Genetic markers in malignant progression of Barrett’s esophagus
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Chapter 1

General introduction and outline of the thesis

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1. Introduction

Barrett’s esophagus (BE) is a well established precursor of esophageal adenocarcinoma (EAC). Although most patients with BE do not progress to EAC, those that do progress may have a poor prognosis. Current management strategies of BE patients include frequent endoscopic surveillance with multiple biopsies. This approach, however, may miss dysplastic lesions or early cancers. Furthermore, given the relatively high prevalence of BE but low incidence of BE that will progress into malignancy, this invasive and expensive approach is not cost-effective. Therefore, there is increasing interest in using biomarkers to identify BE patients with a higher risk for progression towards EAC.

This chapter will provide a general introduction to BE and EAC, describing epidemiology, risk factors and suggested mechanisms involved in the malignant progression of BE. Furthermore, the problems associated with current BE surveillance strategies is explained. Finally, promising prognostic and diagnostic genetic markers that are of potential use in surveillance programs and for clinical management of BE patients as well as techniques for their detection are described.

2. Epidemiology of Barrett’s esophagus and esophageal adenocarcinoma

2.1 Barrett’s esophagus

Barrett’s esophagus (BE) is defined as a metaplastic change in which the normal squamous epithelium of the distal esophagus has been replaced by a specialized columnar epithelium as a result of longstanding gastro-esophageal reflux disease (GERD). Individuals with BE have a 30- to 125-fold higher risk of developing esophageal adenocarcinoma (EAC) compared to the general population. It is estimated that in the general population at least 1 out of 50-100 male patients above the age of 50 has BE. Remarkably, BE is 2 to 3 times more prevalent in males as compared to females, and is mostly diagnosed in Caucasians. Although BE is the predisposing condition for EAC, only 0.5-1% of BE patients will develop EAC. Malignant progression of BE may be a slow and lengthy process characterized by dysplastic lesions before invasive cancer occurs. Based on histopathological features, dysplasia is commonly subclassified into indefinite, low-grade, and high-grade categories.

2.2 Esophageal adenocarcinoma (EAC)

The prevalence of esophageal adenocarcinoma (EAC) is most alarming in Western European countries and the US, representing the fastest and most dramatically
increasing of all malignancies. According to the CBS (Centraal Bureau voor Statistiek: central registry of causes of death) over a 1000 patients die per year from EAC in the Netherlands. This cancer, even after surgical intervention, has a cumulative 2 year survival of less than 20%. EAC only has a good prognosis with long term survival of over 90%, when treated in very early stage, i.e., in case of high grade dysplasia (HGD) or early cancer. Because of this reason, routine periodic endoscopy to detect early malignant changes in patients with BE is important.

3. Key risk factors for BE and EAC

There are many risk factors associated with EAC. These not only include BE and GERD, but also factors that predispose to GERD.

3.1 GERD (Gastro-esophageal reflux disease)
Gastro-esophageal reflux disease (GERD) is a consequence of chronic reflux of stomach contents into the esophagus, and is characterized by erosive esophagitis, strictures and ulcerations of the esophagus. GERD is common in Western populations, with typically 40% of people reporting occasional symptoms of heartburn and regurgitation, and 20% experiencing at least weekly symptoms. A longstanding GERD is a well recognized risk factor for BE. In BE the normal squamous epithelium is replaced by an abnormal metaplastic columnar epithelium, which provides a better resistance to the effects of gastroesophageal reflux. Approximately 10-15% of patients who suffer from GERD will develop BE. A seven - to eightfold increased risk of developing EAC has been reported in individuals with recurrent reflux symptoms (at least once a week) and the risk was greater among individuals with severe and long-lasting symptoms (>20 years duration). The increasing prevalence of GERD in the general population may account for the increasing prevalence of BE and the associated EAC. However, as GERD is relatively common in the general population and only a fraction of individuals with GERD develop BE and EAC, it is likely that molecular and life style factors interact to modulate individual susceptibility to develop BE and progression towards malignancy.

3.2 Obesity, Diet, Tobacco and Alcohol
It has been suggested that obesity is associated with an increased risk for developing EAC. The high body mass index (BMI) could increase the risk of hiatal hernia and provoke reflux through increased intra- abdominal pressure. Indeed, some reports demonstrated that in individuals undergoing endoscopy, obesity was linked to GERD, esophagitis and hiatal hernia. However, obesity
has also been associated with EAC independently of reflux.\textsuperscript{23, 24} Thus, mechanism by which obesity is involved in EAC seems to be more complex then a simple induction of reflux disease.

The link between diet and risk for EAC has been found as well. Studies of fruit, vegetable intake and specific micronutrients are consistent with a protective role of antioxidants against EAC.\textsuperscript{24, 25} Dietary fiber was also associated with a reduced risk of developing EAC.\textsuperscript{25, 26} In contrast, high levels of dietary fat are associated with an increased risk for EAC, possibly through the promotion of GERD.\textsuperscript{27}

Tabacco intake is a reported risk factor for EAC as well.\textsuperscript{28} This could be because smoking may provoke reflux disease.\textsuperscript{21} On the other hand, they are also studies showing no or weak association between smoking and EAC.\textsuperscript{29} Regarding alcohol intake, there is equivocal evidence with studies showing limited or no association between this risk factor and EAC development.\textsuperscript{29, 30}

4. Mechanisms in EAC development

4.1 The role of GERD, inflammation and free radicals

The biological mechanism(s) through which reflux constituents drive carcinogenesis in the human esophagus remains unclear. Reflux seems not only to be involved for development of BE but also may act during the various stages of malignant progression of BE to EAC. The composition of reflux is highly heterogeneous. It can contain oro-esophageal (saliva, esophageal secretion, food), gastric (acid, pepsin, mucous) and duodenal (bile slats, trypsin, cholesterol and lipase) components.\textsuperscript{31} Different components of the reflux might also act at different stages of BE progression. Literature data suggests that there is a correlation of esophageal exposure to both acid and bile with increasing severity of GERD. It appears that acid is needed to activate pancreatic proteolytic enzymes and enhance the capacity of bile salts to penetrate the mucosa of the esophagus.\textsuperscript{14, 32}

Reflex esophagitis and BE are inflammatory conditions. The oxidative stress and free-radical generation associated with inflammation could provide a link between GERD, BE and EAC.

Acid and bile salts and subsequent esophagitis can induce reactive-oxygen species, deplete antioxidants and increase expression of oxidative-stress-related genes.\textsuperscript{33-35} It is known that one of the biological effects of free radicals is their ability to induce DNA damage. Increased DNA strand breaks and the presence of the pro-mutagenic oxidative DNA lesions, have been observed in BE cells.\textsuperscript{36, 37} Indeed, the types of mutations observed in EAC are consistent, although not specific to, oxidative DNA damage.\textsuperscript{38}
4.2 Acquired molecular changes in EAC development

It is generally accepted that the development of EAC follows a no-dysplasia (metaplasia)-dysplasia-adenocarcinoma sequence, which is characterized by the accumulation of multiple genetic (e.g., mutation in specific genes) and epigenetic modification (non-sequence changes that are inherited through cell division). In the development of cancer, the occurrence of sequential genetic changes is responsible for clonal selection and tumor heterogeneity.

Perhaps one of the earliest molecular events is the selection and propagation of the metaplastic clones with specialized intestinal metaplasia. Subsequently, loss of cell cycle check points and genomic instability may contribute to slow clonal expansion probably by increased proliferation. Inhibition of apoptosis occur rather late, in high grade dysplasia of BE. Invasive cancer may be preceded by alteration in cell cycle regulation and hypermethylation of gene promoters are particularly common. In addition, tetraploid/aneuploid fractions of cells are increasing. Further chromosomal losses and gains occur in dysplasia and EAC in addition to mutations in key tumor suppressor genes. The alterations in expression of genes involved in the regulation of apoptosis and in adhesion appear in late sages of BE. In this diagram, molecular changes are indicated at the earliest point observed in the proposed pathway. The frequency of the molecular changes typically increases with BE progression. APC, adenomatous polyposis coli; COX2, cyclooxygenase 2; FASL, FAS ligand; GLUT1, glucose transporter 1; iNOS, inducible nitric oxide synthase; RB retinoblastoma. (adapted from Wild et al)

Figure 1: Molecular events associated with the development of BE and esophageal adenocarcinoma. The development of BE, dysplasia and EAC is associated with many genetic and epigenetic alterations; chromosomal losses of regions harboring genes involved in the cell cycle regulation and hypermethylation of gene promoters are particularly common. In addition, tetraploid/aneuploid fractions of cells are increasing. Further chromosomal losses and gains occur in dysplasia and EAC in addition to mutations in key tumor suppressor genes. The alterations in expression of genes involved in the regulation of apoptosis and in adhesion appear in late sages of BE. In this diagram, molecular changes are indicated at the earliest point observed in the proposed pathway. The frequency of the molecular changes typically increases with BE progression. APC, adenomatous polyposis coli; COX2, cyclooxygenase 2; FASL, FAS ligand; GLUT1, glucose transporter 1; iNOS, inducible nitric oxide synthase; RB retinoblastoma. (adapted from Wild et al)
adhesion \(^\text{41}\), whereas subsequent cumulative genetic abnormalities may result in the
generation of multiple clones of transformed cells with an angiogenetic or metastactic
potential. Examples of molecular events associated with BE and EAC are outlined in
Figure 1 \(^\text{42}\), and also have been reviewed in detail elsewhere. \(^\text{32, 43, 44}\) The sequential
order in which the genetic events occur is not entirely clear and it is presented only to
reflect the potential interplay of different molecular events in BE progression to EAC.
Delineating the genetic abnormalities that occur during BE and EAC development
could be useful in clinical management of patients at a number of levels by offering
opportunities for early treatment and improved survival. \(^\text{42}\) These include rational
approaches to primary and secondary prevention, as well as improved surveillance
of high-risk subgroups of individuals with BE. In addition, the characterization of
genetic changes during BE progression may lead to targeted therapeutic regimens.

5. Endoscopic surveillance of BE

The goal of any cancer surveillance program is the detection of premalignant
lesions or early cancers, when treated, will ultimately result in improved survival.
Endoscopic surveillance of BE patients refer to esophagogastroscopy and biopsy
performed at regular intervals to detect high grade dysplasia (HGD) or cancer at
an early and potentially curable stage. The current guidelines for BE surveillance
are based on the highest grade of dysplasia identified by histology at baseline
and confirmed by two expert gastrointestinal pathologists. \(^\text{45}\) At each surveillance
interval, four-quadrant biopsies every 2cm should target normal appearing BE
mucosa, and any visibly abnormality, such as nodules or ulcers. \(^\text{1}\)

The efficacy and utility of the BE surveillance program is controversial and is
extensively criticized. The problems include the difficulty of identifying neoplastic
lesions with current endoscopic techniques and a high frequency of sampling
errors. \(^\text{46}\) Furthermore, even among expert histopathologists, substantial intra-
and inter-observer variability in grading dysplasia, especially for LGD, is seen,
making accurate diagnosis difficult. The cost-effectiveness of such surveillance
program is questioned as well since there is a low absolute incidence of EAC
among BE patients (0.5%\%/year), rendering surveillance endoscopy not cost-
beneficial. \(^\text{47, 48}\) Cost estimates widely depend on variables as entered in a model,
and the choice of the most optimal screening intervals is sensitive to estimates of
cancer rates in BE patients. \(^\text{49}\) Therefore, one strategy is to limit the surveillance
programs to BE patients that run the highest risk for developing EAC, and this
requires development of more efficient risk estimates.

The proposed new approaches include the use of alternative techniques for tissue
sampling (such as brush cytology or mucosal striping), combined with the evaluation
of predictive biomarkers to identify patients with higher risk of BE progression.
6. Candidate genetic markers to predict malignant progression of BE

To better understand the multistep no-dysplasia-dysplasia-adenocarcinoma sequence and to identify early makers of BE malignant transformation, a variety of molecular studies have been carried out. Although, only a few markers have been evaluated in prospective follow-up studies of BE patients, the examples of candidate biomarkers are numerous. Only genetic markers with a potential prognostic and diagnostic value in the management of BE and EAC patients and those studied in this thesis will be presented in more detail.

6.1 Changes in DNA content: DNA aneuploidy

Cells that contain any other formulation of chromosomes than 2N (diploid) are regarded to be aneuploid. Detection of aneuploidy is based on the nuclear DNA content measurements. Aneuploidy may serve as an important prognostic factor and marker to identify BE patients at risk, since it may appear early in non-dysplasia and has high frequency in HGD and EAC cases. Indeed, the Seattle group, using flow cytometry, has shown in prospective studies that patients with aneuploid or increased G2M cell populations have a higher risk of developing HGD and adenocarcinoma. The same group showed that among patients with non-dysplastic, indefinite for dysplasia (IND) or LGD, the risk of cancer was strongly related to the presence DNA aneuploidy or increased G2M cell populations. It was suggested that the group with no dysplasia, IND and LGD and a diploid DNA content is at lower risk for EAC progression, and therefore could undergo endoscopic surveillance at intervals of up to 5 years. Patients whose baseline biopsies had aneuploidy, increased G2M cells populations or HGD had a 5-year incidence of 43%, 56% and 59%, respectively, prompting a recommendation for more frequent surveillance of that group. Although some studies have confirmed that DNA aneuploidy is a prognostic factor for malignant progression of BE, other groups have reported discordance between histology and DNA ploidy.

6.2 Specific chromosomal abnormalities

Detection of aneuploidy is based on the nuclear DNA content. However, no information is obtained about detailed chromosomal abnormalities in these cells. These types of abnormalities can be studied by comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH) techniques. By applying CGH and FISH, average losses and gains of chromosomal arms and the smallest detectable abnormalities like losses and gains of specific gene loci can be evaluated.
Loss of the Y chromosome, is among the most consistent chromosomal changes found by cytogenetic studies in BE. Y chromosome loss occurs already in the stage of metaplasia and has a high frequency in dysplasia and EAC. \(^{51-53, 59}\) Doak at al showed chromosome 4 and 8 hyperploidy as the earliest and most common alterations identified using FISH on endoscopic cytology brushings. \(^{60}\) Furthermore, LOH (loss of heterozygosity) and CGH studies showed frequent chromosomal losses concerning the chromosomal regions of 4q, 5q, 7q, 9p, 13q, 17p, 18q and gains of 6p, 7, 8q, 11, 12q, 14, 17q and 20q in the metaplasia–dysplasia–sequence of BE. \(^{53, 59, 61-64}\) A recent report using aCGH tested a series of EAC for almost 300 different genomic loci of tumor suppressor and oncogenes, and revealed that from these genes 50 were abnormally expressed in EAC, most of which were not previously noted. \(^{65}\) The study confirmed frequent gains of the 8q24 (c-myc), 17q11 (Her-2) locus, 20q region, and loss of the 9p21 (p16) locus. It remains to be determined whether any of these abnormalities may serve as prognostic markers for BE progression to malignancy.

### 6.3 Tumor suppressor genes

In tumor suppressor genes both gene copies need to be inactivated for the tumor suppressive effect to be lost. One allele of the gene is frequently inactivated by loss of heterozygosity (LOH) while the remaining copy is often inactivated by mutation or promoter (hyper) methylation. LOH can arise via several pathways including deletion, gene conversion, mitotic recombination and chromosome arm or entire chromosome loss.

#### 6.3.1 TP53

The p53 tumor suppressor gene is located on the chromosome 17p13 arm and encodes a TP53 protein that regulates cell cycle progression, DNA repair, apoptosis, and neo-vascularization in both normal and malignant cells via highly complex DNA and protein interactions. \(^{66, 67}\) Deletion of one allele of p53 in combination with a functionally inactivating mutation of the other p53 allele is among the most common combinations leading to inactivation of p53 in human cancers. Loss of heterozygosity (LOH) at the p53 locus (17p13) and p53 mutations seem to be relatively early events in BE neoplastic progression because these may occur in diploid cells before aneuploidy and other LOH events such as 5p, 13p, and 18q occur. \(^{68-70}\)

17p LOH analysis performed on endoscopic biopsies identified BE patients at risk of neoplastic progression, therefore this abnormality was suggested as supplement to histology in determining the frequency of endoscopic examinations during surveillance. \(^{71, 72}\) Since p53 mutations are detectable before development of HGD or adenocarcinoma during BE surveillance, it may also be a
useful marker for BE malignant progression. Reid et al evaluated LOH at 17p13 in conjunction with flow cytometry. The prevalence of 17p LOH ranges from 6% in non-dysplastic BE to 57% in HGD and was a significantly independent predictor of progression to EAC. The 17p LOH was also associated with an increased risk for aneuploidy, increased tetraploid fractions and HGD. Thus, p53 gene alterations are early and frequent events in malignant progression of BE associated adenocarcinoma, thus of potential use as a prognostic marker in BE surveillance programs.

6.3.2 The p16 (CDKN2A)
The p16 gene is localized on the chromosomal region 9p21, and encodes a P16 protein that belongs to a family of CDK inhibitors. The P16 protein inhibits CDK4/6, resulting in reduced phosphorylation of RB1 and inhibition of cell-cycle progression through inhibition of the G1 phase. P16 becomes inactivated by a two-hit mechanism that can involve LOH of 9p21, mutation, homozygous deletion, or CpG island methylation. In Barrett’s esophagus and esophageal adenocarcinoma, point mutations of p16 are relatively uncommon but 9p21 LOH and promoter hypermethylation are frequent mechanisms of p16 inactivation. P16 promotor methylation with or without LOH is already present in high frequency in non-dysplastic BE. It is suggested that p16 gene alterations are the most frequent and earliest known somatic genetic/epigenetic abnormalities in BE occurring in more than 85% of cases in all histological grades of dysplasia. Cells with p16 abnormalities, may undergo clonal expansion and to involve large areas within the BE segment, creating a field in which other premalignant clones may arise and result in development of esophageal adenocarcinoma. P16 alterations may therefore be a useful biomarker to stratify patient’s risk of BE metaplasia progression to esophageal adenocarcinoma. Suspiro et al, reported 9p LOH in 35% if BE patients without dyplasia or cancer and regarded 9p LOH as a useful prognostic marker for risk stratification within endoscopic surveillance programs.

6.4 Proto-oncogenes
Proto-oncogenes are cellular genes, which can be converted into oncogenes by activating mutations or amplifications.

6.4.1 Her-2/neu (c-erbB2)
Her-2 (neu/c-erB2) is a member of the EGF receptor family and encodes a tyrosine kinase cell membrane receptor, normally involved in the signal transduction pathways leading to cell growth and differentiation. Her-2/neu is localized at chromosome 17q11.2 and is activated via amplification. When the Her-2 oncogene is amplified, the Her-2 protein is usually overexpressed. Of interest is that the
Her-2 amplification/overexpression has therapeutic and prognostic implications in breast cancer and other carcinomas. An antibody-based therapeutic approach (transtuzumab/herceptin) targeting the Her-2 protein has proved to be an effective adjunctive treatment for breast cancer.

The amplification of the Her-2 gene and its protein overexpression have been also found in dysplasia and EAC associated with BE and some data suggest that, as in breast cancer, Her-2 alterations correlates with poor prognosis of EAC patients. However, the specific role of the Her-2 status as a marker in BE malignant progression is unclear. The correlation of the Her-2 amplification and/or overexpression within the non-dysplasia-dysplasia-adenocarcinoma sequence of BE, is still obscure. Nevertheless, the available data suggests that evaluation of Her-2 amplification/overexpression is useful to identify HGD/EAC patients and as such may serve as a diagnostic marker for malignant progression of BE. Additionally, this may be used as a prognostic factor and help to select BE candidates for Her-2 targeted therapeutic approaches.

6.4.2 C-myc
The c-myc gene is located on chromosome 8q24 and encodes a transcription factor involved in the regulation of normal cellular proliferation, differentiation and apoptosis. The oncogene is activated via chromosomal translocation or amplification. C-myc amplification has been reported in none of non-dysplasic BE and LGD, but it was found in a range of 11-25% in HGD and 14-44% in EAC patients. Thus, c-myc may be a candidate as a diagnostic marker of HGD or EAC in BE progression.

6.4.3 EGFR
The epithermal growth factor receptor (EGFR), localized at chromosome 7p12-13, plays an important role in tumor cell survival and proliferation. EGFR is amplified and overexpressed in many epithelial cancers, including lung, non-small-cell carcinoma, and colorectal adenocarcinoma. EGFR amplification was found in 8-30% of esophageal adenocarcinoma, which in some studies has been correlated with the occurrence of lymph node metastasis. EGFR amplification was not found in HGD or earlier stages of BE. Thus, EGFR amplification can be considered as a diagnostic marker to identify BE patients with EAC with possible lymph node metastasis.

6.4.4 20q- locus harboring putative oncogenes
It has been shown that an increased copy number of 20q is associated with cellular immortalization, and amplification of 20q13.2 was correlated with genomic instability. Interestingly, different human cancers e.g, breast cancer,
ovarian cancer \(^{104}\) and head-and-neck cancer \(^{105}\) display gain or amplification of this region, suggesting that the gene(s) on 20q plays an important role in carcinogenesis. Falk et al. found the 20q13 locus amplification in 62% of EAC patients. \(^{106}\) Walch et al. also reported this amplification in EAC and additionally in HGD associated with EAC. \(^{107}\) Several candidate genes have been proposed as a potential target gene(s) in this region, e.g. \(\text{NABCI, BTAK, ZNF217, BCASI}\) and it is likely that more than one putative oncogene is involved in the overrepresentation of 20q in BE. \(^{108}\)

### 6.5 Genetic polymorphisms

Individual variations in cancer risk have been associated with specific variant alleles (polymorphisms) of different genes that are present in significant proportions of the normal population. \(^{109-111}\) Recent studies have suggested that genetic polymorphisms may clarify the causes and events involved in esophageal carcinogenesis. \(^{112}\) A variety of genetic polymorphisms may be associated with esophageal carcinogenesis including variants of genes involved in alcohol, folate, and carcinogen metabolism, DNA repair and cell cycle control and oncogene expression. \(^{113}\)

The examples of association between polymorphisms of specific genes and predisposition to esophageal adenocarcinoma are emerging. An association, for instance, was shown between smoking and risk for EAC in the individuals with allele variant of either M1 or M2 of the \(\text{GST}\) (glutathione S-tranferase), carcinogen metabolizing enzyme. \(^{114}\) Another study investigating a Swedish population demonstrated that polymorphism of the \(\text{XPD}\) gene (751Gln allele), involved in DNA repair, is associated with an increased risk for esophageal adenocarcinoma. \(^{115}\) Recently, Moons et al. observed that COX-2 CA polymorphism, previously found to be linked with the COX-2 activity, is more frequently observed in EAC patients compared to BE patients with reflux esophagitis, suggesting a direct link between COX-2 activity and malignant progression. \(^{116}\)

Although there is accumulating evidence of potential links between genetic polymorphisms and BE and EAC susceptibility, data is still limited and frequently inconsistent. The best scientific evidence for this association will come from large cohort studies that simultaneously consider multiple factors that potentially are involved in EAC carcinogenesis, including both, genetic polymorphisms, and environmental factors. Identification of genetic variants that modify the impact of environmental factors will depend on direct exploration of the interaction between genes and environment. \(^{117}\) These types of studies will allow us to estimate the relative contribution of individual genetic variants in the risk stratification for developing both BE (in the general population) and/or EAC (within BE population).
7. Techniques for assessing genetic markers in BE

7.1 DNA cytometry

Nuclear DNA content (ploidy status) may be evaluated using either flow cytometry (FCDA) or image cytometry (ICDA). Both FCDA and ICDA are based on stoichiometrically binding of a dye to the DNA that can be measured quantitatively.

Flow cytometry (FCDA) analyses large numbers of cells and gives meaningful cell cycle data. FCDA, however, is susceptible to false negative results due to errors that are inherent in the technique. Focal lesions are particularly susceptible since the cell suspensions from biopsies are admixed with normal epithelial, inflammatory and stromal cells, leading to dilution of the cells of interest. Same reports indicate that the risk of false negative results in FCDA can be minimized by the use of dual parameter flow cytometry, especially with Ki67/DNA content multiparameter or total protein (SR 101)/DNA content flow cytometry. 40, 57 However, the need for time-consuming, special tissue preparation may limit usefulness of this approach in large clinical settings.

Image cytometry (ICDA) is more specifically targeted to the populations of cells that are of interest (epithelial cells), and can accurately measure rare events. ICDA seems a more convenient method for DNA ploidy analysis than FCDA, and can be applied to tissue sections as well as to disaggregated cytospin preparation or to microscopically identified epithelial cells. 118 Some reports indicate that ICDA is more sensitive than flow cytometry to detect DNA ploidy changes. 119-121 A study by Fang et al. even suggested that aneuploidy as determined by ICDA may be a more sensitive marker than HGD, for identifying subset of BE patients likely to progress to cancer. 122 Thus, ICDA appears to be a convenient and useful adjunct to histology as a marker for BE patients who are at risk for developing adenocarcinoma. However, future multicenter prospective studies with a large sample size are required to validate these findings.

7.2 FISH

Interphase fluorescent in situ hybridization (I-FISH) using fluorescently labeled DNA probes for chromosome- and gene specific loci allows for the visualization and quantization of chromosomal and specific gene aberrations that may correlate with disease progression. The principle of this method is shown in Figure 2. Compared to conventional cytogenetic methodologies and flow cytometry for assessing ploidy, FISH is more sensitive, permits evaluation of larger number of cells, and detects numeric and structural abnormalities of chromosomes. 123-125 Compared to molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and Southern blot analysis for detecting genetic aberrations, FISH is more quantitative and less laborious, and requires fewer samples. Furthermore, FISH ravel cell- to- cell heterogeneity and enables the detection
of minor subpopulations of genetically distinct cells. Importantly, I-FISH does not require a special cell culture process; the technique is directly applicable to cellular material including cytology specimens and tissue sections.

### 7.2.1 FISH as a powerful diagnostic tool in cytology

FISH has been applied successfully in routine cytology specimens for a variety of tumors with different genetic abnormalities such as chromosomal aneuploidy, translocations, and gene deletions or amplifications. Therefore, FISH on cytology specimens has become an important research tool, and some DNA FISH probes are usefully applied for clinical decision making.

### 7.2.3 FISH in diagnosis and prognosis of solid tumors

The value of cytogenetic analysis was not well established in solid tumors until chromosomal and genetic changes were identified by the FISH technique. Numeric chromosomal abnormalities detected by FISH in effusions or fine-needle aspirations from patients with breast cancer have been correlated with tumor stage and clinical outcome of the disease.\(^{126}\) A FISH test also showed to be effective for detection of metastasis in various types of cancers.\(^ {126, 127}\) Gains of chromosome 7 and 8 in prostate cancer, as determined by this method, are associated with poor prognosis and are potential markers for tumor aggressiveness.\(^ {128}\) Recently, the U.S Food and Drug Administration approved detection of Her-2/neu amplification via PathVysion Her-2 assay (Vysis) as a prognostic factor and in the assessment of breast cancer patients for anthracycline therapy. The other example

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**Figure 2: The principle of fluorescent in situ hybridization (FISH) method.** FISH utilizes fluorescently labeled DNA probes which after denaturation of target DNA hybridize to specific peri-centrometric chromosomal regions or chromosomal loci. Number of fluorescent signals within interphase nuclei indicates copy number of chromosomes/genes of interest.
is the UroVysion Multicolor FISH probe set (Vysis), which consists of three alpha-satellite sequence probes (hybridizing to chromosome 3, 7, 17) and a specific locus probe (hybridizing to 9p21). This set of probes can detect bladder cancer-related genetic abnormalities in urinary epithelia cells from bladder washings and urine. The sensitivity of this multicolor FISH to detect urothelial carcinoma has proven to be superior to that of conventional cytologic evaluation. Its disadvantages, however, include labor-intensive screening, interpretative challenges with signal overlap in highly aneuploid samples and focal plane distortions.

Thus, the use of DNA FISH probes in the diagnosis and prognosis of diseases has a great potential clinical value in the field of cytology. However, despite its technical promise, FISH is a multistep procedure, and its reproducibility, normal range, and accuracy in clinical practice still needs to be established.

7.2.4 FISH on cytology as potential prognostic and diagnostic tool in Barrett’s esophagus

Many of the cytogenetic studies on BE applied FISH on biopsies or resection specimens. However, FISH applied on these materials resulted in a considerable amount of artifacts, complexity and insecurity of FISH signal interpretation due to truncation of the nuclei. The other problem is the sampling error when using biopsy specimens that are taken randomly in the BE segment. To overcome this problems, DNA-FISH can be applied on brush cytology specimens which offers many advantages as a method to detect genetic markers, including simplicity, lower cost, and the potential to sample a larger area of the BE epithelium when compared to taking random biopsies. Recent publications have demonstrated that DNA FISH is feasible on BE brush cytology specimens and this methodology may be a promising approach to improve diagnosis and surveillance programs (prognosis) of BE patients. However, because of the laborious and time consuming nature of manual DNA-FISH signal enumeration, screening of large surveillance cohorts of BE patients using this method may not be easily feasible. This disadvantage might be circumvented by using automated DNA-FISH analysis systems, which can provide hands-off, reproducible and objective scores of genetic markers.

Up to date, the number of prospective studies evaluating FISH markers in BE brush cytology are lacking and specific prognostic genetic markers have not been validated for clinical use.

8. Aim and structure of the thesis

The aim of this thesis was to comprehensively evaluate a panel of genetic markers in the no-dysplasia- dysplasia- adenocarcinoma sequence of BE. The ultimate
goal of this research is to evaluate the predictive value of these genetic markers and finally improve the risk stratification of BE patients.

**Chapter 2** describes the validation of a novel automated CytoVison SPOT AX system for assessment of genetic abnormalities detected by FISH on BE brush cytology specimens. Using this system, six DNA FISH probes including probes for chromosome 9, 17, Y and 9p21 (p16), 17q11.2 (Her2/neu), 17p13.1 (p53) loci were prospectively evaluated in a cohort of 151 BE surveillance patients.

**Chapter 3** compares DNA ploidy status as assessed by image cytometry (ICDA) and chromosomal gains by FISH analysis on BE brush cytology specimens, and describes the value of the detected abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC in a BE.

**Chapter 4** describes the frequency of chromosome 17 copy number changes and 17q11.2 (Her-2) locus amplification as well as different evolutionary events leading to the Her-2 amplification in the no-dysplasia- dysplasia- adenocarcinoma sequence.

**Chapter 5** reports the correlation between 17q11.2 (Her-2) locus amplification as assessed by FISH and Her-2 protein overexpression as determined by immunohistochemistry (IHC) in BE patients with various stages of dysplasia on EAC.

**Chapter 6** describes heterogeneity of copy number changes of several oncogene loci including 7p12 (EGFR), 8q24 (c-myc) and 20q13 in the sequence of no dysplasia- dysplasia - adenocarcinoma of BE.

**Chapter 7** describes the association of Y chromosome haplotypes (polymorphisms) with susceptibility to BE. This study for the first time links the Y-haplotypes DE and J with a lower susceptibility for BE in Caucasian men with GERD.
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General introduction and outline of the thesis


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