrast, these features were not correlated in lung or head and neck cancers (28). How p53 gene mutations and p53 immunoreactivity correlated in preneoplastic lesions of Barrett's esophageal mucosa and their associated cancers has not been extensively studied.

The aim of the present study was 1) to characterize expression and mutation of p53 during the progression of Barrett's esophagus to adenocarcinoma, 2) to test the reliability of immunohistochemistry (IHC) as an indicator of p53 mutation, and 3) to evaluate the association of both gene mutation and protein accumulation with pathological findings and survival.

MATERIALS AND METHODS

Tissue Samples

Thirty esophagectomy specimens for adenocarcinoma developed in a Barrett's esophagus between 1986-1996 were selected from the files of the Institute of Pathology at the University of Lausanne. Patients had received neither radiation therapy nor chemotherapy before surgery. The age of the patients (27 males and 3 females) ranged from 46 to 86 years with an average of 65 years. Follow-up was available for 25 patients. Samples were formalin-fixed and paraffin-embedded. All available histological sections, which had been collected by mapping of the lesions as described for gastric carcinomas (29), was re-examined by two pathologists (MCO and CF). Five lesions were selected from each esophagectomy specimen, which resulted in 77 samples encompassing 30 invasive adenocarcinomas, 17 metaplasias, 17 low-grade (LG) dysplasias and 13 high-grade (HG) dysplasias. Of each patient, a sample of normal squamous epithelium was also studied.

Diagnosis and Grading

Hematoxylin and eosin stained slides were screened to identify the different lesions. Barrett's esophagus was diagnosed when metaplastic epithelium (columnar epithelium with goblet cells) was present above the macroscopically identified gastro-esophageal junction. Dysplasia was graded into two categories, low grade and high grade, based of the degree of cytonuclear and architectural atypia (30). Tumors were graded into well, moderately and poorly differentiated adenocarcinoma and the depth of invasion was defined according to the UICC TNM classification of malignant tumors, fifth edition 1997.

Detection of p53 mutation by Non-radioactive Single Strand Conformation Polymorphisms (SSCP)

After deparaffinization in xylol and methanol, esophageal adenocarcinoma and premalignant lesions were carefully removed from the sections by microdissection as described before (31). DNA was prepared by digestion with proteinase K, treated with chelex, extracted with phenol/chloroform, and then precipitated with ethanol (31). The

methods for non-radioactive PCR-SSCP were previously described in detail (32). Primers for exon 5 to 9 of the p53 gene have also been described (33).

Direct DNA Sequencing.

After SSCP, the mutation specific band observed on 30% nondenaturing MDE gel was cut out and reamplified using the same primers. The PCR-amplified DNAs were purified using QIAquick PCR purification Kit (Qiagen, Germany) and the purified product was sequenced on an ABI PRISM 310 automatic sequencer using the dideoxy dye termination method.

Immunohistochemical Analysis.

All the selected tissue samples were examined by immunohistochemistry using the Strept ABComplex/HRP Duet Kit (DAKO, Denmark) and visualized with DAB. The monoclonal mouse anti-human p53 protein (Clone DO-7, DAKO, Denmark) which is known to recognize an epitope between amino acids 19 to 26 and to react with wild type and mutant p53 protein was used in this study (diluted 1:500, 26.8ug/ml). Two staining patterns were distinguished: 1) positive when >10% of nuclei were positive, in clusters or scattered through the tissue, 2) negative when no or only a few cells (<10%) showed a nuclear staining.

Statistics.

The chi-square test was used for univariate analysis and unweighted logistic regression for the multivariate analysis.

RESULTS

p53 Mutation and Protein accumulation in Adenocarcinomas

p53 gene mutations, confirmed by direct DNA sequencing, were found in 17 of the 30 (57%) adenocarcinomas (Table 1). In these tumors, p53 gene mutations were distributed as follows: 7 in exon 5, 1 in exon 6, 6 in exon 7 and 3 in exon 8. No mutation was found in exon 9. There were 15 point mutations and 2 deletions (one of 10bp and another of 19bp). G:C to A:T transition was found rather frequently (7/17, 41%), and among them 5 occurred at CpG sites. Five G:C to T:A transversions (one of them resulting in a stop codon) were also present.

Tissue sections adjacent next to those used for microdissection were analyzed in the same region by IHC. p53 protein accumulation was found in 20 of the 30 adenocarcinomas (67%). Nuclear staining was characteristic and no cytoplasmic staining was noted (Figure 1A).

Of 17 adenocarcinoma cases with p53 mutation, 16 also showed p53 protein accumulation. One case with a 10bp deletion in the p53 gene, resulting in the production of a partially different p53 protein, did not show any staining by IHC (case 7). Nuclear accumulation of p53 protein was found in all 14 cases with missense mutation of the p53 gene. In 4 of the 13 cases which p53 mutations were negative by PCR-SSCP, p53 protein accumulation was observed.

p53 Mutation and Protein accumulation in premalignant lesions

To investigate whether p53 mutations occurred consistently throughout the Barrett's epithelium, SSCP analysis was used to screen premalignant lesions (metaplasia, LG and HG dysplasia) sampled from all 17 cases in which a p53 mutation was found in the adenocarcinoma (Table 1). p53 mutations were detected significantly more frequently ($p<0.01$) in HG dysplastic (10/13; 77%) than in LG dysplastic lesions (5/17; 29%). In 47% (8/17 cases) of tumors with a p53 mutation, the same mutation was also detected in at least one premalignant lesion. In 4 cases, the p53 mutation detected in the premalignant lesions differed from those in the associated adenocarcinomas. No p53 mutation was observed in metaplastic lesions.

Table 1. p53 mutation and overexpression during neoplastic progression in Barrett's esophagus in the 17 cases with p53 mutated tumors.

	Tumor		HG dysplasia		LG dysplasia		Metaplasia	
Case	Exon:codon / mutation	IHC	Exon:codon / mutation	IHC	Exon:codon / mutation	IHC	Exon:codon / mutation	IHC
2	Ex8:289 / CTC→CCC	+	-	+	-	+	-	-
4	Ex7:244 / GGC→AGC	+	-	+	-	+	-	-
5	Ex7:249 / AGG→ACG	+	Ex7:249 / AGG→ACG	+	Ex7:245 / GGC→AGG *	+	-	-
6	Ex7:248 / CGG→CAG	+	Ex7:248 / CGG→CAG	+	-	-	-	-
7	Ex5:174-177 / 10bp del	-	Ex5:174-177 / 10bp del	-	-	-	-	-
8	Ex5:176 / TGC→TTC	+	Ex5:181 / CGC→GGC *	+	-	+	-	-
10	Ex5:149 / TCC→ACC	+	NA	NA	Ex5:149 / TCC→ACC	+	-	-
11	Ex5:175 / CGC→CAC	+	Ex5:175 / CGC→CAC	+	-	+	-	-
13	Ex5:175 / CGC→CAC	+	NA	NA	-	-	-	-
14	Ex7:248 / CGG→TGG	+	Ex7:248 / CGG→TGG	+	-	-	-	-
15	Ex8:266 / GGA→GAA	+	NA	NA	-	-	-	-
16	Ex6:194-200 / 19bp del	+	-	+	-	+	-	-
23	Ex5:176 / TGC→TTC	+	Ex5:176 / TGC→TTC	+	Ex5:176 / TGC→TTC	+	-	-
25	Ex5:186 / GAT→AAT	+	NA	NA	-	+	-	-
27	Ex7:240 / AGT→ATT	+	Ex7:236 / TAC→AAC *	+	Ex7:236 / TAC→AAC *	+	-	-
28	Ex8:282 / CGG→TGG	+	Ex5:175 / CGC→CAC *	+	Ex5:175 / CGC→CAC *	+	-	-
30	Ex7:258 / GAA→TAA	+	Ex7:258 / GAA→TAA	-	-	+	-	-

NA : material not available
* : different p53 mutation than those found in the tumor.

p53 overexpression was detected in 85% (11/13) of specimens with HG dysplasia and in 71% (12/17) of those with LG dysplasia. No p53 protein accumulation was found in the 17 intestinal metaplastic lesions without dysplasia (Figure 1).

Positive p53 staining was found in 13 of 15 (87%) premalignant lesions with a p53 mutation. The non-concordant cases were 2 HG dysplastic lesions, one of which showed a 10 bp deletion which was also IHC negative in the tumor and the other associated with an IHC positive adenocarcinoma which had the same p53 mutation. Of the 15 dysplastic lesions negative by PCR-SSCP, 9 (60%) were p53 positive by IHC (Table 1). Most of them (7/9, 78%) were LG dysplasia (Fig. 1).

p53 Alterations and clinicopathological parameters

Table 2 provides the correlation between alteration of p53 and clinicopathological parameters. p53 mutation and p53 protein accumulation were analyzed according to patient gender, tumor histological type, pathologic stage and survival. The incidence of p53 mutation and nuclear overexpression was higher in patients with lymph nodes metastasis, but the difference was not statistically significant. No significant differences were found according to the gender of patients or pathological characteristics of the specimens. The five patients lacking follow up data were excluded from survival analysis. No differences in survival were observed between the patients with p53 mutations or p53 protein accumulation in esophageal adenocarcinoma and those without.

Table 2. p53 mutation and protein accumulation and correlation with clinical and pathological findings

	p53 mutation*	p53 protein accumulation*
Gender		
Male	16/27	18/27
Female	1/3	2/3
Grade		
1	4/8	4/8
2	4/8	5/8
3	9/14	11/14
T Stage		
Tis	1/2	1/2
T1	3/6	4/6
T2	0	0
T3	12/21	14/21
T4	1/1	1/1
Node Stage		
+	12/19	13/19
-	4/11	6/11
Survival		
more than 1 year	8/14	9/14
less than 1 year	6/11	7/11

*: all $p>0.05$

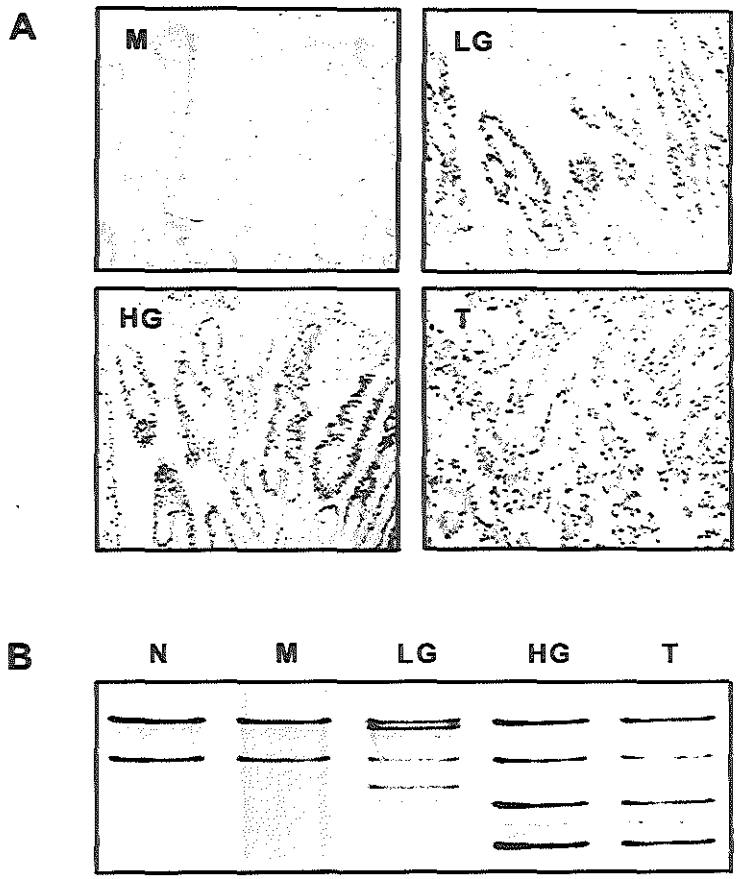

Figure 1. *p53 protein overexpression and p53 mutation in a case with premalignant lesions and adenocarcinoma in Barrett's esophagus (Table 1, case 5).* ***Part A:*** *IHC using the DO-7 antibody (M): metaplasia showing absence immunostaining for p53 : (LG), (HG) and (T): LG dysplasia, HG dysplasia and moderately differentiated esophageal adenocarcinoma showing nuclear immunostaining for p53 (magnification 100×).* ***Part B:*** *PCR-SSCP analysis of exon 7 of the p53 gene. Mobility observed in LG dysplasia differed from those in HG dysplasia and adenocarcinoma (T). No mutation was detected in metaplasia (M). DNA from normal tissues (N) of the same patient was used as a negative control.*

Discussion

Using molecular and immunohistochemical techniques we characterized the spectrum of p53 alterations during neoplastic progression in Barrett's esophagus and assessed their correlation with survival for patients with esophageal adenocarcinoma. In 30 Barrett's esophageal adenocarcinoma, p53 mutations were found in 17 cases (57%) while p53 protein accumulation was detected in 20 cases (67%). These results correspond to the incidence of p53 alterations in Barrett's esophageal adenocarcinomas reported by Kubba et al (23), who reviewed publications on the subject over the last 10 years. Previous studies have shown that the spectrum of p53 gene mutations is dependent on the cancer type (34, 35). Most of the mutations occur in exon 5-9, which encodes the DNA binding domain (36). The variation found in p53 mutations concern the position of mutational hot spots as well as the mutation type such as missense mutations (transition or transversion) and null mutations (nonsense, deletions, insertions, and splicing junction mutations). In esophageal squamous cell carcinoma as in lung carcinoma, a high frequency of G:C to T:A transvertions has been observed (37-39). This pattern has been linked to cigarette smoking or the occupational exposure to polycyclic aromatic hydrocarbons (40). In Barrett's esophageal carcinoma, however, the mutation profile is different. In our series, in addition to G:C to T:A transvertions (5/30 cases), G:C to A:T transitions were found frequently (7/30), and most of them occurred at CpG dinucleotides. Mutational hotspots at CpG dinucleotides may reflect endogenous mutagenic mechanisms, such as deamination of 5-methylcytosine to thymidine followed by a faulted repair procedure.

On histological section, the simplest and most practical approach is the use of IHC to detect p53 protein accumulation. Few studies have compared the results of p53 mutation analysis to p53 overexpression by IHC in esophageal adenocarcinoma. The reported discordance between these two approaches ranged between 21 and 41% of cases (18,41). The number of discordant cases in the present study (20%) is comparable to the data reported in the literature. We found that of 20 adenocarcinomas with p53 protein accumulation, 16 (80%) had p53 gene mutations. The discrepancy between IHC and mutation analysis was not due to sampling error : in this study, all the IHC analysis was done in tissue section adjacent to those used for microdissection for p53 gene mutation analysis.

The results confirm that p53 gene mutation and/or p53 protein accumulation are common in Barrett's adenocarcinoma. One aim of this study was to determine the step at which p53 mutation occurs during neoplastic progression. p53 mutations were detected more frequently ($p<0.01$) in HG dysplasia surrounding the tumor than in LG dysplasia. Furthermore, the mutations found in HG dysplasia were generally identical to those of the corresponding tumors, whereas different mutations were commonly detected in LG dysplasia. The finding of identical p53 mutations in HG dysplastic lesion and the associated tumor suggest that this genetic alteration occurs during the progression from LG to HG dysplasia. Some of the p53 mutations detected in the premalignant lesion, mainly in LG dysplasia, were different from those in adjacent adenocarcinomas which indicates that, while the entire esophageal epithelium of an individual is exposed to dietary or environmental carcinogens, lesion may arise from independent mutated cell clones at several different sites, some of which may eventually progress to carcinoma. It appears that p53 mutation is important in the later steps of the progression to carcinoma.

p53 overexpression occurred at a similar high frequency in the analyzed HG and LG dysplastic lesions. A high discordance rate between detection of p53 alteration by SSCP and IHC was observed in LG dysplastic lesions (7/17, 41%). The high incidence of p53 overexpression in LG dysplasia could be a consequence of the accumulation of wild-type p53 and/or the presence of p53 mutated clones, too small to be detected by SSCP analysis. In fact, only a few glands were positive by IHC in some LG dysplastic lesions in which no p53 mutations were detected. Neither p53 mutation nor p53 overexpression was observed in metaplastic lesions without dysplasia. It appears that in Barrett's esophagus p53 is not affected in the nondysplastic mucosa. These results indicated that p53 staining may have potential value for confirming a suspected diagnosis of LG dysplasia, as reported by other investigators (42).

The prognostic significance of p53 alterations in esophageal adenocarcinoma have been studied by many groups. Casson et al (20) reported a series of 61 patients in which p53 alterations (gene mutation and protein accumulation) occurred mainly in poorly differentiated adenocarcinoma which showed significantly reduced post-operative survival. However, Duhaylongsod et al (43) and Coggi et al (44) failed to find any association between p53 gene mutation or protein accumulation and survival in patients with esophageal adenocarcinoma. In this study, we found no correlation between p53 mutation or nuclear overexpression and clinicopathological parameters.

p53 alterations were not associated with a worse patient survival. However, it should be emphasized that these findings are based on a relatively limited number of cases, and interpretation of results concerning the association between p53 alterations and prognosis need to be confirmed in larger prospective multicenter studies (20).

In conclusion, we found that p53 alterations are common and occur mainly during the transition from LG to HG dysplasia in the neoplastic progression of Barrett's esophagus. A minor discordance between p53 mutation and p53 accumulation was observed in tumor and HG dysplastic lesions, but this discordance was major in LG dysplastic lesions. Neither p53 mutation nor p53 protein accumulation had independent prognostic value in patients with Barrett's adenocarcinoma.

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CHAPTER V

Nuclear Accumulation of β-Catenin is a Common and Early Event during Neoplastic Progression of Barrett's Esophagus

Yan-Song Bian, MD, Maria-Chiara Osterheld, MD, Fred T. Bosman, MD, PhD, Charlotte Fontolliet, MD, and Jean Benhattar, PhD

Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

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ABSTRACT

The aim of this study was to characterize expression and mutation of β-catenin in the progression of Barrett's esophagus to adenocarcinoma. β-catenin behaves as an oncoprotein which can lead to carcinogenesis when the APC/β-catenin/Tcf signal transduction pathway is disrupted. Immunohistochemical analysis of β-catenin was performed on paraffin-embedded tissue from 30 cases with adenocarcinomas and premalignant lesions. To determine whether there is a correlation between β-catenin nuclear accumulation and exon 3 mutation of this gene, mutational analysis by PCR-SSCP were also carried out on DNA extracted from the same 30 adenocarcinomas. As a result, the prevalence of reduced expression of β-catenin on the membrane, with or without nuclear staining, increased significantly from low grade (LG) to high grade (HG) dysplasia. Focal nuclear staining for β-catenin was present in 19 cases of adenocarcinoma, and nuclear staining was significantly associated with progression from metaplasia to LG dysplasia. In addition, in glands with clear histological transition from metaplasia to LG dysplasia, nuclear accumulation of β-catenin was found only in the LG dysplasic areas. No mutation in exon 3 of β-catenin gene was detected in adenocarcinomas. These results demonstrate that disturbance of the APC/β-catenin pathway, as indicated by nuclear accumulation of β-catenin, is a common and early event during neoplastic progression in Barrett's esophagus.

INTRODUCTION

Barrett's esophagus represents a peculiar form of healing in the lining of the distal esophagus which occurs in response to chronic gastro-esophageal reflux.[1-2] The most important clinical significance of this disease is the associated predisposition to adenocarcinoma of the esophagus and gastric cardia.[3-4] Recently, there has been controversy regarding diagnostic criteria for Barrett's esophagus. Some authorities have defined the condition according to an arbitrary extent of esophageal columnar lining whereas others have felt that the presence of specialized intestinal metaplasia anywhere in the esophagus establishes the diagnosis.[5-6] Studies have clarified that intestinal metaplasia was not only the most common and distinctive type of epithelium detected within the columnar metaplastic mucosa, but also the one with greatest malignant potential. Therefore, Barrett's esophagus has come to be defined by the histological presence of intestinal metaplasia.[7-8] Cancer arising in Barrett's esophagus may result through a multistep progression from metaplasia through dysplasia to carcinoma, accompanied by a sequence of genetic alterations, the nature of which is just beginning to be explored.[9-11] An increased understanding of molecular alterations during this neoplastic progression might allow improved diagnostic techniques and finally lead to improved management of this disease.

β-catenin mediates cell to cell adhesions together with transmembrane glycoprotein E-cadherin. It is an oncoprotein which can lead to carcinogenesis when the APC/β-catenin/Tcf signal transduction pathway is disrupted.[12-15] Investigation of β-catenin in this pathway might provide valuable insight into the mechanisms of cellular transformation and tumor progression. In non-transformed cells, the mitogenic signal Wnt-1 from outside the cell is absent, GSK-3β is active and together with wild-type APC bind to the free β-catenin. In this multiprotein complex GSK-3β phosphorylates β-catenin, leading to its dissociation from the complex and to its degradation in the cytoplasm.[14,16,17] Residual β-catenin hold cells together by binding tightly both to E-cadherin at adherent junctions and to the actin cytoskeleton, providing polarized non-motile cells.[18] In transformed cells the presented Wnt-1 signal inhibits the activity of GSK-3β or the multiprotein complex is not correctly formed (i.e. mutation of APC or β-catenin). As a

result, β-catenin can no longer be degraded resulting in an excess of free (monomeric) β-catenin. Then the cytoplasmic concentration rises, monomeric β-catenin binds to other newly synthesized proteins, especially transcription factors such as lymphocyte enhancing factor (LEF) and T cell factor (Tcf-4), and is transferred to nucleus.[15,19] Nuclear proteins in combination with β-catenin can activate or suppress individual gene promoters, resulting in down regulation of E-cadherin and positive regulation of epithelio-mesenchymal transition (EMT), which appears to be closely associated with invasion.[20]

Several studies have examined the role of the E-cadherin/β-catenin complex in maintenance of cell-cell adhesion and the disturbance of β-catenin in the Wnt pathway in various types of tumor.[15,21-25] A significant percentage of gastric adenocarcinomas and squamous cell carcinomas of the esophagus have shown abnormal expression of the E-cadherin/β-catenin complex.[21] In colorectal cancer the nuclear accumulation of β-catenin has been found frequently, predominantly at the invasion front.[26] In a recent report, a strong reduction of the expression of β-catenin was observed in dysplasia and adenocarcinoma in Barrett's oesophagus.[23] However, little is known about the nuclear accumulation of β-catenin during the progression from Barrett's esophagus to adenocarcinoma. Furthermore *APC* gene mutations are rarely observed in adenocarcinomas in Barrett's esophagus,[27,28] and so far no information is available on β-catenin gene mutations.

In the present study, we therefore examined the expression of β-catenin by immunostaining in the various steps of progression of Barrett's esophagus to adenocarcinoma, mainly focusing on membrane reduction and nuclear accumulation. Furthermore, mutations in exon 3 of β-catenin gene were analyzed by the PCR-SSCP method.

MATERIALS AND METHODS

Tissue Samples

Thirty esophagectomy specimens with an adenocarcinoma developed in a Barrett's esophagus were selected from our files between 1986-1996 in the Institute of Pathology at the University of Lausanne. No patients had received either radiation therapy or chemotherapy before surgery. All available material was re-examined by two pathologists (MCO and CF), which included mapping of the lesions as described for gastric carcinomas.[29] Samples were formalin-fixed and paraffin-embedded. Hematoxylin-eosin slides were screened to identify different lesions. Five histological lesions were selected and a total 87 samples from thirty different patients were summarized as follows: 30 corresponded to invasive adenocarcinoma, 19 to metaplasia, 19 to low-grade dysplasia and 19 to high-grade dysplasia. All samples of histologically normal squamous epithelium were also studied.

Diagnosis and Grading

Hematoxylin and eosin stained slides were screened to identify the different lesions. Barrett's esophagus was defined as the occurrence of columnar epithelium with incomplete intestinal metaplasia (specialized epithelium). Dysplasia was graded into two categories, low grade, and high grade, based of the degree of cytonuclear and architectural atypia similar to the grading of dysplasia in ulcerative colitis.[30]

Immunohistochemistry

Paraffin sections of 4 μm were cut and mounted on coated slides. Immunohistochemistry was performed using an avidin-biotin-peroxidase complex (ABC) method.[31] In brief, slides were dewaxed and rehydrated in xylene-ethanol series, endogenous peroxidases were blocked with 0.3% hydrogen peroxide solution and incubated for 20 minutes. Antigen retrieval was performed by microwave (750W) for 20 minutes in sodium citrate buffer (0.01M, pH 6.0). After three washes in PBS, slides were incubated with normal goat serum IgG (1:100) for 30 minutes to prevent non-specific binding. Then, they were incubated with the monoclonal mouse anti-human β-catenin antibody (Clone 14,

Transduction Laboratories, Lexington, KY) at a concentration of 2.5μg/ml (1:500) and at room temperature for 4 hours. The Strept ABComplex/HRP Duet Kit (DAKO, Denmark) was used according to the manufactures' instructions and the results were visualized with DAB. After rinsing in distilled water, sections were counterstained with hematoxylin, dehydrated and overslipped. A colorectal neoplasm with a high expression of membrane β-catenin immunoreactivity was used as a positive control, while negative control slides were obtained by using PBS buffer instead of the primary antibody. Results were quantified by two independent observers.

Expression of β-catenin was graded to five different categories on the membrane and in the nucleus (as shown in Table 1), relative to the staining intensity of normal squamous epithelium present on the same slide.

Detection of β-catenin mutation by Non-radioactive Single Strand Conformation Polymorphism (SSCP)

After deparaffinization in xylene of sections from paraffin-embedded esophageal adenocarcinoma, tumor tissue was carefully removed by microdissection. DNA was prepared by digestion with proteinase K, extraction with phenol and phenol/chloroform, and precipitation with ethanol. Exon 3 of the β-catenin gene was amplified by PCR using the following primers: catenin-3A: TAGTCACTGGCAGCAACAGTCTT, and catenin-3B: AAAATCCCTGTTCCCACTCATAC. The PCR reaction was carried out for 40 cycles using the following amplification profile: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 73°C for 75 sec. Correct amplification was controlled by electrophoresis on a 2% agarose gel. The size of the PCR product was 146 bp. Five microliters of PCR product was denatured in 10 μl of 50 mM NaOH and 1 mM EDTA at 50°C for 10 min. After the addition of 1.5 μl of formamide dye, the samples were immediately analyzed in a 30% Mutation Detection Enhancement acrylamide gel (AT Biochem, Malvern, PA) in 0.5X TBE, with a constant voltage of 20 V/cm for 5 h at 20°C. After electrophoresis, gels were released from plates, stained for 20 min in the dark with a SYBR Gold gel stain (Molecular Probes, Eugene, OR, USA), diluted 1:10'000 in TBE buffer (90 mM Tris-HCl, 2 mM EDTA, 80 mM boric acid), and visualized with UV light using a CCD camera.

RESULTS

β-Catenin Expression in normal esophageal epithelium and metaplastic lesions

All histologically normal esophageal epithelia showed uniform membrane staining for β-catenin (**Image 1A**). In squamous epithelium the immunoreactivity of the membrane increased from luminal border to the basal layers. In metaplastic mucosa, cells of the crypts and glands strongly expressed β-catenin homogeneously on cell-cell boundaries. No nuclear accumulation was found in normal squamous epithelium cells. β-catenin expression in Barrett's esophagus (metaplasia) without dysplasia showed staining patterns similar to those in the normal squamous epithelia. In only one case (5%) a reduced expression on the membrane was noticed. No nuclear staining was found in metaplastic lesions.

β-Catenin expression in dysplastic lesions

Focal nuclear staining for β-catenin was observed in areas of low-grade dysplasia and high-grade dysplasia with a relatively high frequency, respectively 53% and 42% of cases (**Table 2**). Furthermore, in the section, a histological transition from metaplasia to low-grade dysplasia was noted sometimes in the same gland. Nuclear accumulation of β-catenin was only found in the low-grade dysplasia zones (**Image 2**).

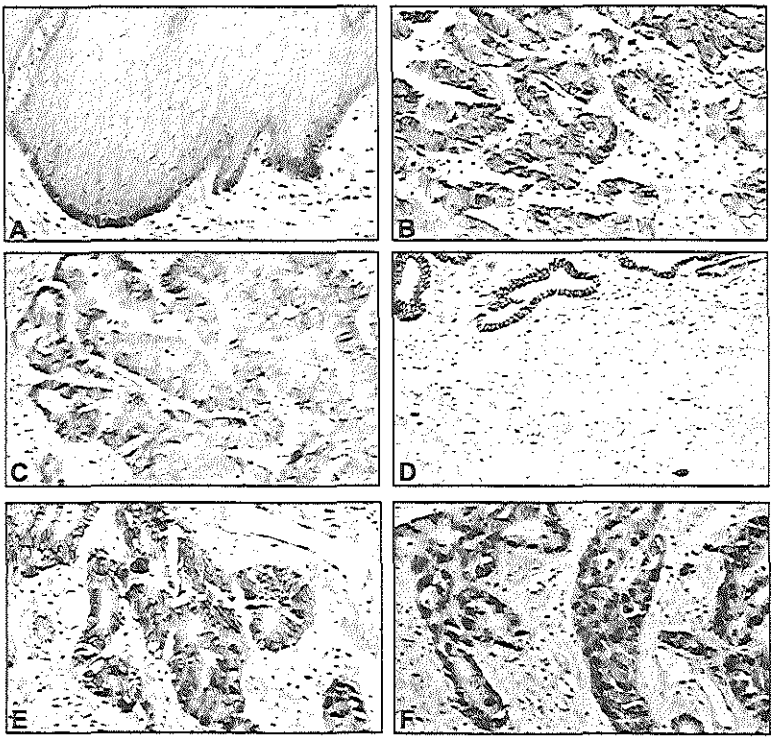

Image 1. β-catenin expression in normal esophageal squamous epithelium and Barrett's esophageal adenocarcinoma (A-F). In normal esophageal squamous epithelium, β-catenin were expressed at the boundaries (A). In adenocarcinoma, β-catenin expression showed different patterns: No change in expression compared with the expression in normal esophageal epithelium (B). Reduced expression on the membrane (C). No staining (D). Nuclear accumulation with no change expression on the membrane (E). Nuclear accumulation with reduced expression on the membrane at the deepest part (F).

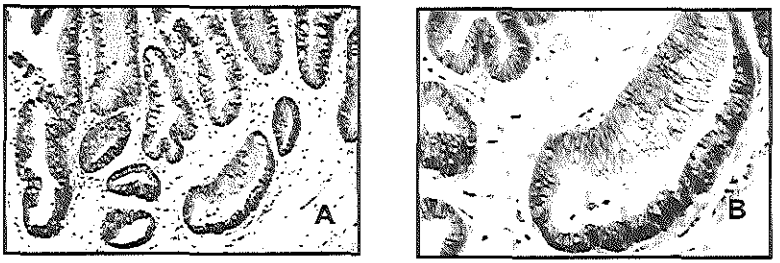

Image 2. β-catenin expression in metaplasia and low grade dysplasia. In one gland with transition from metaplasia to low-grade dysplasia, nuclear accumulation of β-catenin is only seen in the dysplastic cells (A & B).

Table 2. Nuclear expression of β-catenin in Barrett's esophageal adenocarcinoma and premalignant lesions

nuclear expression	Metaplasia	LG Dysplasia	HG Dysplasia	ADC
No	19	9 (47%)	11 (58%)	11 (37%)
Yes	0	10 (53%)	8 (42%)	19 (63%)
Total	19	19	19	30

β-Catenin expression and mutational analysis in adenocarcinoma

In adenocarcinomas β-catenin showed different patterns of expression (**Image 1B-F** and **Table 1**). In 7% (2/30) of cases a pattern similar to normal esophageal mucosa was observed. In 30% (9/30) of tumors a reduced expression on the membrane, without accumulation in the nuclear was noted. Among them, complete absence of staining on tumor cells was observed in 4 cases. Reduction of β-catenin expression on the membrane was almost uniformly distributed in the analyzed lesions. Focal nuclear staining for β-catenin was present in 19 (63%) cases together with normal (4 cases) or reduced (15 cases) expression on the membrane. Nuclear accumulation of β-catenin was mainly found in the deepest invasive part or in scattered cells. Tumor cells in the center of the tumor mass often retained membranous staining without nuclear accumulation. For cases without

an invasive part on the slide, either no or only few tumor cells with nuclear accumulation of β-catenin were noted.

To assess the prevalence of β-Catenin mutations in esophageal adenocarcinomas we carried out non-radioactive SSCP analysis of PCR products using primers within exon 3 of β-catenin. Of the same 30 adenocarcinomas analyzed for expression of β-catenin by Immunohistochemistry, none revealed a β-catenin mutation (**Image 3**).

Table 1. Expression of β-catenin in Barrett's esophageal adenocarcinoma and premalignant lesions

β-catenin expression*	Metaplasia	LG Dysplasia	HG Dysplasia	Adenocarcinoma
M++ / N-	18	7	2	2
M+ / N-	1	2	6	5
M- / N-	0	0	3	4
M++ / N+	0	9	4	4
M+ / N+	0	1	4	15
Total	19	19	19	30

** M++ / N-: no change in expression compared with normal esophageal epithelium (**Image 1B**) ; M+ / N-: reduced expression on the membrane(**Image 1C**); M- / N-: no staining (**Image 1D**); M++ / N+: nuclear positive with no change expression on the membrane(**Image 1E**); M+ / N+: nuclear positive with reduced expression on the membrane(**Image 1F**). M: membrane; N: nuclei*

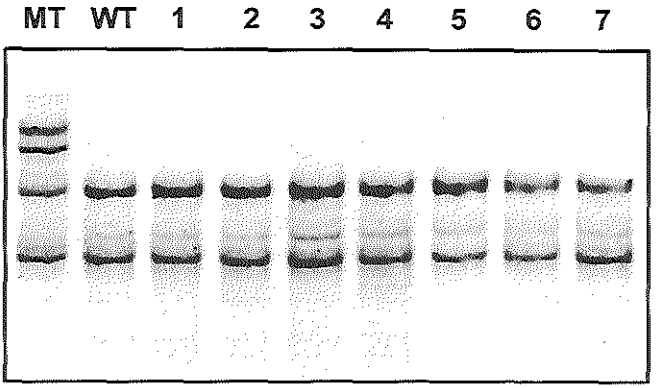

Image 3. *SSCP analysis of the PCR amplification of β-catenin exon 3 in seven Barrett's adenocarcinomas. Lane MT shows a typical alteration observed in β-catenin gene (colon cancer cell line HCT116 with a 3-base deletion). Lane WT shows the wild-type pattern (colon cancer cell line SW480).*

β-Catenin expression during neoplastic progression of Barrett's esophagus

Nuclear accumulation of β-catenin was significantly associated with transition from metaplasia to low-grade dysplasia ($p<0.0001$). The prevalence of reduced or absence expression of β-catenin on the membrane, with or without nuclear staining, increased significantly ($p<0.0001$) from low-grade dysplasia to high-grade dysplasia with an occurrence of 5% (1/19) for metaplasia, 16% (3/19) for LG dysplasia, 68% (13/19) for HG dysplasia and 80% (24/30) for adenocarcinoma.

DISCUSSION

β-catenin is known to act as a key regulator in the cadherin mediated cell adhesion system.[32] Alteration of β-catenin can induce disorders in this system, and is associated with tumor invasion and metastasis in many cancers, including oesophageal carcinoma.[21-24] In addition to simply supporting cell-cell adhesion, a role for β-catenin in the Wnt signal transduction pathway has been discovered.[15] More detailed investigation of β-catenin in this pathway might provide valuable insights into the mechanisms of cellular transformation and tumour progression in Barrett's esophagus. In the present study, we characterized expression of β-catenin, focusing on membrane reduction and nuclear accumulation, during neoplastic progression in Barrett's esophagus using immunohistochemical analysis. Increased expression of β-catenin in the cytoplasm was not considered in this study. Indeed, in our series, interpretation of cytoplasmic staining by different investigators gave controversial results. All normal squamous epithelia showed uniform membranous staining for β-catenin, and no nuclear staining was detected. The prevalence of decreased or absence expression of β-catenin on the membrane, with or without nuclear staining, increased significantly ($p<0.0001$) from LG 16% (3/19) to HG dysplasia 68% (13/19). The reduction of β-catenin expression on the membrane is important for cell adhesion, invasion and metastasis, but its mechanism remains only partly resolved. Focal nuclear staining for β-catenin was observed in 19 (63%) cases of adenocarcinoma and this was statistically significantly associated with progression from metaplasia to LG dysplasia ($p<0.0001$). Our results show that nuclear accumulation of β-catenin is frequent and occurs relatively early during the neoplastic progression in Barrett's esophageal adenocarcinoma. No nuclear staining for β-catenin was found in metaplastic lesions and this was confirmed by 2 cases, unambiguously showing transition from metaplasia to LG dysplasia in the same gland. Indeed, in these glands, nuclear accumulation of β-catenin was found only in the LG dysplastic areas but never in the regions of metaplasia. These findings are similar to those reported by other groups, where nuclear staining for β-catenin was observed in adenocarcinomas.[23,33] Nevertheless, some contradictory results were obtained concerning dysplasia. Indeed, presence of β-catenin in

nucleus was found to be either restricted to high grade dysplastic cells in one study [33] or even a rare event for the other study.[23]

In adenocarcinomas, nuclear accumulation of β-catenin was found mainly in the deepest part or in scattered cells, as observed in colorectal cancers.[26] For those cases without invasive part on the slide either no or only few tumor cells with nuclear accumulation of β-catenin were noted. Therefore, we guess that there should be some more cases with nuclear accumulation of β-catenin in Barrett's esophageal adenocarcinoma than we found here. To strengthen this hypothesis, non concordant nuclear staining for β-catenin expression has been observed in three cases in which different parts of the adenocarcinoma were analyzed. In order to know if this observation is mainly due to intratumoural genetic heterogeneity, p53 mutation analysis was performed. Our results indicate that, although different parts of a tumor showed heterogeneous nuclear expression of β-catenin, they all harbored the same p53 mutation (data not show). However, lack mutational heterogeneity for p53 does not imply that other genetic alterations are homogeneous for these tumors. Nevertheless, micro-environmental changes may be the most likely explanation for the variation in β-catenin nuclear expression, but the possibility of genetic influences cannot be excluded.

It is tempting to speculate that the nuclear expression of β-catenin is the result of disorders in the APC/β-catenin/Tcf signal pathway.[14,16,17] Mutations of APC gene have been found in 60% to 80% of colorectal cancers and they occur relatively early during colorectal tumorigenesis.[34] Mutations or deletions of the β-catenin gene have been found in 10% of colorectal cancers without APC gene mutations.[35,36] β-catenin accumulation in the cytoplasm and nuclei has been found associated with APC mutation both in colorectal and desmoid tumors.[25,37] However, APC gene mutations were found to be uncommon in esophageal cancers,[27,28] suggesting that the nuclear accumulation of β-catenin in Barrett's adenocarcinomas could be a result of β-catenin mutations. To answer this question, using PCR-SSCP analysis, we studied exon 3 of the β-catenin gene, a frequent site for mutation. No mutations in exon 3 of β-catenin gene were detected in our 30 adenocarcinomas. It has been recently established from a series of genetic, cellular, and biochemical investigations that the frizzle (Fz) gene family of seven transmembrane proteins serves as receptor for

Wnt signaling.[38,39] Recently a novel member of the human Fz gene family has been cloned from human esophageal carcinoma. Expression of the FzE3 cDNA in esophageal carcinoma cells appeared to stimulate complex formation between APC and β-catenin, and was followed by nuclear translocation of β-catenin.[40] Therefore, in Barrett's adenocarcinomas mechanisms other than APC or β-catenin mutations might explain the disruption of the APC/β-catenin pathway. These will include Fz gene transcription, deletion of exon 3 of the β-catenin gene and GSK3β or Axin gene mutations.

In summary, the results of our study have shown that abnormal expression of β-catenin is common during neoplastic progression in Barrett's esophagus and is strongly associated with development of neoplasia. Nuclear accumulation of β-catenin is also common and occurs relatively early, already in LG dysplasia. Disruption of the APC/β-catenin/Tcf signal pathway appears not to be a result of mutations in exon 3 of the β-catenin gene. Further studies will be necessary to know why the APC/β-catenin/Tcf signal pathway is disrupted and what is the role of these disturbances in carcinogenesis in Barrett's esophagus.

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CHAPTER VI

p16 Inactivation by methylation of the CDKN2A promoter occurs early during neoplastic progression in Barrett's esophagus.

Yan-Song Bian, Maria-Chiara Osterheld, Charlotte Fontolliet, Fred T. Bosman and Jean Benhattar

Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

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ABSTRACT

Background & Aims: The potential role of p16 inactivation by *CDKN2A/p16* promoter hypermethylation and/or loss of heterozygosity (LOH) of the *CDKN2A* gene were investigated in neoplastic progression of Barrett's esophagus. *Methods*: *CDKN2A* promoter hypermethylation was studied by methylation sensitive single-strand conformation analysis (MS-SSCA) and sequencing using bisulfite modified DNA in Barrett's esophageal adenocarcinomas, premalignant lesions, as well as normal squamous esophageal epithelium. All the lesions of interest were sampled by microdissection from paraffin-embedded fixed tissue sections. *Result*: No methylation of the *CDKN2A* promoter was found in normal esophageal squamous cell epithelia while methylation was detected in 18 of 22 (82%) adenocarcinomas and 10 of 33 (30%) premalignant lesions, including 4 of 12 (33%) samples with intestinal metaplasia only. LOH at the *CDKN2A* gene locus was found in 68% of adenocarcinomas and 55% of premalignant lesions. Of 28 samples without p16 immunoreactivity, 25 (89%) showed *CDKN2A* promoter hypermethylation with or without LOH of *CDKN2A*. Only 2 (8%) samples expressing p16 protein were found to be methylated; these showed a mixture of completely methylated and unmethylated *CDKN2A* promoter. In 7 of 19 (37%) informative samples without LOH of *CDKN2A*, the *CDKN2A* promoter was found to be methylated at both alleles. Loss of p16 protein expression was strongly associated with *CDKN2A* promoter hypermethylation ($P < 0.00001$) but not with LOH ($P = 0.33$). *Conclusions*: Our results indicate that methylation of the *CDKN2A* promoter is the predominant mechanism for p16 inactivation. This hypermethylation is a very common event in esophageal adenocarcinoma and occurs as early as metaplasia.

INTRODUCTION

Barrett's esophagus is an acquired condition where, in the distal esophagus, the normal squamous epithelium is replaced by metaplastic columnar epithelium.[1,2] The molecular mechanisms involved in the development of Barrett's esophagus and its subsequent progression to dysplasia and carcinoma have been extensively studied but are still not completely understood.[3-5] Better understanding of the molecular alterations during this process might allow improved tumor control and prevention and also lead to better management of this disease.

Inactivation of *CDKN2A/p16* tumor suppresser gene is one of the most frequent genetic abnormalities in human neoplasia.[6] The *CDKN2A* gene, located on 9p21, encodes a cell cycle regulatory protein which inhibits cyclin-dependent kinases 4 and 6, preventing the phosphorylation of pRb protein and the release of transcription factor E2F. This blocks cell cycle progression from G1 to S phase.[7-9] Alterations of the *CDKN2A* gene lead to its inactivation, resulting in deregulation of cell proliferation and the occurrence of genomic instability.[10] In various tumor types, the *CDKN2A* gene has been shown to be inactivated by different mechanisms. Homozygous deletions of the *CDKN2A* gene have been reported to be a common mechanism of inactivation, both in tumor cell lines and primary malignancies,[11] A high frequency of point mutations in combination with LOH of 9p21 was detected in squamous cell carcinoma of the esophagus and this combination has been presumed as predominant mechanism for p16 inactivation.[12] In Barrett's esophagus, 9p21 LOH was found to be a relatively frequent abnormality and associated with early lesions in the progression from Barrett's esophagus to adenocarcinoma. However, inactivation of the *CDKN2A* gene by mutation of the remaining allele was reported in only 23% of the patients and homozygous deletions of *CDKN2A* were not detected in esophageal adenocarcinomas.[13] Recently *CDKN2A* promoter was found to be frequently hypermethylated in esophageal adenocarcinoma.[14,15] Promoter hypermethylation, which goes along with transcriptional silencing of this tumor supressor gene, has been observed in many human cancers.[16] However, the combination of 9p loss and *CDKN2A* gene promoter hypermethylation has not been comprehensively studied. Consequently, the question as to whether loss of p16 protein expression is caused by *CDKN2A* promoter hypermethylation or by LOH of 9p21 remains to be clarified.

Likewise, when p16 inactivation occurs during neoplastic progression of Barrett's esophagus and whether or not promoter hypermethylation alone is sufficient for *CDKN2A* gene inactivation are still open questions.

In order to correlate 9p21 allelic loss or methylation of the *CDKN2A* promoter with specific lesions, microdissection of morphologically defined tissue sections is the only reliable approach. Accurate grading of premalignant lesions is difficult in frozen section due to freezing artifact. Thus, only microdissection of paraffin-embedded tissue can ensure purity of the lesion to be studied and guarantees the specificity of the molecular events encountered. Although other methods for methylation analysis exist[17-20] for reasons of sensitivity and specificity we prefer a new method of screening for DNA methylation changes, the methylation sensitive single-strand conformation analysis (MS-SSCA).[21-23] The combination of bisulfite modification and PCR results in the conversion of unmethylated cytosines to thymines whereas methylated cytosines remain unchanged. This sequence conversion induces methylation-dependent alterations of single strand conformation, which can be detected by SSCA. We have previously demonstrated that MS-SSCA can be performed on as little as 1 ng of genomic DNA extracted from microdissected paraffin-embedded formalin-fixed tissue sections.[23] This method not only allows semi-quantitative analysis of the methylation status but also establishes the clonal nature of the methylation pattern.

In this study, we analyzed *CDKN2A* promoter hypermethylation, LOH at *CDKN2A* gene locus as well as expression of p16 protein by MS-SSCA, LOH analysis and immunohistochemistry (IHC) in order to investigate if and how p16 is inactivated during neoplastic progression of Barrett's esophagus and with which histological lesions p16 inactivation is associated.

PATIENTS AND METHODS

Tissue Samples and Cell lines

Twenty two esophagectomy specimens with an adenocarcinoma in Barrett's esophagus, occurring between 1986-1996, were selected from our files. Samples had been formalin-fixed and paraffin-embedded. Concurrent premalignant lesions were found in 12 cases. All available material, which included histological mapping of the lesions as described for gastric carcinomas[24] was re-examined by two pathologists (MCO and CF). Samples were taken from histologically distinct lesions, including intestinal metaplasia (12 samples), low-grade dysplasia (12 samples), high-grade dysplasia (11 samples) and adenocarcinoma (22samples). In addition, 10 samples of normal squamous esophageal epithelium were analyzed. Human placenta and a cultured colon cancer cell line SW620 were used as control, for the methylation analysis.

Hematoxylin and eosin stained slides were histologically screened to identify the different lesions. Barrett's esophagus was diagnosed when metaplastic epithelium (columnar intestinal epithelium with goblet cells) was present. Dysplasia was graded into low grade and high grade, based upon the degree of cytonuclear and architectural atypia.[25]

Microdissection and DNA extraction

After deparaffinization in xylene and rinsing in methanol, tissue sections were stained with 0.01% toluidine blue, selected areas were microdissected and DNA was extracted as previously described.[26] Only the lesion of interest was retained and all other areas, as well as stromal cells, were eliminated. By this procedure, microdissected areas were determined to contain at least 95% of a defined epithelial cell type. DNA was prepared by digestion of the tissue samples with proteinase K, treatment with chelex, extraction with phenol/chloroform, and precipitation with ethanol. Extracted DNA was resuspended in 20 µl of 10 mM Tris-HCl pH 8.0 solution and kept at -20°C until use. Genomic DNA from frozen tissues and cell lines was extracted using Qiagen DNeasy™ Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions.

LOH at *CDKN2A* gene locus

Two polymorphic markers hMp16α-I1 and D9S942, which localize to 9p21 at intron 1 and exon 1 (between 1α and 1β) of the *CDKN2A* gene were used to screen for allelic loss.[27] Genomic DNA from normal and microdissected different lesions was analyzed. The used primers were: hMp16α-I1 sense: 5'-CAGGCAGAGAGCACTGTGAG-3', antisense: 5'-CACATTCTGCGCTTGGATATAC-3'; D9D942 sense: 5'-AAAGCAAGATTCCAAACAGTAAA-3', antisense: 5'-CATCCTGCGGAAACCATTAT-3'. The PCR reaction was carried out for 40 cycles with 2μl of extracted genomic DNA in a total volume of 20 μl. The following amplification profile was used: 94°C for 30 sec, 54°C for 45 sec and 72°C for 75 sec. Analysis was performed on a 6% denaturing polyacrylamide gel containing 8 M urea. Allellic loss of a marker was considered to be present when the heterozygous microsatellite demonstrated absence or at least 80% decrease in intensity of one band as compared with the normal esophageal mucosa control.

MS-SSCA and DNA sequencing

One μg of genomic DNA in the case of cell lines, or all remaining DNA of microdissected tissues after LOH analysis, was modified in 40 μl water with sodium bisulfite as previously described.[23] In brief, genomic DNA was first heated at 100°C for 10 min and then denatured for 10 min at 37° by addition of 2.7 μl of freshly prepared 3N NaOH. After addition of 28 μl of 10 mM hydroquinone and 500 μl of 4.8M sodium bisulfite, the mixture was incubated at 55°C for 4 hr. The modified DNA was purified by passing it through a column from the Qiagen DNeasy Tissue Kit. After washing with the wash buffer contained in the kit, DNA modification was completed on the column by addition of 500 μl of 0.2 M NaOH/90% EtOH. The reaction was performed for 10 min at room temperature, washing was done twice, and DNA was eluted into 50 μl of a 10 mM Tris-HCl solution and stored at -20°C.

A 194 bp fragment of the *CDKN2A* gene promoter (-280 to -86 from the ATG transcription start codon, Genebank accession number X94154), containing 11 CG dinucleotides, was amplified by PCR using the following primers specific to the upper modified strand: 5'-GGGGGAGATTTAATTTGG-3' (sense) and 5'-CAACCCCTCCTCTTTCTT-3' (anti-sense). After pre-incubation for 5 min at 95°C,

the PCR reaction was carried out in the presence of 5% DMSO, for 35 (with DNA from cell lines) or 40 (with DNA from fixed tissues) cycles using the following amplification profile: 94°C for 30 sec, 54°C for 45 sec and 72°C for 75 sec. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. Single strand conformation analysis was performed with 5 µl of PCR product as previously described.[23]

After separation of the PCR product on 6% nondenatured polyacrylamide gel for 90 min, the band of the correct size was excised and reamplified using the same primer. The PCR-amplified DNAs were purified using QIAquick PCR purification Kit (Qiagen, Germany) and the purified product was sequenced on an ABI PRISM 310 automatic sequencer.

Immunohistochemistry

Paraffin sections of 4 µm were cut and mounted on coated slides. Immunohistochemistry was performed using an avidin-biotin-peroxidase complex (ABC) method.[28] In brief, slides were dewaxed and rehydrated in a xylene-ethanol series, endogenous peroxidases were blocked by incubation in 0.3% hydrogen peroxide solution (20 min). Antigen retrieval was performed in a microwave (750W) for 20 minutes in sodium citrate buffer (0.01M, pH 6.0). After three washes in PBS, slides were incubated with normal goat IgG (1:100) for 30 minutes to prevent non-specific binding. Then, they were incubated overnight at 4°C with the monoclonal IgG1 mouse anti-human p16 antibody (Clone G175-405, PharMingen, San Diego, CA, USA) at a concentration of 1.0 µg/ml (1:500). The Strept ABComplex/HRP Duet Kit (Dako, Denmark) was used according to the manufacturer's instructions and the results were visualized with diaminobenzidine. After rinsing in distilled water, sections were counterstained with hematoxylin, dehydrated and coverslipped. An esophageal squamous cell carcinoma case overexpressing p16 was always included as a positive control while staining the seem section with normal mouse serum at a 1:1000 dilution instead of the primary antibody was used as a negative control. Results were quantified by two independent observers (CF and MCO). Only nuclear staining was regarded as positive. Inflammatory cells and reactive stromal cells were used as positive internal controls.[29,30] Two staining patterns were distinguished: 1) Positive : when at least 10%

of nuclei were positive, in clusters or scattered throughout the tissue, 2) negative : when no or only few cells (<10%) showed nuclear staining.

CDKN2A gene mutation analysis

PCR amplification for exon 2 of the *CDKN2A* gene was performed using two primer pairs: part A: 5'-ACCCTGGCTCTGACCATTCTGT-3' (sense) and 5'-GGCATCGCGCACGTCCAGC-3' (anti-sense); part B: 5'-GGGCTTCCTGGACACGCTGGT-3' (sense) and 5'-GGAAGCTCTCAGGGTACAAATTCTCA-3' (anti-sense). The PCR reaction was carried out in the presence of 5% DMSO, for 40 cycles using the following amplification profile: 94°C for 30 sec, 58°C for 45 sec and 72°C for 75 sec. After separation of the PCR product on 30% MDE gel (AT Biochem, Malvern, PA) in 0.5X TBE (45 mM Tris-HCl, 1 mM EDTA, 40 mM boric acid), with a constant voltage of 20 V/cm for 5 h at 20°C, the mutational band was excised and reamplified using the same primer. The PCR-amplified DNAs were purified using QIAquick PCR purification Kit and the purified product was sequenced on an ABI PRISM 310 automatic sequencer.

Statistical analysis

Statistical significance of differences in levels of methylation was established using the Fisher exact test.

RESULTS

CDKN2A gene promoter hypermethylation in Barrett's esophageal adenocarcinoma and premalignant lesions

Methylation analysis of the *CDKN2A* promoter region by MS-SSCA showed two patterns of band mobility, one corresponding to unmethylated and the other to completely methylated CpG sites (Fig 1A). Some cases showed a mixture of both patterns (Fig 1A, lane M). The *CDKN2A* promoter methylation status was obtained in all 22 adenocarcinoma samples and in 33 of 35 samples from premalignant lesions. Extracted DNA of poor quality did not allow evaluation of only two samples.

In the 10 normal squamous cell esophageal epithelia, the *CDKN2A* promoter was not methylated. Of the carcinomas 16 showed complete methylation, 4 showed no methylation and 2 showed a mixture of methylated and unmethylated *CDKN2A* promoter (Table 1). Of the 33 premalignant lesions 10 showed methylation, including 3 of 10 HG dysplasia (30%), 3 of 11 LG dysplasia (27%) and 4 of 12 intestinal metaplasia (33%). The difference in frequency of methylation between premalignant lesions than in adenocarcinomas was significant, 30 and 82% respectively ($P < 0.0002$; Table 2). Direct sequencing confirmed that in samples showing complete methylation by MS-SSCA all the CpG were indeed fully methylated at cytosine residues, whereas in unmethylated samples all cytosines had been converted to thymines. In the cases of a mixture of the two patterns, sequencing showed single CpG sites to contain both cytosine and thymine; in addition, the cytosine/thymidine ratio seemed to be identical for all the 11 CpG sites analyzed. Partially methylated alleles (which means that only some of the 11 CpG sites within the analyzed *CDKN2A* promoter region are methylated) were not observed but these are difficult to detect by MS-SSCA unless they represent a quantitatively important subclone, which appeared not to be the case in our material.

Methylation status was also analyzed in 12 carcinoma cases with concurrent premalignant lesions. In 5 cases with *CDKN2A* promoter methylation in the carcinoma, methylation was also detected in dysplastic mucosa and in three cases also in metaplasia (e.g. case 6, Fig. 1A). In 3 cases methylation was only found in the carcinoma (Table 1, cases 7, 11 and 13), while in 2 cases methylation was found in dysplastic or metaplastic mucosa but not in the concurrent carcinoma (Table 1, cases

16 and 22). In 2 cases methylation was observed neither in the tumor nor in the analyzed metaplastic or dysplastic lesions (Table 1, cases 5 and 18). In most tumor samples (16/18, 89%), *CDKN2A* promoter methylation was complete. A mixture of unmethylated and methylated *CDKN2A* promoter was found more commonly in premalignant lesions (5/10, 50%).

LOH at *CDKN2A* gene locus in Barrett's esophageal adenocarcinoma and premalignant lesions

For LOH of *CDKN2A*, 3 of 22 cases (9 samples) were not informative. In the 19 informative cases, 29 of 48 samples (60%) showed LOH for at least one marker. LOH was found in 5 of 10 (50%) intestinal metaplasia samples, 4 of 10 (40%) with LG dysplasia, 7 of 9 (78%) with HG dysplasia and 13 of 19 (68%) with adenocarcinoma (e.g. case 6, Fig. 1B).

Among the 29 samples with LOH at the *CDKN2A* locus, *CDKN2A* promoter hypermethylation of the remaining allele was found in 14 (48%), most were adenocarcinomas (11/14, 79%). Also in 2 of 7 samples with HG dysplasia and 1 of 5 with intestinal metaplasia, *CDKN2A* promoter hypermethylation together with LOH of *CDKN2A* was observed. In 9 of the 19 (47%) samples without LOH of the *CDKN2A* locus, methylation was detected, and MS-SSCA revealed that in 7 of them, only the complete methylation pattern was present, which could rule out that biallelic methylation was present in these samples. Thus, methylation of *CDKN2A* was not always associated with allelic loss of the *CDKN2A gene locus*.

We found that most of the metaplastic lesions (8/12, 67%) had *CDKN2A* abnormalities (methylation and/or LOH at *CDKN2A* gene locus), suggesting that these develop early during the development of Barrett's esophageal adenocarcinoma.

Table 1. Genetic analysis versus IHC staining for p16 in Barrett's esophagus

	Tumor			HG dysplasia			LG dysplasia			Metaplasia		
Case	Met	LOH	IHC	Met	LOH	IHC	Met	LOH	IHC	Met	LOH	IHC
1	+++	LOH	-									
2	+++	LOH	-	-	LOH	+	+++	-	-	-	-	UI
3	+++	LOH	-									
4	+++	-	-	++	-	-	-	-	+	-	-	+
5	-	LOH	+	-	LOH	+	-	LOH	+	-	LOH	+
6	+++	LOH	-	++	LOH	-	-	LOH	-	++	LOH	-
7	++	LOH	-	UI	LOH	+	-	LOH	+	-	LOH	+
8	+++	-	-									
9	+++	-	-									
10	+++	LOH	-									
11	++	LOH	-	NA	NA	NA	-	-	+	-	LOH	+
12	+++	NI	-	-	NI	+	+	NI	+	+++	NI	-
13	+++	LOH	-	-	LOH	+	-	LOH	+	-	LOH	-
14	+++	LOH	-									
15	+++	-	-									
16	-	NI	-	-	NI	+	++	NI	+	-	NI	+
17	+++	LOH	-									
18	-	LOH	+	-	LOH	+	-	-	+	-	-	+
19	+++	LOH	-									
20	+++	-	-	+++	LOH	-	-	-	+	++	-	-
21	+++	NI	-									
22	-	-	+	-	-	+	UI	-	+	+++	-	UI

NA : none available
NI : none informative
UI : uninterpretable
Met (*p16* promoter methylation) +++ : CpG sites completely methylated in all cells. ++ : CpG sites completely methylated in 50-90% of the cells. + : CpG sites completely methylated in 10-50% of the cells. - : CpG sites unmethylated in all cells.
LOH : Loss of heterozygosity at the *p16* gene locus.

Table 2. *p16* Gene Promoter Hypermethylation, Allelic Loss of *p16* and absence of p16 expression in Barrett's esophagus

Sample	*p16* methylation	LOH of *p16*	Loss of p16 staining
Metaplasia	4/12 (33%)	5/10 (50%)	4/10 (40%)
LG dysplasia	3/11 (27%)	4/10 (40%)	2/12 (17%)
HG dysplasia	3/10 (30%)	7/9 (78%)	3/11 (27%)
Adenocarcinoma	18/22 (82%)	13/19 (68%)	19/22 (86%)
Total	28/55 (51%)	29/48 (60%)	28/55 (51%)

Table 3. Correlation between p16 expression and *p16* promoter methylation and LOH of *p16*

	p16 methylation	LOH of *p16*
p16 expressed	2/25	12/20
p16 not expressed	25/28	17/24
Total	27/53[a]	29/44[b]

[a] $P < 0.00001$ by χ^2 test
[b] $P = 0.33$ by χ^2 test

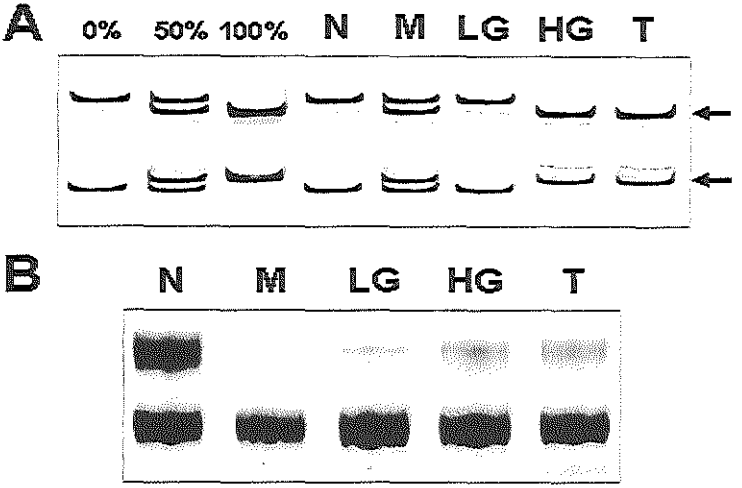

Figure 1. *CDKN2A promoter hypermethylation and LOH of CDKN2A in a case with premalignant lesions and adenocarcinoma in Barrett's esophagus (Table 1, case 6).* ***Panel A:*** *methylation analysis by MS-SSCA. Unmethylated mobilities observed in LG dysplasia (LG) differed from those completely methylated in adenocarcinoma (T) and HG dysplasia (HG). A mixture of methylated and unmethylated pattern was detected in metaplasia (M). No methylation was found in normal tissues (N). DNA from placenta (no methylation of CDKN2A promoter) and a colon cancer-derived cell line SW620 (complete methylation of CDKN2A promoter) were used as a positive control. Arrows indicated the methylated bands.* ***Panel B :*** *The detection of LOH in CDKN2A gene locus (hMp16α-I1) : LOH was noted in all premalignant lesions (M, LG, HG) and adenocarcinoma (T). DNA from normal tissues (N) of the same patient was used as a negative control.*

Expression of p16 protein correlates with *CDKN2A* gene promoter methylation but not with LOH of the *CDKN2A* gene

p16 protein expression was studied by IHC in tissue sections adjacent to those used for microdissection. Interpretable staining was obtained in all 22 adenocarcinoma samples and 33 of the 35 samples with a premalignant lesion. Two cases had to be excluded from the analysis due to technical problems. In all p16 negative cases, strong nuclear staining was observed in reactive stromal fibroblasts and lymphocytes, which were served as internal control (Fig. 2). Absence of p16 expression was found significantly more frequently ($P < 0.0001$) in adenocarcinoma (19 of 22, 86%) than in premalignant lesions (9 of 33, 27%). In premalignant lesions, absence of p16 expression was observed in 3 of 11 (27%) samples of HG dysplasia, 2 of 12 (17%) of LG dysplasia and 4 of 10 (40%) of metaplasia (Table 2).

Of 28 p16 negative samples, 25 (89%) showed *CDKN2A* promoter hypermethylation with or without LOH of *CDKN2A*. Of 25 p16 positive samples, only 2 were found to show hypermethylation with a mixture of completely methylated and unmethylated patterns (Table 1, LG dysplastic lesions, cases 12 and 16). LOH of *CDKN2A* was observed in 17 of 24 (71%) p16 negative samples, and in 12 of 20 (60%) p16 positive samples (Table 3). Loss of p16 protein expression strongly correlated with *CDKN2A* promoter hypermethylation ($P < 0.00001$) but not with LOH at the *CDKN2A* locus ($P = 0.33$).

Three p16 negative cases, without *CDKN2A* promoter hypermethylation, were analyzed for *CDKN2A* mutations. One LG dysplastic (case 6) and one metaplastic sample (case 13) with LOH of *CDKN2A* showed a mutation in exon 2 of the *CDKN2A* gene (CGA→TGA at codon 50 in case 6; CAC→TAC at codon 75 in case 13). In one p16 negative carcinoma without promoter hypermethylation (tumor 16), no *CDKN2A* mutation was identified.

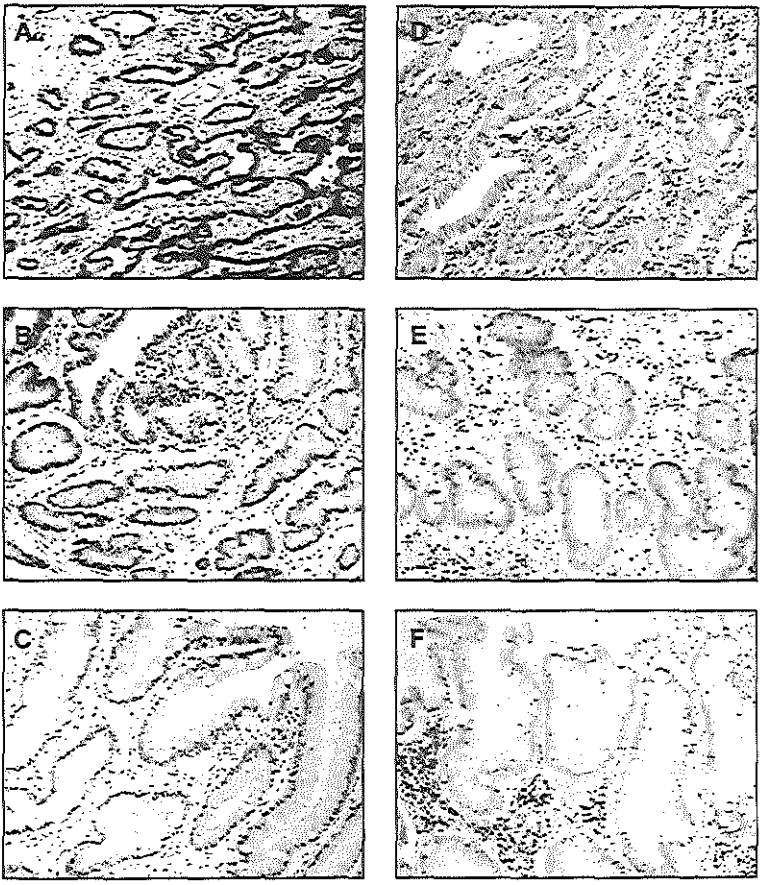

Figure 2. *IHC analysis of p16 expression in premalignant lesions and adenocarcinoma in Barrett's esophagus using G175-405 mAb. Metaplasia (A), dysplasia (B) and adenocarcinoma (C) without CDKN2A promoter hypermethylation showing nuclear immunostaining for p16. Similar lesions (D, E and F) with promoter methylation showing absence of immunostaining for p16, in contrast to background stromal and inflammatory cells which were positive (magnification X100).*

DISCUSSION

p16 is a key regulator at the G1-S checkpoint in the cell cycle and alteration of its function plays an important role in carcinogenesis.[6] Inactivation of the *CDKN2A/p16* gene by different mechanisms has been found frequently in human cancers.[11-14] In esophageal adenocarcinoma, inactivation of the *CDKN2A* gene does not frequently involve point mutation or homozygous deletion.[13] In the present study, a high prevalence of *CDKN2A* promoter hypermethylation was found in esophageal adenocarcinomas (82%) with or without LOH at the *CDKN2A* gene locus and this was associated with absence of immunohistochemical p16 expression. Our observations demonstrate that the *CDKN2A* gene is the tumor suppressor gene most commonly inactivated in Barrett's esophageal adenocarcinoma and that hypermethylation of the *CDKN2A* promoter is the most commonly occurred mechanism of this inactivation.

To study *CDKN2A* promoter methylation, two complementary methods using bisulfite modified genomic DNA, MS-SSCA and sequencing, both employing primers without CpG to avoid selective amplification of either methylated or unmethylated DNA, were used. By MS-SSCA method identical methylated alleles derived from a cell clone can be detected with a lower limit of sensitivity of 5-10% of the cell population.[23,31] In contrast, sequencing reveals the proportion of cytosine and 5-methyl-cytosine for any single CpG in the total DNA population. In order to assure the pathological nature of the samples tested, the different lesions (intestinal metaplasia, grades of dysplasia and adenocarcinoma), as well as normal squamous cell esophageal epithelia, were sampled from formalin-fixed paraffin-embedded tissues by microdissection. Using MS-SSCA only two methylation patterns were detected in this study: complete methylation and non-methylation of the analyzed *CDKN2A* promoter region. Results were confirmed by direct sequencing. A mixture of both methylated and unmethylated *CDKN2A* promoter was also observed, but the percentage of methylated DNA was 50% or greater in all but one of these samples. Carcinoma samples mostly showed only hypermethylated promoter (16/18, 89%), while a mixture of unmethylated and methylated *CDKN2A* promoter was observed commonly in premalignant lesions (5/10, 50%) indicating that methylation of *CDKN2A*

may be present in subpopulations of premalignant cells that will expand during tumor progression.

It has been suggested that DNA methylation may differ among genes, tumor types, patients with the same tumor type, or even individual tumor cells within the same patients.[32] In colorectal cancers, partial methylation of *hMLH1* has been reported,[33] while in malignant melanomas, the CpG sites of *CDKN2A* gene promoter were found either fully methylated or completely unmethylated.[34] Our results suggest that partially methylated alleles do not occur or do not clonally expand in esophageal adenocarcinoma. Nevertheless, due to the relatively low sensitivity of MS-SSCA analysis, it is also possible that we failed to detect small populations of cells with a partially methylated *CDKN2A* allele.

Previous studies have shown that biallelic inactivation of the *CDKN2A* gene is highly prevalent in human cancers.[8,9,11,16] Concurrent loss of 9p21 and *CDKN2A* promoter hypermethylation has been reported in several types of tumors, including esophageal adenocarcinoma.[11,26] In the present study, inactivation of p16 by deletion of one allele and methylation of the second allele was observed in 85% (11/13) of adenocarcinomas, but in only 19% (3/16) of premalignant lesions which displayed an allelic loss of the *CDKN2A* gene locus. We found 7 samples with complete biallelic methylation and without LOH at the *CDKN2A* locus, indicating that gene inactivation can also result from hypermethylation only. Similar results have been reported for NSCLC cell lines, 5 of 7 of which showed monoallelic *CDKN2A* promoter hypermethylation and two showed biallelic hypermethylation.[16]

In our series, loss of immunohistochemical p16 expression was strongly correlated with promoter hypermethylation ($P < 0.00001$) but not with LOH of *CDKN2A* gene locus ($P = 0.33$). The high frequency of promoter hypermethylation and its strong correlation with loss of immunoreactivity suggest that *CDKN2A* promoter hypermethylation might be the most important mechanism for p16 inactivation during neoplastic progression in Barrett's esophagus. IHC analysis appears to be a reliable and practical alternative to a comprehensive genetic analysis of *CDKN2A* status and allows rapid screening of *CDKN2A* abnormalities, especially in small precursor lesions. Of 25 samples with a hypermethylated *CDKN2A* promoter, 2 nonetheless showed p16 IHC staining.

Microdissection and PCR were repeated for these two cases and identical results were obtained. Thus DNA or PCR contamination can be excluded. As the IHC showed that almost all the LG dysplastic cells were positive for p16, a possible explanation in these two samples could be that only one allele of the *CDKN2A* gene was completely methylated. Coexistence of methylated and unmethylated alleles of the *CDKN2A* gene has been reported in the HCT116 colon cancer cell line [35] and has also been demonstrated for some other genes, such as E-cadherin and *hMLH1*.[36,37] Absence of p16 staining without *CDKN2A* hypermethylation was observed in only 3 samples (a carcinoma, a LG dysplasia and a metaplasia). A mutation was detected in the two preneoplastic samples but not in the carcinoma, suggesting that the combination of *CDKN2A* mutation with LOH of the *CDKN2A* locus, though rare, can be an alternative mechanism for inactivation of the *CDKN2A* gene.

LOH of the *CDKN2A* gene locus was observed not only in 71% of the informative samples without p16 protein expression, but also in 60% of those with p16 expression. Although allelic loss of *CDKN2A* occurs frequently and early in Barrett's esophagus, our results suggest that *CDKN2A* might not be the target of this event. Tumor suppressor gene(s) other than *CDKN2A* on 9p21 locus may also play an important role in Barrett's esophageal carcinogenesis. In addition, the apparent occurrence of both DNA methylation and loss of *CDKN2A* locus in adenocarcinoma could not be due to the presence of tumor suppressor genes but rather to structural alterations of chromosomes.[38] This chromosomal instability could be associated with either short telomeres of tumor cell chromosomes, partial inactivation of DNA repair pathways,[39] or demethylation of chromosomal segments,[40] and could play a significant role in allelic losses during tumor progression.

In a diagnostic perspective, it is important to know at which step promoter hypermethylation occurs in the evolution of Barrett's esophagus towards adenocarcinoma. We found hypermethylation of *CDKN2A* promoter in 82% of the adenocarcinomas and in 30% of the premalignant lesions, of which a relatively high percentage concerned metaplasia. These results suggest that this epigenetic event occurs early during neoplastic progression in Barrett's esophagus. Several studies have demonstrated molecular alterations in nondysplastic Barrett's mucosa in cases of adenocarcinoma. Allelic loss, mainly at chromosome 5q (APC locus), 18q (DCC locus) and 3p (FHIT locus) were

clearly demonstrated in Barrett's metaplasia.[41-43] Hypermethylation of promoter region of the *APC*, *CDKN2A*, estrogen receptor and *TIMP3* genes were also observed in metaplasia.[5,44,45] However, in these studies no correlation between the methylation status of a gene and its expression was conducted. For this reason, in the present study, we correlated the expression of p16 with *CDKN2A* promoter methylation using a relatively new approach for methylation analysis (MS-SSCA) on microdissected paraffin-embedded tissues.[23] This microdissection technique ensures enrichment and purity of the lesion to be analyzed, and thus, attests for the true occurrence of *CDKN2A* hypermethylation in Barrett's metaplasia. All the pre-malignant lesions analyzed in this study were obtained from esophagectomy samples from patients with adenocarcinoma. Thus, these lesions could not be representative of Barrett's esophagus in general and may reflect those lesions that are at particular risk of progressing to adenocarcinoma.

In conclusion, we found the *CDKN2A* gene to be inactivated frequently in esophageal adenocarcinomas. Inactivation by promoter hypermethylation may occur already at a very early stage of neoplastic progression in Barrett's esophagus and appears to be the most common mechanism for p16 inactivation in Barrett's esophageal adenocarcinoma.

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CHAPTER VII

Concluding remarks and summary

7.1 Concluding remarks

The majority of adenocarcinomas developed in Barrett's esophagus are diagnosed at an advanced stage, which is associated with poor prognosis; the detection of preneoplastic lesions seems to be imperative to ensure an effective clinical treatment. Endoscopic recognition and histological classification of dysplastic lesions are difficult. Efforts are required to further understand the pathogenesis of columnar metaplasia and its progression to cancer. We studied genetic alterations during neoplastic progression from Barrett's esophagus to adenocarcinoma, using tissues of different lesions microdissected from formalin-fixed, paraffin-embedded sections, specially focused on expression and mutation of p53 and p16 gene transcriptional silencing. A role of disruption of APC/β-catenin/Tcf pathway was also studied.

Molecular analysis of genetic changes has been limited by the diversity of cell types present in specimens. The genomic DNA of non-neoplastic cells may complicate interpretation of the results of molecular analysis, depending on the ratio of neoplastic to non-neoplastic cells and the sensitivity of the assays. To solve this problem, an inverse tissue microdissection technique is described in our study (Bénédicte et al, 2000) which has been developed to allow analysis of invasive lesions as small as 1 mm^2 on formalin-fixed, paraffin-embedded tissue sections. Unwanted cells are completely removed before sampling of target cells, an optimized DNA extraction and subsequent polymerase chain reaction (PCR) complete the procedure. Using this approach, 15 PCR reactions can be performed on each microdissected-area and, therefore, the study of several genetic alterations can be carried out on the same small microdissected-area without contamination by normal cellular DNA.

Hypermethylation of the 5'CpG island in the promoter region is one of the mechanisms for inactivation of tumor suppressor genes in human neoplasia (Laird et al, 1994; Merlo et al, 1995). Detection of DNA methylation has recently attracted considerable attention. Many methods have been established for methylation analysis. However, the demands of a large quantity and good quality of genomic DNA hinder routine analysis of samples, especially from paraffin-embedded fixed tissues. We improved a recently established method of screening for methylation changes named methylation sensitive single-strand conformation analysis (MS-SSCA) (Bianco et al, 1999; Burri et al, 1999). We found that MS-SSCA is rapid, specific, semi-quantitative and works very well with DNA extracted from microdissected fixed tissues. MS-SSCA can detect methylation changes in as little as 1 ng of DNA. This technique is also

sensitive and allows the detection of 10% or even less of fully methylated p16 gene promoter in excess of unmethylated DNA. The advantage of this method is to not only allow the analysis of methylation status within a lesion but, when microdissection is performed, also to give further information about the clonality of this genetic alteration.

p53 inactivation has been shown to contribute to the development of Barrett's esophageal adenocarcinoma. However, despite the increasing amount of information, the precise role of p53 alterations in the natural history of the progression from benign to malignant lesions in Barrett's and the potential clinical significance of these observations remain unclear (Kubba et al, 1999). We analyzed 77 samples from 30 esophagectomy specimens with Barrett's esophagus and adenocarcinoma of patients in longitudinal clinical follow-up and found that p53 mutations occurred mainly during the transition from low-grade to high-grade dysplasia but not in nondysplastic Barrett's mucosa. Mutation analysis of p53 by PCR-SSCP and p53 accumulation by immunohistochemistry was mostly concordant in adenocarcinomas and high-grade dysplastic lesions, but frequently discordant in low-grade dysplastic lesions. No correlation between p53 gene mutation or p53 accumulation and clinicopathological findings was observed in our study.

β-catenin mediates cell-cell adhesions in a complex with transmembrane glycoprotein E-cadherin. It is an oncoprotein which can contributes to carcinogenesis when the APC/β-catenin/Tcf signal transduction pathway is disrupted. (Su et al, 1993; Rubinfeld et al, 1993; Peifer, 1997). More detailed investigation of β-catenin in this pathway might provide valuable insights into the mechanisms of cellular transformation and tumour progression in Barrett's esophagus. In the present study, we found that the prevalence of reduced expression of β-catenin on the membrane, with or without nuclear staining, increased significantly from LG to HG dysplasia. Focal nuclear staining for β-catenin was present commonly in adenocarcinoma, and nuclear staining was significantly associated with progression from metaplasia to LG dysplasia. In addition, in glands with clear histological transition from metaplasia to LG dysplasia, nuclear accumulation of β-catenin was found only in the LG dysplasic areas. No mutation in exon 3 of β-catenin gene was detected in adenocarcinomas. Our results demonstrate that disturbance of the APC/β-catenin pathway, as indicated by

nuclear accumulation of β-catenin, is a common and early event during neoplastic progression in Barrett's esophagus (Bian et al, 2000)

p16 is a key regulator at the G1-S checkpoint in the cell cycle and loss of its function plays an important role in carcinogenesis (Sherr, 1996). Inactivation of p16 gene by different mechanisms has been frequently found in human cancers (Cairns et al, 1995; Igaki et al, 1995; Wong et al, 1997). In esophageal adenocarcinoma, inactivation of p16 gene does not frequently involve point mutation and homozygous deletion (Barrett et al, 1996). To investigate whether p16 promoter hypermethylation is the predominant mechanism for p16 inactivation during the neoplastic progression from Barrett's esophagus to adenocarcinoma, we analyzed p16 promoter hypermethylation by MS-SSCA in 22 Barrett's esophageal adenocarcinomas. In 12 cases, premalignant lesions adjacent to the tumor as well as normal squamous cell esophageal epithelium were also studied. No methylation of the p16 promoter was found in the normal squamous cell esophageal epithelia while methylation was detected in 82% (n=22) of the adenocarcinomas and in 30% (n=33) premalignant lesions, including 33% (n=12) samples with intestinal metaplasia. p16 promoter hypermethylation of the remaining allele was found in 48% (n=29) samples which showed LOH of one p16 allele. In 37% (n=19) samples, the p16 promoter was found to be methylated at both alleles. Immunohistochemical analysis of premalignant lesions and adenocarcinomas showed that absence of p16 protein was strongly associated with p16 promoter hypermethylation ($p<0.0001$) but not with LOH of the p16 locus ($p=0.45$). Our results demonstrate that the p16 gene is the most commonly inactivated tumor suppressor gene detected so far in Barrett's esophageal adenocarcinoma. Inactivation of p16 by promoter hypermethylation could occur at a very early stage in the multistep process of neoplastic progression in Barrett's esophagus, probably from intestinal metaplasia. Absence of p16 expression correlated with methylation but not LOH of p16 gene locus in Barrett's esophageal adenocarcinoma and premalignant lesions. Therefore, methylation of p16 gene promoter is obviously the most common mechanism for p16 inactivation in the pathogenesis of Barrett's esophagus.

Our results indicate that a wide variety of biologic events and mechanisms appear to have roles in the development and progression of Barrett's esophagus adenocarcinoma and associated preneoplastic lesions. Different biomarkers at different stages of this particular carcinogenesis model were observed: p16 gene

promoter hypermethylation and LOH of p16 gene locus occur extremely early, probably from the transition from normal epithelium to metaplasia, and these may contribute to the initiation and/or progression from Barrett's metaplasia to adenocarcinoma. Subsequently, disruption of APC/β-catenin/Tcf signal pathway indicated by nuclear accumulation of β-catenin was significantly associated with progression from metaplasia to LG dysplasia. Loss of cell cycle check points by p53 mutation or p16 promoter hypermethylation may contribute to slow clonal expansion perhaps by increasing proliferation from LG to HG dysplasia. Inhibition of apoptosis, in which p53 mutation may be involved, occurs late apparently only in a select proportion of cells with high-grade dysplasia. Invasive cancer may be preceded by alterations of cell cycle regulation and cell to cell adhesion especially involving p16 gene hypermethylation and abnormalities of cadherin/catenin complexes (Figure 1).

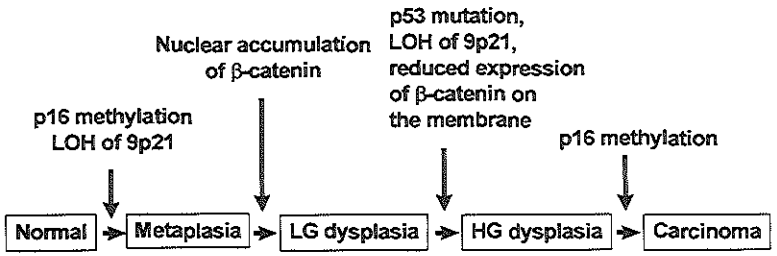

Figure 1. Genetic model of Barrett's esophageal carcinogenesis. *This model is based on the concept of an metaplasia-dysplasia-adenocarcinoma sequence. Different genetic alterations occur at the variant step of the progression.*

The results of our study permit an improvement of the understanding of the role of genetic alterations in the carcinogenesis of Barrett's esophagus. These new information could open the way to a possible application of these biomarkers for prevention, prognosis and treatment of Barrett's adenocarcinomas and preneoplastic lesions. Additional studies are needed to determine the value of each biomark as a independent factor in assessing the malignant potential of Barrett's esophagus.

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7.2 Summary

Genome instability is a hallmark of neoplastic progression in Barrett's esophagus, and the progression to cancer has been shown to involve an accumulation of genetic and cell cycle abnormalities. The interest of this study lies on the multiplicity of molecular alterations during neoplastic progression in Barrett's esophagus examined on the same material. **Chapter 2** described an inverse microdissection technique which has been developed to allow analysis of invasive lesions as small as 1 mm^2 on formalin-fixed, paraffin-embedded histological tissue sections. An optimized DNA extraction and subsequent polymerase chain reaction (PCR) complete the procedure. Using this approach, the study of several genetic alterations can be carried out on the same small microdissected-area without contamination by normal cellular DNA. In **Chapter 3,** methylation sensitive single-strand conformation analysis (MS-SSCA) was applied for methylation analysis of human p16 promoter region, using genomic DNA obtained from either frozen, fixed or microdissected archival paraffin-embedded fixed tissue sections. Our results demonstrated that MS-SSCA is a rapid, specific, and semi-quantitative approach, permits the detection of 10% or less of cells harboring a methylated p16 promoter. In addition, when microdissection was performed, the clonality of this genetic alteration could be identified. In **Chapter 4,** using microdissection, different lesions (intestinal metaplasia, dysplasia and adenocarcinoma) as well as normal squamous cell esophageal epithelia were sampled from formalin-fixed paraffin-embedded tissues. We found p53 mutations occurred mainly during the transition from low-grade to high-grade dysplasia in the neoplastic progression of Barrett's esophagus but not in the nondysplastic Barrett's mucosa. Mutational analysis of p53 by PCR-SSCP and p53 accumulation by immunohistochemistry were mostly concordant in adenocarcinoma and high-grade dysplastic lesions, but frequently discordant in low-grade dysplastic lesions. No correlation between p53 gene mutation or p53 accumulation and clinicopathological findings was observed. We characterized expression and mutation of β-catenin in the progression of Barrett's esophagus to adenocarcinoma in **Chapter 5.** A prevalence of reduced expression of β-catenin on the membrane, with or without nuclear staining, increased significantly from LG to HG dysplasia. Focal nuclear staining for β-catenin was present in 63% cases of adenocarcinoma, and nuclear staining was significantly associated with progression from metaplasia to LG dysplasia. In addition, in glands with clear histological transition from metaplasia to

LG dysplasia, nuclear accumulation of β-catenin was found only in the LG dysplasic areas. No mutation in exon 3 of β-catenin gene was detected in adenocarcinomas. These results demonstrate that disturbance of the APC/β-catenin pathway, as indicated by nuclear accumulation of β-catenin, is a common and early event during neoplastic progression in Barrett's esophagus and this disruption is obviously not the result of β-catenin gene mutation. In experiments of **Chapter 6,** we analyzed p16 promoter hypermethylation by MS-SSCA in Barrett's esophageal adenocarcinomas, premalignant lesions adjacent to the tumor as well as normal squamous cell esophageal epithelium. No methylation was found in the normal squamous cell esophageal epithelia while methylation was detected in 82% adenocarcinoma and 30% premalignant lesions including 33% intestinal metaplasia. p16 promoter hypermethylation of the remaining allele was found in 48% samples who had LOH of p16 locus, most of them were adenocarcinomas (79%). In 37% samples, p16 promoter was found to be methylated at both alleles. Immunohistochemical analysis showed that absent expression of p16 protein was strongly associated with p16 promoter hypermethylation ($p<0.0001$) but not with LOH of p16 locus ($p=0.45$) indicating that p16 inactivation is a very common event in esophageal adenocarcinoma and occurs in the pathogenesis of Barrett's esophagus as early as in intestinal metaplasia. Although allelic loss of 9p21 also occurs frequently and early in Barrett's esophagus, it seems that p16 inactivation is not the target of this genetic event. In contrast, methylation of the p16 promoter is obviously the predominant mechanism for this inactivation.

SAMENVATTING

Instabiliteit van het genoom is een essentiëel kenmerk van tumorcellen en verantwoordelijk voor tumorprogressie, ook bij Barrett's oesofagus. Wij hebben gepoogd inzicht te krijgen in de genetische veranderingen die een rol spelen bij de progressie van Barrett's oesofagus naar adenocarcinoom van de oesofagus. Wij bestudeerden daartoe moleculair genetische veranderingen in de adenocarcinomen in operatiepreparaten, en in de lesies die voorafgaan aan het adenocarcinoom in hetzelfde materiaal.

In hoofdstuk 2 wordt beschreven hoe handmatige microdissectie, bestaande uit het wegschrapen van ongewenste weefselbestanddelen, moleculaire analyse mogelijk maakt van lesies niet groter dan 1 mm^2 in in formaline gefixeerde, in paraffine ingebedde weefselcoupes. DNA extractie en de PCR reactie werden daartoe eveneens geoptimaliseerd. Met deze techniek kunnen kleine, cellulair relatief homogene weefselmonsters worden onderzocht.

In hoofdstuk 3 wordt een onderzoek beschreven naar hypermethylering van de p16 promoter, met behulp van een methylering-gevoelige single stand conformatie analyse (MS-SSCP). Deze techniek maakt methyleringsanalyse mogelijk op in paraffine ingebed microdissectie materiaal. Wij vonden dat MS-SSCA een specifieke en gevoelige techniek is die semikwantitatieve informatie over promotormethylering verschaft, als tenminste 10% van de cellen in het monster promotermethylering vertoont. Het resultaat geeft ook aanwijzingen over het bestaan van cel subclonen met verschillen in methylering.

In hoofdstuk 4 wordt een onderzoek beschreven naar expressie van het p 53 gen en mutatie analyse van het gen in aldus verkregen materiaal, waarbij zowel intestinale metaplasie als verschillende graden van dysplasie en adenocarcinoom werden bestudeerd. P53 mutaties werden vooral gevonden bij de progressie van laaggradige naar hooggradige dysplasie. In niet-dysplastisch Barrett's oesofagus slijmvlies werden geen p53 mutaties gevonden. Een positief resultaat in de mutatie-analyse (uitgevoerd met behulp van SSCP) was concordant met immunohistochemische overexpressie van p53 in hooggradige dysplasie en adenocarcinoom maar discordant in laaggradige dysplasie. Noch p53 mutaties noch p53 overexpressie correleerde met klinisch-pathologische bevindingen.

In hoofdstuk 5 wordt een onderzoek beschreven naar mutaties in en expressie van β-catenine tijdens de progressie van Barrett's oesofagus naar adenocarcnoom. De celmembraan gebonden expressie van β-catenine nam af van laaggradige naar hooggradige dysplasie, terwijl de celkern expressie toenam. Kernexpressie werd ook frekwenter gevonden in laaggradige dysplasie dan in metaplasie zonder dysplasie. In weefsels met haarden van metaplasie en dysplasie werd kernkleuring voor β-catenine alleen gevonden in de metaplastische haarden. Expressie van β-catenine in de celkern werd gevonden in 63% van de adenocarcinomen. In geen van deze gevallen werd een mutatie gevonden in exon 3 van het β-cateninegen. Deze gegevens bevestigen dat in de progressie van Barrett's oesophagus de APC/β-catenine signaaltransductie vroeg is verstoord, hetgeen zich uit in de ophoping van β-catenine in de celkern. Dit is niet het gevolg van β-catenine mutaties.

Deze techniek werd gebruikt in het onderzoek naar p16 promotermethylering in adenocarcinomen in Barrett's oesophagus, premaligne afwijkingen en in normaal oesophagusslijmvlies, zoals beschreven in hoofdstuk 6. Methylering werd nimmer gevonden in normaal slijmvlies. In 82% van de adenocarcinomen en in 30% van de premaligne lesies, waaronder 33% van de gevallen van intestinale metaplasie, werd methylering gevonden. Van de gevallen met allelverlies van p16, bijna allemaal (79%) adenocarcinomen, toonde 48% methylering van het overblijvende p16 allel. In 37% van de onderzochte monsters toonden beide allelen promotermethylering. Immunohistochemisch onderzoek wees uit dat hypermethylering van de promoter correleert met verlies van p16 expressie, hetgeen niet het geval is bij p16 allelverlies.
Deze resultaten wijzen uit dat p16 inactivering veel voorkomt in oesophagus adenocarcinomen, en in de progressie van Barrett's oesophagus naar carcinoom vroeg optreedt. Allelverlies in het 9p21 locus wordt ook freqkwent gevonden maar inactivatie van het p16 gen is niet het (enige) doelwit van deze genetische afwijking. Promoterhypermethylering is het dominante mechanisme voor p16 inactivering.

摘 要

食道腺癌是常见的恶性肿瘤之一，临床预后很差。深入了解该肿瘤发生发展过程中相关癌基因及抑癌基因的作用，对于阐明食道腺癌发病的分子机理，指导临床治疗，提高患者预后具有重要意义。本研究对食道腺癌由癌前病变向恶性肿瘤演进过程中各个阶段相关癌基因及抑癌基因的变化进行了探讨。

第一章 综述肿瘤分子生物学在食道腺癌研究中的进展概况。

第二章 重点描述了显微切割技术在恶性肿瘤及癌前病变分子病理学研究中的重要性。我们建立的新方法与优化后的DNA提取技术及PCR反应相结合，可对取自1mm^{2}石蜡包埋切片的组织进行多基因分析。该技术大大降低了来源于正常及非特异性细胞的DNA污染，提高了研究结果的可靠性。

第三章 应用显微切割技术，我们对食道腺癌恶性演进不同阶段（肠上皮化生，低度异型，高度异型及腺癌）p53基因突变及蛋白表达情况进行了研究。结果显示：该基因突变作为晚期现象，在食道腺癌发生发展过程中具有重要作用。基因突变主要发生在由低度异型向高度异型的演进过程中。在腺癌及高度异型病变中，该基因突变与蛋白表达之间具有良好的对应性；在低度异型阶段，两者对应性较差。在肠上皮化生阶段既无基因突变又无蛋白表达。将p53基因突变及蛋白表达与临床病理学指标进行对比未发现两者之间有任何联系，说明该指标无助于临床预后判断。

第四章 采用免疫组织化学及PCR－SSCP的方法，我们对食道腺癌恶性演进不同阶段（肠上皮化生，低度异型，高度异型及腺癌）β-Catenin蛋白异常表达及该基因exon3突变进行了对比研究。结果发现：β-Catenin 蛋白异常表达普遍存在于食道腺癌的发生发展过程中，其中β-Catenin 在细胞核内的积聚是早期现象，主要发生在肠上皮化生向低度异型的演进过程中。位于细胞膜上的β-Catenin表达减弱或消失主要发生在低度异型向高度异型演进过程中。在食道腺癌中未发现有该基因的突变，提示APC/β-Catenin/Tcf径路的异常并非由β-Catenin 基因突变引起。

第五章 我们建立了一种分析DNA甲基化的新方法（MS－SSCA）。用该方法检测DNA甲基化的敏感度达10％，可用于检测不同来源的DNA（冰冻组织，石蜡包埋组织以及经显微切割后的固定组织）。

第六章 应用MS－SSCA，我们对食道腺癌恶性演进不同阶段（肠上皮化

生，低度异型，高度异型及腺癌〕p16基因启动子区甲基化情况进行了研究，并将p16基因启动子区甲基化，9p21区同源染色体丢失（LOH）与p16蛋白表达进行了比较。研究结果发现：p16基因启动子区甲基化普遍存在于食道腺癌中且发生极早，在正常上皮向肠上皮化生阶段既已出现。 p16基因启动子区甲基化而非9p21区同源染色体丢失（LOH）与p16蛋白表达缺失直接相关。由此推测p16基因启动子区甲基化是p16蛋白失活的主要原因。

CURRICULUM VITAE

August 12, 1967	Born in Qiqihar, Heilongjiang Province, China
1985-1991	M.B. China Medical University, Shenyang, China
1991-1994	Research assistant, Beijing Institute of Otorhinolaryngology, Beijing Tongren Hospital, Beijing, China
1994-1997	M.Sc. Capital University of Medical Sciences, Beijing, China
1997- 2001	M.D./Ph.D. candidate, University Institute of Pathology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

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