Genetic markers in malignant progression of Barrett’s esophagus
Rygiel, A.M.

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Genetic Markers in Malignant Progression of Barrett’s Esophagus
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Chapter 1

General introduction and outline of the thesis

A. M. Rygiel, M. P. Peppelenbosch, K. K. Krishnadath
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esophagus (BE) is defined as a metaplastic change in which the normal squamous epithelium of the distal esophagus has been replaced by a specialized columnar epithelium as a result of longstanding gastro-esophageal reflux disease (GERD).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
General introduction and outline of the thesis

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 trough 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, oesophageal 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}

Reflux 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

![Diagram](image)

*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 \(^{41}\), 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 \(^{42}\), and also have been reviewed in detail elsewhere. \(^{32, 43, 44}\) 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. \(^{42}\) 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. \(^{45}\) At each surveillance interval, four-quadrant biopsies every 2 cm should target normal appearing BE mucosa, and any visibly abnormality, such as nodules or ulcers. \(^{1}\)

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. \(^{46}\) 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. \(^{47, 48}\) 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. \(^{49}\) 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. Doak et al showed chromosome 4 and 8 hyperploidy as the earliest and most common alterations identified using FISH on endoscopic cytology brushings. 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. 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. 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. 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.

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. 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 then 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. Susprio 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 epidermal 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 \(NABCI\), \(BTAK\), \(ZNF217\), \(BCAS1\) and it is likely that more then 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 \(GST\) (glutathione S-tranferase), carcinogen metabolizing enzyme. \(^{114}\) Another study investigating a Swedish population demonstrated that polymorphism of the \(XPD\) 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).
Chapter 1

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. 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. Some reports indicate that ICDA is more sensitive than flow cytometry to detect DNA ploidy changes. 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. 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. 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 ravelles 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 cytogenic 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. A FISH test also showed to be effective for detection of metastasis in various types of cancers. 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. 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

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 pericentrometric 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 probe 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.
REFERENCES

General introduction and outline of the thesis

Chapter 1


General introduction and outline of the thesis


Chapter 1


General introduction and outline of the thesis


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General introduction and outline of the thesis


Efficient automated assessment of genetic abnormalities detected by Fluorescent in situ hybridization on brush cytology of a Barrett’s esophagus surveillance population


Cancer. 2007 May15; 109 (10):1980-8
Chapter 2

ABSTRACT

**Background & Aims:** Automated assessment of genetic abnormalities detected by Fluorescent in situ hybridization (FISH) in brush cytology of Barrett’s esophagus (BE) may enhance the clinical applicability of this methodology. The aims were to validate a novel automated CytoVison SPOT AX system for assessment of FISH abnormalities in BE brush cytology, and subsequently use this method for screening of a BE surveillance cohort.

**Methods:** FISH with DNA probes for chromosome 9, 17, Y and 9p21(p16), 17q11.2(Her2/neu), 17p13.1(p53) loci was applied on brush cytology of a surveillance cohort of 151 BE patients. Validation of the automated system was performed by comparison of the automated FISH results with manual scores of the first 60 cases.

**Results:** We found 98 % concordance between manual and automated FISH analysis with kappa values 0.49 - 1 for the different probes. Loss of the 17p13.1 (p53) was seen in only 5% in ND (no dysplasia) and 9% in LGD (low grade dysplasia), but increased to 46% in HGD (high grade dysplasia) (p<0.005, Fisher’s exact test). Chromosome 9 and 17 polysomy was 6% in ND, 21% in LGD and 62% in HGD (p<0.05). Ten percent of ND cases had loss of the Y chromosome, which increased to 27% in HGD (p<0.05). Amplification of the17q11.2 (Her2/neu) was detected in 62% of HGD (p<0.001).

**Conclusions:** CytoVison SPOT AX is an objective, efficient system for analysis of DNA-FISH on BE brush cytology and applicable to analyze large populations of BE patients. In our cohort, loss of the 17p13.1 (p53), Y chromosome loss, polysomy of chromosome 17 and 9 correlate with increasing grade of dysplasia in BE.
INTRODUCTION

Barrett’s esophagus (BE) is a metaplastic condition of the distal esophagus, which is associated with an increased risk for developing esophageal adenocarcinoma (EAC). The malignant transformation of BE is assumed to follow the sequence of no dysplasia (ND), low grade dysplasia (LGD) and high grade dysplasia (HGD) before invasive EAC occurs. For several decades, in regions of Western Europe and the United States the incidence of EAC has been increasing rapidly. Since long term survival of EAC patients is highly dependent on early diagnosis, detection of BE patients at high risk for developing HGD or EAC has become crucial. The present surveillance strategies based on biopsy and histopathological staging for dysplasia in order to identify a subpopulation with a higher risk of malignant progression have been proven to be insufficient. The major difficulty is the inter- and intra-observer variation when classifying biopsies for grade of dysplasia and the sampling error that may occur when using random biopsies. Despite the vigorous protocols that are applied for extensive and systematic sampling, malignant lesions may still be missed. Moreover, the transformation of BE into EAC may not necessarily follow the assumed sequence of histopathological events. Thus, novel surveillance strategies should aim on improving the risk stratification of BE patients, for instance by using objective, genetic markers to assess neoplastic progression.

During the past years, research on BE has focused on identifying biological markers in the metaplasia-dysplasia-adenocarcinoma sequence, and numerous cytogenetic abnormalities have been described. Among the most important cytogenetic changes contributing to BE progression are chromosomal numerical aberrations, Y chromosome loss, the p53 and p16 tumor suppressors locus losses, as well as amplification of the Her2/neu oncogene. One of the methods to detect these cytogenetic abnormalities is fluorescent in situ hybridization (FISH), which utilizes fluorescently labeled DNA probes to peri-centromeric chromosomal regions or unique chromosomal loci to detect cells with numerical or structural chromosomal changes. Earlier studies with DNA-FISH for the detection of genetic abnormalities in BE frequently used biopsies or resection specimens. An alternative way to obtain material for FISH analysis is by brush cytology. The advantage of brush cytology includes simplicity, lower cost, and the potential of this method to sample a larger area of the BE epithelium when compared with taking random biopsies. Recent studies have demonstrated that DNA-FISH can successfully detect cytogenetic changes in interphase nuclei of brush cytology specimens of BE patients. As such, DNA-FISH on brush cytology seems to be a promising method to screen BE patients for cytogenetic abnormalities associated with dysplasia and/or malignancy. However, because of the laborious and time consuming nature of manual DNA-FISH signals
enumeration, screening of large cohorts of BE patients using this method is not easily feasible. This disadvantage might be circumvented by using an automated DNA-FISH analysis system, which can provide hands-off and objective scores of genetic abnormalities, and give an opportunity to apply DNA-FISH for screening of large BE surveillance populations.

Therefore, the main objectives of this study were to validate the novel CytoVison SPOT AX counting system by determining the degree of concordance between manual and automated counting of FISH signals in BE brush cytology specimens, and to further evaluate the FISH abnormalities in the premalignant stages of BE in brush cytology of a surveillance population. For these purposes, we applied FISH on brush cytology specimens of 151 BE surveillance patients using DNA probes for the peri-centromeric regions of chromosome 9, 17, Y and the locus specific regions of 9p21 (p16), 17p13.1 (p53) and 17q11.2 (Her2/neu).

MATERIALS AND METHODS

Patients
A surveillance cohort of 151 Barrett’s esophagus patients, who underwent routine surveillance by endoscopy and biopsy at the Academic Medical Center in Amsterdam between 2002 and 2006, were included. The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material. Only patients that had proven incomplete intestinal type of metaplasia in biopsies, taken during and prior to surveillance were included. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. During endoscopy, the brush cytology specimens were taken prior to biopsies. The brushes of the normal squamous epithelium were taken from patients without dysplasia at least 3 cm above the BE segment, and were used for control purposes. Biopsies for routine histological examination were taken at least per 2 cm in 4 quadrants and of all suspected visible lesions using the protocol of Reid et al. 5

Brush Cytology
Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S. Directly before brushing, the mucosal surface was sprayed with acetylcysteine (50mg/ml) for dissolving the mucus layer. After the procedure the brushes were inserted in a vial with 20ml of 5% acetylcysteine in 0.9% of NaCl, mixed gently to obtain a homogeneous cell suspension, and then cytospin slides were processed. The cell suspension from the brush was poured into a 50 ml conical tube and centrifuged at 2100 rpm for 10 minutes at 4 ºC.
The majority of supernatant was discarded leaving the pellet in 5ml of solution and the cells were agitated to generate a cell suspension. A Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to generate a single layer of the cells on a glass slide. First, 50 µl of phosphate-buffered saline (PBS) was loaded to the cytospin chambers and centrifuged for 1 min at 550 rpm at room temperature (RT). Subsequently, up to 150 µl of cells suspension was loaded into the cytospin chambers and centrifuged 2 min at 550 rpm at RT. The cytospin slides were dried overnight at RT, and then stored at -80°C until FISH analysis was performed.

**Fluorescent in situ hybridization (FISH)**

We used directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 9, 17, Y and the locus specific probes (LSI) for regions of 9p21 (p16), 17p13.1 (p53) and 17q11.2-q12 (Her2/neu), obtained from Vysis (Downers Grove, IL). Dual color probes were used combining CEP 9 SpectrumGreen/LSI p16 (9p21) SpectrumOrange and a single probe for LSI p53 (17p13.1) SpectrumOrange. The rest of the probes were combined into one set, which contained: CEP 17 SpectrumGreen, LSI Her2/neu (17q11.2-q12) SpectrumOrange and CEP Y SpectrumAqua. DNA-FISH was performed according to the manufacturer’s instructions provided by Vysis with slight modifications. Briefly, the cytospin slides were immersed in 2x standard saline citrate (SSC) at 37 °C for 10 min, and then treated with 0.005% pepsin (Sigma, UK) in 0.01N HCl, pH 2. A five minute wash in phosphate-buffered saline (PBS) at RT arrested the enzymatic treatment, and slides were fixed in 5% of formaldehyde at RT for 10 min. After one wash in PBS at RT for 5 min, slides were dehydrated in ethanol series and air-dried. Then, 2 µl of probe from the mixture consisting of 7 µl of hybridization buffer, 1 µl of each probe, and water to a 10 µl volume was applied to each cytospin slide. The specimen and probes were co-denatured in 80 °C for 3 minutes, and slides were incubated at 37 °C for 48 hours. Hereafter, slides were washed in 0.4x SSC at 70 °C for 5 min, 2x SSC/0.1% Nonidet P-40 at RT for 30 seconds, dehydrated in ethanol series and then air-dried. Subsequently, DAPI (4',6-diamidino-2-phenylindole) diluted 1:1000 in mounting medium for fluorescence (Vetashield, Vector Laboratories, Inc., Burlingame, CA), was applied to counterstain the nuclei.

**FISH analysis by the CytoVision SPOT AX system**

The CytoVision Spot AX work station (Applied Imagining, Newcastle UK) combines an automated Olympus BX61 fluorescent microscope with SPOT™ counting software. It is a high throughput system for objective counting of fluorescent signals within interphase nuclei. The Spot software incorporates trainable DAPI classifier that based on the average size and circularity of the
nuclei excludes untypical shaped cells (damaged or smeared cells) from the analysis. The system automatically scans the slide area, and captures the signals in 3-dimensions (Z-Stack utility) that help to distinguish overlapping spots. The system captures simultaneously up to 5 different probes and then counts number of fluorescent signals per nucleus. The counting result is displayed as a number of cells with normal and abnormal spot counts for each of the probe used in the analysis. Within abnormal cells those with signal loss (<2), gain or amplification signal (>2) are distinguished. The output of the analysis is a gallery of images of each nucleus, which can be used for interactive review.

For each case between 100 to 200 interphase nuclei of BE cells were scored per slide. At the end of each analysis the gallery of cell images was manually reviewed to correct for counting errors. Incorrectly counted cells were excluded from the analysis. The counting errors appeared to be due to a high hybridization background, overlapping cells, autofluorescent signals, and cells out of focus. The percentage of incorrectly counted cells observed during the automated analysis varied from 5 - 35%. The automated analysis was performed blindly with respect to results from manual FISH analysis and histology.

**Manual enumeration of FISH signals for validation of the automated system**

In order to validate the automated FISH analysis system, the first 60 BE cases were as well evaluated by manual analysis. These cases were evaluated without prior knowledge of histology findings or findings by the automated analysis. Per slide 100 interphase nuclei of BE cells were evaluated using fluorescent microscope (Olympus BX61, Germany). Damaged cells and cells with indistinct and blurry signals were not scored. In first instance, the slides were analyzed by a single experienced scorer (A.M Rygiel). After comparison of the manual and automated FISH results, all discordant FISH abnormalities were blindly reviewed by a second experienced scorer (K.K Krishnadath).

**Criteria for determining FISH abnormalities**

To establish the frequencies of artifacts, resulting from background hybridization variation, the probes used in the study were applied to normal squamous epithelium obtained from 20 BE patients without dysplasia. Signals from 100 and 200 interphase nuclei of the squamous cells were evaluated by manual and automated FISH analysis, respectively. From these counts, cutoff values were calculated for each probe and each method separately as the mean percentage of cells with signal gain or loss plus 3xSD (standard deviation). A BE case was considered abnormal when the number of cells with abnormal counts, for any probe, was equal or greater then the cutoff value. The cutoff values of each probe for both FISH analysis methods are presented in Table 1.
Automated FISH analysis of a Barrett’s surveillance cohort

<table>
<thead>
<tr>
<th>FISH analysis method</th>
<th>abnormality</th>
<th>CEP9</th>
<th>CEP17</th>
<th>CEPY</th>
<th>LSI p16</th>
<th>LSI p53</th>
<th>LSI Her2/neu</th>
</tr>
</thead>
<tbody>
<tr>
<td>手工</td>
<td>Losses</td>
<td>5</td>
<td>11</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Gains</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>自动</td>
<td>Losses</td>
<td>8</td>
<td>11</td>
<td>7</td>
<td>17</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Gains</td>
<td>5</td>
<td>4</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1: The cutoff values (%) of the FISH probes for manual and automated FISH analysis.

Statistical analysis
For validation of the automated system, an overall percentage of concordance between manual and automated FISH analysis was assessed for all six probes hybridized for the 60 test cases. Sensitivity of the automated FISH analysis to detect an abnormal case (at least one abnormality present) was determined for 26 patients classified as abnormal by manual FISH analysis, considered here as the gold standard. Likewise, specificity of the automated analysis was calculated using the 34 cases without any abnormalities as assessed by manual FISH analysis. The kappa value ($\kappa$) was determined for each probe separately to adjust for chance of agreement; $\kappa=1$ implies perfect agreement and $\kappa=0$ suggests that the agreement is not better then that obtained by chance. Kappa was judged as providing agreement which is: good if $\kappa>0.80$; substantial if $0.61<\kappa<0.80$; moderate if $0.41<\kappa<0.60$; fair if $0.21<\kappa<0.41$; poor if $\kappa<0.20$. Differences in frequencies of abnormalities were compared using Fisher’s exact test and $p$-values <0.05 were considered statistically significant.

RESULTS
Patients and histopathology
Of 151 BE patients 123 were males and 28 females, median age was 60 (range 26-84), median length of BE segment was 3 cm (range 1-13cm). The studied cases included 114 patients with no dysplasia (ND), 11 patients with low grade dysplasia (LGD), 13 patients with indefinite for dysplasia (IND), and 13 patients with focal or diffuse high grade dysplasia (HGD). For validation of the automated FISH analysis system, 60 BE cases with ND (n=42), LGD/IND (n=10) and HGD (n=8) were analyzed by both manual and automated FISH analysis.

Validation of the automated FISH analysis system
FISH abnormalities as assessed by manual and the automated analysis are shown in Table 2. Figure 1 depicts examples of BE cell images with FISH abnormalities captured by the automated system. Twenty six (43%) and 25 (41%) out of 60 BE patients had abnormal FISH findings by manual and automated FISH analysis,
respectively. We observed a high overall concordance (calculated for all 6 probes) of 98% between these two methods. The sensitivity and specificity of the automated FISH analysis to detect an abnormal case (presented with at least one abnormality) were 96% and 100%, respectively. Table 3 shows the comparison of manual and automated FISH analysis as well as the kappa values ($\kappa$), indicating the degree of agreement for each probe. We found perfect agreement, between manual and automated FISH analysis, for assessment of the trisomy and/or tetrasomy of chromosome 9 and amplification of the 17q11.2 ($\text{Her2/neu}$) ($\kappa =1$). Further, we observed good agreement for detection of losses of the 9p21 ($p16$), 17p13 ($p53$) loci and the Y chromosome loss ($\kappa>0.80$) with a low level of discrepancies of 1-2%. Moderate agreement was found for assessment of the trisomy and/or tetrasomy of chromosome 17 with 3% of discrepancy between the two methods ($\kappa =0.49$).
Overall, there were 6 discordant FISH abnormalities (Table 2), which were subsequently blindly reviewed by a second experienced scorer (K.K Krishnadath). Two out of 6 discordant FISH results obtained by manual FISH analysis turned out to be false positive. These were loss of the 17p13.1 (p53) and loss of the Y chromosome (Table 2; case no. 5 and 17, respectively). Three other abnormalities: a trisomy and/or tetrasomy of chromosome 17 (Table 2; case no. 8 and 15) and loss of the 9p21 (p16) (Table 2; case no. 9) were missed by the automated system.
Chapter 2

In one case loss of the Y chromosome was detected by automated FISH analysis but missed by the manual method (Table 2; case no.4).

### Cytogenetic abnormalities in the cohort of BE patients

Using the same DNA probe set and the automated FISH analysis system, the frequency of FISH abnormalities in brush cytology of the rest of surveillance population was determined. FISH outcomes of the total BE surveillance cohort (n=151) were compared with grade of dysplasia as assessed by routine histopathology of biopsies taken at the same endoscopic procedure as brush cytology (Table 4).

<table>
<thead>
<tr>
<th>FISH abnormalities</th>
<th>No.(%) Manual</th>
<th>Automated</th>
<th>Kappa (κ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9p21 (p16) loss</td>
<td>17 (28)</td>
<td>16 (27)</td>
<td>0.96</td>
</tr>
<tr>
<td>17p13.1(p53) loss</td>
<td>6 (10)</td>
<td>5 (8)</td>
<td>0.90</td>
</tr>
<tr>
<td>CEPY loss</td>
<td>4(8)</td>
<td>5 (10)*</td>
<td>0.88*</td>
</tr>
<tr>
<td>CEP 9 tris/tetrasomy</td>
<td>2 (3)</td>
<td>2 (3)</td>
<td>1</td>
</tr>
<tr>
<td>CEP 17 tris/tetrasomy</td>
<td>3 (5)</td>
<td>1 (2)</td>
<td>0.49</td>
</tr>
<tr>
<td>17q11.2 (Her2/neu) amplification</td>
<td>7 (12)</td>
<td>7 (12)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3:** Comparison of manual and automated FISH analysis in the assessment of genetic abnormalities in cytology specimens of 60 BE patients. The kappa indicates an agreement between manual and automated FISH analysis (see Statistical analysis); * - percentage and κ value for CEPY loss was estimated for 48 males.

<table>
<thead>
<tr>
<th>FISH abnormalities</th>
<th>ND</th>
<th>IND</th>
<th>LGD</th>
<th>HGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>9p21 (p16) loss</td>
<td>34/114 (30)</td>
<td>4/13 (31)</td>
<td>4/11 (36)</td>
<td>6/13 (46)</td>
</tr>
<tr>
<td>17p13.1(p53) loss</td>
<td>5/114 (5)</td>
<td>0</td>
<td>1/11(9)</td>
<td>6/13 (46)</td>
</tr>
<tr>
<td>CEPY loss</td>
<td>9/89 *(10)</td>
<td>0</td>
<td>0</td>
<td>3/11* (27)</td>
</tr>
<tr>
<td>CEP 9 loss</td>
<td>2/114 (2)</td>
<td>0</td>
<td>0</td>
<td>1/13 (8)</td>
</tr>
<tr>
<td>CEP 17 loss</td>
<td>1/114 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CEP 9 tris/tetrasomy</td>
<td>3/114 (3)</td>
<td>0</td>
<td>1/11 (9)</td>
<td>2/13 (15)</td>
</tr>
<tr>
<td>CEP 17 tris/tetrasomy</td>
<td>4/114 (4)</td>
<td>2/13 (15)</td>
<td>2/11 (18)</td>
<td>6/13 (46)</td>
</tr>
<tr>
<td>CEPY gain</td>
<td>1/89 *(1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17q11.2 (Her2/neu) amplification</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8/13 (62)</td>
</tr>
</tbody>
</table>

**Table 4:** Frequencies of cytogenetic abnormalities, detected by DNA-FISH in cytology specimens (n=151), compared with histological grade of dysplasia. * indicates total numbers of males in certain patients group.
Automated FISH analysis of a Barrett’s surveillance cohort

Figure 2 depicts the most commonly seen genetic abnormalities in our BE cohort, compared between ND, IND/LGD and HGD cases. The most frequently observed cytogenetic abnormality in ND was loss of 9p21 (p16), seen in 30% of the cases. Loss of 9p21 (p16) was further detected in 33% of IND/LGD and 46% of HGD cases (p>0.05). Five percent of ND cases displayed loss of 17p13.1 (p53). Then, none of IND and only 9% of LGD patients had this abnormality, which significantly increased to 46% in cases with HGD (p<0.005). Trisomy and/or tetrasomy of chromosome 17 and 9 were present in 4% and 3% of ND cases, respectively. Trisomy and/or tetrasomy of chromosome 17 was observed in 17% of IND/LGD and 46% of HGD cases (p>0.05). Chromosome 9 trisomy and/or tetrasomy was present in IND/LGD and HGD patients in 9% and 15%, respectively (p>0.05). The combined frequencies of the trisomy and/or tetrasomy of chromosome 17 and 9, indicating aneusomy and/or aneuploidy, were 6% in ND cases. These frequencies significantly increased to 21% in IND/LGD and to 62% in HGD patients (p<0.05). Ten percent of ND cases displayed loss of the Y chromosome. This abnormality was not detected in any case of IND and LGD, but was present in 27% of HGD (p<0.05). The amplification of the 17q 11.2 loci (Her2/neu) was observed only in HGD cases with a high frequency of 62% (p<0.001). Losses of chromosome 9 and 17 as well as gain of chromosome Y were very rare findings in both ND and dysplasia patients (>3%).

Figure 2: Frequency of the most commonly found FISH genetic abnormalities compared between grades of dysplasia in BE; ND (n=114), IND/LGD (n=24) and HGD (n=13).
DISCUSSION

We validated the automated CytoVison SPOT AX system for analysis of FISH results in BE brush cytology specimens, using DNA probes for chromosome 9, 17, Y and the locus specific regions of 9p (p16), 17p (p53) and 17q (Her2/neu). Upon validation, we observed a high concordance of 98% between the automated FISH analysis and manual method. We also demonstrated that the automated FISH analysis has an excellent sensitivity (98%) and specificity (100%) to detect an abnormal case when we considered manual analysis as the gold standard. Moreover, we have shown good to excellent agreement, between these two FISH analysis methods, for each of the used probes separately as indicated by \( \kappa \) values. Excellent and good agreement was achieved for assessment of trisomy and/or tetrasomy of chromosome 9 (\( \kappa =1 \)), 17q11.2 (Her2/neu) amplification (\( \kappa =1 \)), for loss of 9p21 (p16) (\( \kappa =0.96 \)), loss of 17p13.1 (p53) (\( \kappa =0.90 \)), and for Y chromosome loss (\( \kappa =0.88 \)). Moderate agreement was only observed for the assessment of the trisomy and/or tetrasomy of chromosome 17 (\( \kappa =0.49 \)). In total, there were six discordant cases between manual and automated FISH analysis. Of the six discordant cases, automated FISH analysis truly failed to assess only 3 of them, loss of the 9p21 (p16) in one case, and a trisomy and/or tetrasomy of chromosome 17 in two cases. Discrepancies in the assessment of these abnormalities were due to clumping of cells, meaning that an inhomogeneous cell distribution may negatively influence the performance of the automated system. Overlapping nuclei from the cells clumps are excluded from analysis by the DAPI classifier in the SPOT software, but as long as cells in the clumps can be distinguished from each other, automated assessment of these clumps is still possible. For the automated analysis, it is therefore important to use a good quality of FISH samples with sufficient evenly spread cells. Most important is the revision of the gallery of images as provided by the system for each case, which is required to exclude incorrectly counted cells.

From this part of the study, we conclude that the CytoVision SPOT AX counting system can be successfully applied to assess genetic abnormalities detected by DNA-FISH in brush cytology specimens of BE patients. This automated system is highly applicable to analyze large quantities of slides and omits the manual scoring, which is tedious and time-consuming. Therefore, the application of this system gives us an opportunity to extend the use of FISH on brush cytology to larger quantities of cells, what may be of importance especially when cytology sample is taken from a long BE segment.

Additionally, this imaging system provides an objective, consistent way to score FISH signals, and enables revision of once analyzed samples, as all images are digitally stored. The user may, if needed, easily repeat the entire review in a rapid manner through the use of images galleries, whereas similar manual
verification is labor intensive and not always possible (loss of FISH signal intensity in time). The automatic collection of cell images have also the advantage that the investigator can compare the morphology of cells within one, or among different BE patients, which might be useful for standardization of diagnostic decision and quality control in the future. In the past, several software programs have been developed to score fluorescent spots in interphase nuclei, however, their feasibility has been never tested on large sample sizes, and in contrast to the CytoVision SPOT AX system none of them could simultaneously analyze more than one fluorochrome in a 3-dimensional mode (Z-stack utility) \(^\text{21, 22}\). To our knowledge, this is the first report demonstrating the feasibility of an automated high throughput system for accurate assessment of FISH signals in brush cytology specimens of BE patients.

In the second part of the study, we proceeded with screening of a cohort of BE patients using the automated system and the same panel of FISH probes. We evaluated the frequencies of cytogenetic abnormalities in brush cytology specimens of a surveillance cohort of 151 BE patients, and compared the results with the histopathological grade of dysplasia. We found loss of the 9p21 (\(p16\)) in a substantial portion of ND cases (30%), which confirms that this alteration occurs early in BE \(^\text{14, 23, 24}\). In our cohort, loss of the 9p21 (\(p16\)) did not correlate significantly with increasing stage of dysplasia (\(p>0.05\)), which may be due to our small sample size of the population with dysplasia. Since loss of the 9p21 (\(p16\)) had a relatively high frequency in ND patients compared to LGD and HGD cases, this abnormality itself may not be indicative as a marker for dysplasia in BE. Loss of 17p13.1 (\(p53\)) which was a relatively rare event in ND (5%) and LGD (9%) cases but was found in 46% of cases with HGD significantly correlated with increasing dysplasia grade (\(p<0.005\)). Thus, loss of the 17p13.1 (\(p53\), may be indicative for the presence of dysplasia in BE, and in fact it has been shown earlier that loss of the \(p53\) precedes dysplasia and is associated with increased risk for progression to HGD as well as to EAC \(^\text{14, 19, 25, 26}\). Ten percent of the ND patients had loss of the Y chromosome, which is comparable to other in situ hybridization studies \(^\text{12, 13, 19}\). We did not observe loss of this chromosome in IND and LGD patients as shown previously by Krishnadath \textit{et al} \(^\text{12}\) and Doak \textit{et al} \(^\text{19}\). This abnormality was found in 27% of our HGD cases, which is also lower then documented in earlier studies, where loss of the Y chromosome was documented in all or the majority of HGD patients. In these studies, however, loss of the Y chromosome appeared to be mostly focal and confined to a relative low percentage of cells \(^\text{12, 19}\). Previously Walch \textit{et al} \(^\text{16}\) demonstrated that in the metaplasia-dysplasia-adenocarcinoma sequence a polysomy of chromosome 17 was already present in 50% of LGD patients, but was not observed in ND cases. Our study shows that polysomy (trisomy and/or tetrasomy) of chromosome 17 and 9 are detectable by DNA-FISH in a low frequency of 6% in ND. The combined frequency of these abnormalities
further increases through IND/LGD (21%) to HGD (62%) (p<0.05), and may have a predictive value for malignant transformation in BE. The polysomy of these chromosomes may reflect aneuploidy, which is indicative for BE progression as showed by DNA flow cytometry studies demonstrating that an increased tetraploidy/aneuploidy correlate with BE malignancy \(^{27-31}\). We further observed amplification of the 17q11.2 (\textit{Her2/neu}) in over 60% of HGD patients, which is concordant with previous FISH studies, demonstrating a high prevalence of \textit{Her2/neu} amplification only in HGD or EAC cases \(^{15,16,18}\). Our data indicate that the 17q11.2 (\textit{Her2/neu}) amplification is a late event in BE progression, and therefore may be a useful, specific marker to detect HGD in BE. In conclusion, this part of the study has evaluated cytogenetic abnormalities in the premalignant stages of BE of 151 surveillance patients. In general, we found that the genetic abnormalities as detected by FISH on cytology specimens are in agreement with FISH genetic abnormalities found in biopsy specimens as reported in literature. Since brush cytology has a potential to sample the entire surface of BE epithelium, theoretically we may assume that compared to random biopsies, this method diminishes sampling errors. This would be reflected in a higher number of genetic abnormalities assessed in brush cytology then in randomly taken biopsies. In this study, however, we did not compare the cytology FISH results to FISH results obtained from biopsy specimens and therefore cannot draw this conclusion. A future study comparing these two methodologies will enlighten us on this matter.

In summary, our study demonstrates that automated assessment of cytogenetic abnormalities, detectable by DNA-FISH in brush cytology of a BE surveillance population, is feasible and reliable. Using this method, we detected several important cytogenetic abnormalities in BE patients without dysplasia, which correlate with increasing grade of dysplasia. Future follow-up of the surveillance cohort is, however, required to prove the true predictive value of these abnormalities. We believe that potential of the automated FISH analysis for accurate assessment of important genetic changes can improve the efficacy of future surveillance programs.
REFERENCES


Assessment of chromosomal gains as compared to DNA content changes is more useful to detect dysplasia in Barrett’s esophagus brush cytology specimens

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Submitted


ABSTRACT

**Background & Aims:** Abnormal DNA ploidy status has been suggested as a prognostic factor for Barrett’s esophagus progression into esophageal adenocarcinoma (EAC). The aim was to compare image cytometry DNA analysis (ICDA) and fluorescent in situ hybridization (FISH) in the assessment of DNA ploidy status in BE brush cytology specimens, and to determine the value of these abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC.

**Methods:** Brush cytology specimens of 90 BE patients were examined using ICDA (the DNA index) and FISH with peri-centromeric probes for chromosome 7 and 17. The results of ICDA and FISH were compared with each other, and with dysplasia grade or EAC as determined by histology and cytology.

**Results:** FISH and ICDA detected abnormalities in 41% (37/90) and 22% (19/90) of the BE cases, respectively. These two methods were concordant in 76% of the cases. Gains of chromosome 7 and/or 17 as detected by FISH were present in 13% of non-dysplasia cases which further increased with dysplasia stage and EAC, while aneuploidy as determined by ICDA was detected predominantly in high grade dysplasia (HGD) and EAC. Using FISH results combined with cytology, we were able to identify IND or LGD (indefinite - or low grade dysplasia) with a sensitivity and specificity of 75% and 76%, respectively. FISH alone detected HGD or EAC with a high sensitivity and specificity of 85% and 84%, which was superior to that of cytology alone.

**Conclusions:** FISH seems to be more sensitive than ICDA to detect chromosomal abnormalities in BE brush cytology specimens. FISH detects chromosomal gains in early stages of BE and represents a valuable adjunct to conventional cytology to detect dysplasia or EAC.
INTRODUCTION

Barrett’s esophagus (BE) is the metaplastic replacement of the normal squamous epithelium of the lower esophagus by the columnar epithelium developing as a consequence of longstanding gastroesophageal reflux disease (GERD). BE is a premalignant condition that predisposes to the development of esophageal adenocarcinoma (EAC), a tumor with increasing frequency in most Western countries. EAC develops through a multistep process which is characterized by increasing grade of dysplasia (intraepithelial neoplasia). Therefore, routine endoscopy with biopsies for histopathological evaluations of BE patients is considered mandatory. It appears, however, that the present endoscopic and histopathologic evaluations of BE are insufficient for effective identification of high-risk patients at early stage. For the clinical management of BE patients it is, therefore, of great importance to identify specific markers associated with development of EAC and its precursor lesions in BE.

Parallel to the metaplasia-dysplasia-adenocarcinoma sequence, numerous studies demonstrated the accumulation of genetic abnormalities from normal cells to invasive malignant cells. Some of these genetic abnormalities i.e., DNA ploidy changes have been suggested to be of potential help in the surveillance of BE patients. There is accumulating evidence that DNA aneuploidy, defined as any other formulation of chromosomes then 2N, is a early key event in carcinogenesis and may be a cause rather then a consequence of malignancy. Indeed, the Seattle group using flow cytometry, has shown in a prospective study that patients with DNA aneuploidy have an increased risk of developing HGD or EAC. The same group demonstrated that among patients with non-dysplasia, indefinite for dysplasia, LGD, the risk of cancer was strongly related to the presence of DNA aneuploidy or increased tetraploid populations. Although several studies confirmed that DNA aneuploidy is a prognostic factor for malignant transformation of BE, other groups reported discordance between histology and DNA ploidy, which at partially may be due to technical issues.

DNA aneuploidy is usually measured by flow cytometry analysis (FCDA). The sensitivity of this method, however, is limited in diagnosing DNA content abnormalities, because it analyses both affected epithelial cells as well as stromal cells in the sample. Moreover, the need for time-consuming, special tissue preparation also limits the usefulness of DNA FCDA in clinical practice. Image cytometric DNA analysis (ICDA) seems to be a more convenient method for DNA ploidy analysis. This technique was previously applied to measure DNA content in disaggregated cytospin preparations or in microscopically identified epithelial cells. Some reports indicate that ICDA is more sensitive then FCDA to detect DNA ploidy changes. Both FCDA and ICDA are based on stoichiometrically binding of a dye to the DNA that can be measured quantitatively. An alternative
means of estimating ploidy status can be performed by DNA fluorescence in situ hybridization (FISH), which can accurately detect copy numbers of specific chromosomes using (peri-)centromeric DNA probes. DNA FISH can be applied successfully on interphase nuclei in tissues and cellular specimens. Such interphase DNA FISH analysis can easily identify chromosomal gains and losses in populations of (epithelial) cells. Previously, we and others successfully applied DNA-FISH on brush cytology specimens to detect a variety of cytogenetic abnormalities in BE.21-23 The advantage of brush cytology, for sampling of cells in BE patients, includes simplicity, lower cost and the potential to sample a larger area of the BE epithelium when compared to taking random biopsies. Furthermore, cytology samples are more reliable for the enumeration of FISH signals and assessment of ploidy status by ICDA, since there is no artifact caused by nuclear truncation, as is the case when using tissue sections.24

The purpose of the present study was first, to compare the DNA ploidy status as assessed by ICDA to chromosomal gains by DNA-FISH analysis on BE brush cytology specimens. The secondary aim was to determine the value of the observed abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC in BE.

**MATERIALS and METHODS**

**Patients**
A cohort of 90 Barrett’s esophagus patients, who underwent endoscopy at the Academic Medical Center in Amsterdam between 2002 and 2007 were included. Only patients that had proven (incomplete) intestinal type of metaplasia in biopsies, taken during and prior to surveillance were included. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. During endoscopy, the brush cytology specimens were taken prior to biopsies. The brushes of the normal squamous epithelium were taken from each patient at least 3 cm above the BE segment, and were used for control purposes. Biopsies for routine histological examination were taken immediately after brushing and at least per 2 cm in 4 quadrants and of all suspected visible lesions using the protocol of Reid et al.25 The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material.

**Brush Cytology preparation**
Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S. Directly before brushing, the mucosal surface was sprayed with acetylcysteine (50mg/ml) for dissolving the mucus layer. Separate brush samples from Barrett’s and normal squamous mucosa were taken from each
Chromosomal gains in a BE patient. After the procedure the brushes were inserted in a vial with 20ml of 5% acetylcysteine in 0.9% of NaCl and mixed gently to obtain a homogeneous cell suspension. Subsequently, a Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to first generate a single layer of the Barrett cells on the top of the glass slide. A second cytospin spot of the squamous epithelial cells of the same patient was made on the bottom of the same slide. These squamous spots served as internal control for FISH and the ploidy analysis procedures. The cytospin procedure was performed as described previously. After the procedure the cytospin slides were dried at RT, and then stored at -80°C until further processing.

**Cytological evaluation**
The brush cytology samples were thawed and stained with Giemsa. Subsequently, two investigators (F. ten Kate and J.G. de Groot) independently scored the brush cytology samples for dysplasia. Any discrepancies in diagnosis were discussed until consensus was reached. BE was defined when goblet cells or cylindrical cells were identified. For the cytological diagnosis of dysplasia or adenocarcinoma, we used widely accepted standards for nuclear atypia such as increased nuclear size, elongated rather then oval nuclear shape and loss of polarity. Cases were categorized into four groups: 1) no dysplasia, 2) indefinite for dysplasia, including inflammatory cases with reactive changes, 3) LGD and 4) HGD or EAC.

**Fluorescent in situ hybridization (FISH)**
We used directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 7 and 17 (SpectrumGreen each) obtained from Vysis (Downers Grove, IL). DNA-FISH was performed on BE brush cytology according to the manufacturer’s instructions provided by Vysis as described previously.

**Criteria for determining FISH ploidy abnormalities**
Enumeration of the number of hybridization signals per nucleus after applying centromeric DNA FISH probes on interphase nuclei reveals the copy number of the corresponding chromosome (Figure 1). Although FISH by itself cannot measure the entire ploidy status of the cells, abnormal ploidy status can be indicated by a change in the copy numbers of the individual chromosomes within the interphase nuclei, which is mostly indicated by chromosomal gains. In this study, 100 to 200 interphase nuclei of BE cells, after FISH with two peri-centromeric probes (CEP 7 and 17), were scored per slide by an experienced scorer (A.M. Rygiel) using Olympus BX61 fluorescent microscope (Germany). The cases were evaluated without prior knowledge of histology and cytology findings. Damaged cells and cells with indistinct and blurry signals were excluded from the analysis. Chromosomal gains by FISH were defined when the sample contained ≥ 3 % of the nuclei with three or more signals for the peri-centromeric probes (CEP 7 or 17). The cutoff value of
≥ 3% was obtained from counts in the normal squamous epithelia taken from 20 BE patients without dysplasia and calculated as the mean percentage of squamous nuclei with signal gain plus 3xSD (standard deviation) as described previously.  

**Feulgen staining**

Each brush cytology slide, apart of the spot with BE cells, contained also a separate cytopsin spot generated from normal squamous epithelium, which served as the internal diploid control. The slides were stained with Feulgen dye using ImagePath Blue Feulgen Stain kit (ImagePath System, Inc., Edwardsburg, Michigan), according to manufacturer’s protocol. The staining involves treatment with concentrated hydrochloric acid that strips the cells of non-nuclear substances, and hydrolyses chromatin. The Feulgen dye then stoichiometrically binds to the nucleic acids, imparting a blue color. The intensity of the blue color is directly proportional to the amount of DNA content.

**Image cytometry**

After Feulgen staining, DNA ploidy analysis was performed using the Ariol system with software version 3.0 (Applied Imagining, New Castle, UK). The analysis was performed in a blinded manner according to histology and cytology findings. For each brush cytology slide, two separate areas of BE and squamous cytopsin spots were scanned (40 x magnification), digitalized and stored in the same file (Figure 1). Subsequently, the nuclei of interest from the squamous and the BE area (cylindrical or/and atypical cells) were marked for analysis. For the BE cells, we used widely accepted standards for nuclear atypia such as increased nuclei size, elongated rather then oval nuclear shape, loss of polarity, and marked these cells for DNA quantification. BE or adenocarcinoma nuclei were quantified for DNA content and compared with the DNA content of normal squamous cells from the same slides. The digitalized images of nuclei were converted into a series of pixels, which were quantified on the basis of the integrated optical density (IOD), reflecting the DNA content. Overlapping nuclei, nuclear debris and other artifacts that escaped auto-detection and removal by the system were deleted by the operator. At least 100 BE cells and 100 control squamous cells (range 100-650 and 100-375, respectively) within the same brush cytology slide were quantified. The DNA content histogram was automatically plotted in a separate window when using the ploidy software (Figure 1). The mean integrated optical density (IOD) of control squamous cells was assigned as a DNA index (DI) of 1, which served as an internal diploid (2N) standard and reference for DI calculation of the BE cells. DI values of 0.9 -1.1 were considered to be within the diploid range. Aneuploidy was defined when more then 10% of BE cells had DI of main peak greater then 1.2. The cutoff of less than 10% of the coefficient of variation (CV) for diploid and aneuploid peak was applied.
Statistical analysis
Differences in frequencies of abnormalities were tested using a chi-square test (2-sided) and statistical significance was set at a $P$ value of $<0.05$. The statistical analyses were conducted using SPSS software (version 12.0; SPSS, Inc, Chicago, IL). Sensitivity and specificity of the different methodologies (FISH, ICDA or cytology) were determined for patients classified as abnormal (dysplasia or EAC) and normal (non-dysplasia or IND/LGD, depending on the analysis) by histology, respectively, which was considered the gold standard.

RESULTS

Histopathology and Cytology
In this prospective study, 90 BE patients were included. Of these 90 BE patients 86 were men and 6 women, median age was 60 (range 30-84), median length of the BE segment was 3 cm (range 1-13cm). The BE population included 38 patients with ND, 19 patients with IND or LGD, 18 patients with HGD and 15 patients with EAC as assessed by histopathology. Brush cytology specimens of 85 of the 90 BE cases were available for cytology diagnosis. There were 45 cases of ND, 20 cases of indefinite for dysplasia or LGD, 20 cases of HGD or EAC as classified by cytology diagnosis. Table 1 compares cytology and histology results.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Cytology No.</th>
<th></th>
<th></th>
<th>Total nr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND</td>
<td>LGD/IND</td>
<td>HGD/EAC</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>34 (91%)</td>
<td>3</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>IND/LGD</td>
<td>7</td>
<td>8 (50%)</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>HGD/EAC</td>
<td>4</td>
<td>9</td>
<td>19 (60%)</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 1: Correlation of cytology and histology diagnosis of BE. ND- no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia, EAC- esophageal adenocarcinoma

Concordance between FISH and ICDA
Gains of chromosome 7 and 17 were determined by FISH analysis and overall DNA ploidy status was assessed by ICDA analysis in brush cytology specimens of the 90 BE patients. In the FISH analysis, gains of chromosome 7 and 17 were detected on average in 30 % of cells (median 16, range 3-87). The number of signal gain for each of the chromosomes varied from 3 to 6 signals per cell. In the ICDA analysis, the median DNA index (DI) value for control cells and normal BE cells (diploid peak) was 1.0 (range 0.9-1.1) with median coefficient of variation (CV) of 4.5 (range 2.97-7.24) and for abnormal cells (aneuploidy peak) the median DI value was 1.8 (range 1.4-2.0) with a median CV of 6.3 (range 3.47-9.5). On average aneuploidy detected in 40% of cells (median 30, range 11-79%).
Figure 1: DNA content analysis by ICDA and detection of chromosomal gains by FISH.

(A) ICDA analysis. Brush cytology specimen containing a cytospin spot with BE cells (top) and a spot with squamous cells (bottom) were stained with Feulgen’s stain. The final classification of DNA ploidy is depicted in a DNA histogram generated from the DNA content data using the Ariol system (Applied Imaging, New Castle, UK). The histogram is representative for one BE case with DNA content aneuploidy. In the histogram, the results for the control squamous cells are shown in black (control peak), results for BE cells in green (diploid peak) and in red (aneuploid peak). The y axis shows the number of cell nuclei, and the x axis shows the nuclear DNA content defined as DNA index (DI).

(B) FISH analysis. BE brush cytology specimens after FISH with peri-cetromeric probes for chromosome 7 (CEP 7, top) and 17 (CEP 17, bottom). The graph shows frequency of BE nuclei with CEP7/17 gains and is representative for the same case as shown in panel A. The y axis shows percentages of BE nuclei, and the x axis shows the number of signals for CEP 7 and CEP 17. For color figure, see page 144.
Chromosomal gains in a BE

Table 2 illustrates the overall comparison of FISH and ICDA results. FISH and ICDA detected abnormalities in 41% (37/90) and 22% (19/90) of the BE cases, respectively (p<0.0001, chi-square test). Overall these two methods were concordant in 76% (68/90) of the cases. There were 22 discordant cases, which are listed in Table 3. Of the 22 discordant cases there was 1 case with ND and 1 case with EAC classified as aneuploid by ICDA but was found to be normal by FISH. The remaining 20 discordant cases were classified as diploid by ICDA but chromosomal gains were detected by FISH. In most of these cases (18 out of 20), however, only gain of a single chromosome (chromosome 7 or 17) was detected. Nine of the 22 discordant cases displayed chromosomal gains in a relatively small fraction of cells (3-5%), which was equal or close to the cutoff value of 3%.

Correlation of cytology, FISH and ICDA results with histology

Figure 2 compares the frequencies of gains of chromosome 7 and/or 17 detected by FISH with ICDA results in the no-dysplasia–dysplasia-adenocarcinoma stages of BE. In general, FISH detected chromosomal gains in 13% (5/38) of the ND and 21% (4/19) of the IND/LGD cases, which then increased to 83% (15/18) in the HGD and 87% (13/15) in the EAC cases (p<0.0001). ICDA showed aneuploidy in 3% of ND (1/38), in none of the IND/LGD, in 50% (9/18) of the HGD and in 60% (9/15) of the EAC cases (p<0.0001; Figure 2).

We further compared results of cytology, FISH and ICDA to the histological classification of BE as assessed in the biopsy specimens. Table 4 compares the different approaches for assessing the dysplasia stage in BE using cytological diagnosis and the abnormalities as found by FISH and ICDA. In the first approach, the sensitivity to detect any dysplasia or EAC was 77%, 62% and 35% for cytology, FISH and ICDA, respectively. In the second approach, the accuracy of the assays to distinguish IND/LGD from ND was compared. The sensitivity hereto was 50%, 21% and 0 for cytology, FISH and ICDA, respectively. In these two approaches, the specificity for all three methods was comparably high varying from 87% to 97%. In the third approach, the accuracy of the assays to distinguish HGD/EAC from IND/LGD and ND was determined. The sensitivity to detect EAC or HGD was 60%, 85% and 54% for cytology, FISH and ICDA, respectively with corresponding specificities of 77%, 84% and 98%. Interestingly,
FISH and ICDA detected abnormalities in 11 and 9 cases out of 13 cases with HGD/EAC, respectively, which were misdiagnosed by cytology. Finally, since FISH and cytology results were the most sensitive assays, we combined these two methodologies and found even higher sensitivities of 85% to detect any dysplasia, 75% to distinguish IND/LGD from ND, and 90% to detect HGD or EAC. However, the specificity of this combined assay decreased to 76%, 76% and 60%, respectively (Table 4).

Table 3: Discordant cases between FISH and ICDA in the detection of aneuploidy. Gain – more than two copies of chromosome 7 and/or 17 (the number of signal gain varied from 3 to 6 signals per cell), DI – DNA index, D – diploid (DI 0.9-1.1), A – aneuploidy (DI >1.1). FISH – fluorescent in situ hybridization, ICDA – image cytometry DNA analysis, ND- no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia, EAC - esophageal adenocarcinoma.
<table>
<thead>
<tr>
<th>Histology</th>
<th>ICDA</th>
<th>FISH</th>
<th>Cytology*</th>
<th>FISH and Cytology*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>N</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>Any dysplasia or EAC</td>
<td>18 (35%)</td>
<td>34</td>
<td>32 (62%)</td>
<td>20</td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
<td>37 (97%)</td>
<td>5</td>
<td>33 (87%)</td>
</tr>
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<td>IND/LGD</td>
<td>0</td>
<td>19</td>
<td>4 (21%)</td>
<td>15</td>
</tr>
<tr>
<td>HGD or EAC</td>
<td>18 (54%)</td>
<td>15</td>
<td>28 (85%)</td>
<td>5</td>
</tr>
<tr>
<td>IND/LGD or ND</td>
<td>1</td>
<td>56 (98%)</td>
<td>9</td>
<td>48 (84%)</td>
</tr>
</tbody>
</table>

Table 4: ICDA, FISH and conventional cytology in the identification of different stages of dysplasia in BE. (*) the analysis was performed for 85 cases with known cytology diagnosis. FISH - fluorescent in situ hybridization,

ICDA- image cytometry DNA analysis, ND- no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia,

HGD- high grade dysplasia, EAC- esophageal adenocarcinoma
In this study, we compared FISH to image cytometry DNA analysis (ICDA) in the assessment of DNA content changes in BE brush cytology specimens. We demonstrated that FISH is a more sensitive method to detect abnormal fractions of cells with single chromosomal gains compared to ICDA. Moreover, we showed that detection of chromosomal gains by FISH alone or combined with cytology diagnosis is a very sensitive assay to detect dysplasia in BE patients.

We first compared FISH and ICDA analysis in assessing the ploidy abnormalities in BE brush cytology specimens and found an overall concordance of 76%. FISH, however, detected abnormalities in a higher number of BE cases as compared to ICDA (41% vs. 22%). Further, the majority of discordant cases (20 of 22) were diploid by ICDA but showed chromosomal gains by FISH indicating that this method is a more sensitive assay than ICDA to detect DNA content abnormalities. In nine of the 22 discordant cases FISH detected gains in a small fraction of cells of 3 to 5%, which was close to the cutoff value. Thus, at least part of the discrepant results may be a reflection of ICDA limitation to detect small populations of aneuploid cells. Remarkably, in most of the discordant cases (19 out of 22), gain of only one out of two investigated chromosomes was detected by FISH. In three of these cases this single gain was

Figure 2: Frequency of abnormalities as detected by FISH and ICDA in the histologically defined stages of no dysplasia-dysplasia-adenocarcinoma of BE (n=90). The BE cohort included 38 patients with ND, 19 patients with IND or LGD, 18 patients with HGD and 15 patients with EAC as assessed by histopathology.
Chromosomal gains in a BE

even confined to significant fraction of cells of 20-41%. Although we assume that the chromosomal gains are a reflection of a general aneuploid status of the specimen, it is possible that, in certain BE cases, gains of chromosome 7 and 17 are selective events that are independent of gross ploidy changes. Our results suggest also that gains of single chromosomes may result in a too small DNA content change to be detected by ICDA. A similar observation has been previously described in a study comparing ploidy results between FISH, flow cytometry and ICDA on fresh touch preparation and paraffin-embedded samples of prostate tumors.28 This study showed a case having 40% of cells with gain of a single chromosome (chromosome 8) by FISH, which was still assessed as diploid by ICDA and flow cytometry, supporting our conclusion that FISH is more sensitive to detect these subtle DNA content changes. It should be noted, however, that the limitation of the sensitivity of FISH depends on the selection of appropriate and informative centromeric probes, especially in cases when a limited number of chromosomes may be polysomic. Overall DNA content aneuploidy is generally related to genetic instability29,30, while this may not be true in case of single chromosomal gains. Therefore, one can also argue that the abnormal count of FISH signal for a single chromosome in an overall ‘diploid’ cell is not comparable to a general aneuploid state and should be considered as a separate entity.

It has been shown that DNA content abnormalities, as assessed by flow cytometry, identifies BE patients at higher risk for developing HGD or EAC.14,15,27 Therefore, in our study, we correlated chromosomal gains and overall DNA ploidy results as detected by FISH and ICDA with histological grade of dysplasia and EAC. We showed that the abnormalities as detected by FISH using centromeric probes for chromosome 7 and 17, is already present in 13% of ND cases and that its frequency significantly increases with increasing dysplasia reaching 21% in IND/LGD, 83% in HGD and 87% in EAC cases (p<0.0001). This observation is similar to that of a study by Doak et al. showing that aneuploidy of other chromosomes (chromosome 4 and 8) as detected by FISH on brush cytology, are early changes present in the non-dysplastic BE correlating with increasing grade of dysplasia.31 In our study, however, ICDA detected aneuploidy only in 3% of ND cases, in none of IND or LGD cases and in only 50% of HGD and 60% of EAC cases. This finding does not correlate well with the results of a recent ICDA study on paraffin BE biopsy sections, which showed aneuploidy in 13 % of ND, 60-73% of LGD/HGD and 100 % of EAC cases.27 This discrepancy could be due to differences of standardization of the image cytometry methods to determine aneuploidy (i.e. different cutoff values to define aneuploidy, different image cytometry systems), the use of tissue section (overlapping and truncated nuclei) instead of brush cytology or an observer variation when classifying the grade of dysplasia in BE.32

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Brush cytology has potential advantages over endoscopic biopsy for diagnosing BE and monitoring for dysplasia since it has ability to sample a greater area of the BE mucosa surface, and may therefore detect lesions that are missed by biopsy protocols. Indeed, in our study, cytology diagnosed IND/LGD in 3 cases and in 1 case HGD, which were classified as ND and IND/LGD by histology, respectively. On the other hand, cytology misclassified 13 cases, which were regarded as HGD/EAC by histology. (Table 1) In general, we found that conventional cytology has a good sensitivity to detect any dysplasia (77%) and moderate sensitivity to detect IND/LGD (50%) or HGD/EAC (60%) with specificities of 91%, 91% and 77%, respectively. The sensitivity of the cytology to detect HGD/EAC in our study is however lower than demonstrated in other reports. Although these studies suggest that cytology may be as accurate as histology to detect HGD or EAC, the small number of cases investigated in these studies makes it difficult to draw definitive conclusion. The sensitivity of routine cytology to detect IND or LGD, in our study, was higher than in the study by Saad et al. where cytology detected only 20% of cases with LGD. This discrepancy may be due to the interobserver and intraobserver variations and the expertise of the pathologist for scoring dysplasia in cytological specimens. Still, for cytology to become a useful surveillance tool to identify BE patients at higher risk for cancer development, its sensitivity and specificity for dysplasia detection lesions has to be improved, which might be accomplished by adding biomarkers i.e. DNA ploidy status to routine cytology diagnosis. Therefore, in this study, we compared the sensitivity of the abnormalities as determined by FISH and image ICDA in cytology samples with routine cytology diagnosis in the detection of dysplasia or EAC in BE. The histology diagnosis was considered as the gold standard. The sensitivity to detect any dysplasia or EAC by FISH or ICDA was lower compared to routine cytology (62% and 35% vs. 77%, respectively). Cytology was also more sensitive to detect IND or LGD cases compared to FISH and ICDA assays (50% vs. 21% and 0%, respectively). In these analyses, the specificity for all three methods was comparably high varying from 87% to 97%. We found, however, that chromosomal gains assessed by FISH have a higher sensitivity and specificity to detect HGD or EAC as compared to routine cytology (85 % vs. 60% and 84% vs. 77 %, respectively). Moreover, FISH detected gains in 11 of 13 HGD/EAC cases that were misdiagnosed by cytology. Upon combining results of FISH and cytology diagnosis we observed a further increase in sensitivities up to 90% to discriminate between the different stages of dysplasia in BE (Table 4). In case histology is truly the ‘gold standard’, the combination of these two assays to detect HGD/EAC show a specificity of 60%, yielding a considerable number of false positives. On the other hand the false positive cases may reflect a population of BE patients that are at higher risk for developing dysplasia. Future follow up of these cases will enlighten us on this matter. At present we advocate that FISH combined with cytology is a highly promising method for the identification of dysplasia and/or EAC in BE.
In summary, we showed that FISH is more sensitive technique than ICDA to detect chromosomal changes in early stages of BE and therefore might be of value to identify BE groups at high risk for EAC development. Additionally, we demonstrated that assessment of chromosomal gains by FISH represents a valuable adjunct to conventional cytology to detect dysplasia and/or EAC in BE.
REFERENCES

Chromosomal gains in a BE


Chapter 3


Sequential chromosomal events preceding Her-2 proto-oncogene amplification in Barrett’s esophagus


Submitted
Chapter 4

ABSTRACT

**Background & Aims:** Her-2 proto-oncogene amplification is an important marker in Barrett’s esophagus associated esophageal adenocarcinoma (EAC), correlating with poor prognosis of EAC patients. However, studies showing the sequential genetic events preceding Her-2 amplification in Barrett’s esophagus (BE) in the no dysplasia-dysplasia-adenocarcinoma-sequence are lacking. The aim was to study sequential chromosomal events that may lead to Her-2 locus amplification in BE and EAC.

**Methods:** Her-2 locus (17q11.2) and chromosome 17 copy numbers were evaluated by DNA fluorescent in situ hybridization (FISH) in 183 brush cytology specimens of BE and EAC patients. DNA image analysis was applied to determine DNA ploidy status in all cases displaying chromosome 17/Her-2 abnormalities (n=37). Analysis of coexisting subclones was performed of all cases with Her-2 amplification (n=15).

**Results:** Low and high level of Her-2 locus amplification occur only in high grade dysplasia (45%) and EAC (30%), whereas gain of chromosome 17 is present already in no dysplasia (3.5%) cases, and its frequency increases significantly with dysplasia stage and is high in EAC (82%). In most of the cases, the subclonal analysis suggested that either selective gain of chromosome 17 or DNA content aneuploidy precedes the Her-2 amplifications. Yet, a subset of cases did not show any of these preceding genetic abnormalities.

**Conclusions:** Gains of chromosome 17, either due to selection or as a result of overall DNA content aneuploidy, precedes Her-2 amplifications in BE with HGD or EAC, however, in certain cases Her-2 amplification may as well occur without prior chromosome 17 gains or DNA content aneuploidy. The different evolutionary pathways of Her-2 amplification may be related to different behavior of the EAC, while the early gains of chromosome 17 in BE might serve as prognostic marker in future surveillance programs.
INTRODUCTION

Patients with Barrett’s esophagus (BE) are at higher risk for developing esophageal adenocarcinoma (EAC) than the general population.\textsuperscript{1, 2} Invasive EAC usually is preceded by dysplasia\textsuperscript{3}, but adenocarcinoma develops in only a minority of patients with BE.\textsuperscript{4, 5} Because long-term survival of EAC patients is highly dependent on early diagnosis, identification of BE patients with an increased risk for the development of EAC is of great importance.\textsuperscript{5, 7} The present endoscopic and histopathological evaluations of BE have been proven to be insufficient for effective identification of high risk patients.\textsuperscript{8-10} A better understanding of the molecular biology of BE may allow improvement of cancer risk stratification of BE patients.\textsuperscript{3, 11}

Abnormalities in the expression of adhesion molecules and growth factor ligands or receptors of the epidermal growth factor (EGF) family may occur early during cancer development.\textsuperscript{3} Her-2 (neu/c-erB2) proto-oncogene is a member of the EGF receptor family, frequently involved in the pathogenesis of various cancers.\textsuperscript{12} Her-2 is a tyrosine kinase cell membrane receptor, which is normally involved in the signal transduction pathways leading to cell growth and differentiation.\textsuperscript{13} Her-2 is frequently amplified and overexpressed in human cancers such as breast, lung, and colon cancer.\textsuperscript{12} Of interest is that the Her-2 amplification/overexpression has therapeutic and prognostic implications in breast cancer and other carcinomas.\textsuperscript{14} An antibody-based therapeutic approach (trastuzumab/herceptin) targeting the Her-2 protein has proved to be an effective adjunctive treatment for breast cancer.\textsuperscript{15-17} Importantly, the amplification of the Her-2 gene and its protein overexpression have been also found in dysplasia and EAC associated with BE\textsuperscript{18-20}, and some data suggest that, as in breast cancer, the Her-2 alterations correlate with poor prognosis of EAC patients.\textsuperscript{21, 22} In general, however, there is limited insight in the genetic events that precede Her-2 gene amplification during BE malignant progression. Identification of such precursor genetic changes may not only provide important insight in the evolutionary pathways leading to Her-2 amplification, but may also lead to discovery of markers for early detection of malignancy in BE. BE is a premalignant lesion that may serve as a unique \textit{in vivo} human model to study clonal evolution of Her-2 amplification. Patients with BE are monitored periodically to detect early and late malignant changes (dysplasia), and will only be treated in case of progression into high grade dysplasia and/or EAC. This provides us the unique opportunity to investigate the different sequential genetic events which lead to amplification of the Her-2 gene, as these occur in the non dysplasia-dysplasia-adenocarcinoma sequence.
One of the most widely used assays to determine Her-2 gene copy number is DNA fluorescent in situ hybridization (FISH). Most DNA-FISH cytogenetic studies have been performed on biopsies or resection specimens of BE and EAC patients. DNA-FISH is, however, also feasible on BE brush cytology specimens. The advantage of brush cytology includes simplicity, lower cost and the potential to sample a larger area of the BE epithelium when compared to taking random biopsies. Furthermore, cytology samples are more reliable for the enumeration of FISH signals and analysis of subclones within the sample, since there is no artifact caused by nuclear truncation, as is the case when using tissue sections.

The aim of this study was to gain more insights in the sequential chromosomal events that may lead to Her-2 amplification in BE and EAC. Hereto, we determined the frequencies of chromosome 17 gains and Her-2 locus amplification (17q11.2) in the different stages of dysplasia and EAC using FISH on brush cytology specimens of 183 BE and EAC cases. We then analyzed co-existing clones in all cases with Her-2 and/or chromosome 17 copy number changes with respect to overall ploidy status as determined by DNA image analysis. Upon analysis of these clones, we propose a model that describes three evolutionary pathways that lead to the amplification of the Her-2 locus in BE and EAC. Our results gives important inside in the precursor abnormalities as these occur during the evolution of Her-2 gene amplification in BE. These abnormalities may be used as important clinical tools for further evaluation with respect to the biological behavior of BE and EAC and outcome of these patients.

MATERIALS and METHODS

Patients
Barrett’s esophagus (BE) patients and BE associated adenocarcinoma patients (EAC), who underwent endoscopy at the Academic Medical Center in Amsterdam between 2002 and 2007 were included. Only patients that had proven (incomplete) intestinal type of metaplasia in biopsies were enrolled for this study. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. During endoscopy, the brush cytology specimens were taken prior to biopsy. The brushes of the normal squamous epithelium were taken from each patient at least 3 cm above the BE segment, and were used for control purposes. Biopsies for routine histological examination were taken at least per 2 cm in 4 quadrants, and of all suspected visible lesions using the protocol of Reid et al. The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material.
Brush Cytology
Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S. Directly before brushing, the mucosal surface was sprayed with acetylcysteine (50mg/ml) for dissolving the mucus layer. Separate brush samples from BE or adenocarcinoma and normal squamous mucosa were taken from each BE patient. After the procedure the brushes were inserted in a vial with 20 ml of 5% acetylcysteine in 0.9% of NaCl and mixed gently to obtain a homogeneous cell suspension. Subsequently, Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to first generate a single layer of the Barrett or adenocarcinoma cells on the top of the glass slide. A second cytospin spot of the squamous epithelial cells of the same patient was made on the bottom of the same slide. These squamous spots served as internal control for FISH and the ploidy analysis procedures. The cytospin procedure was performed as described previously. The cytospin slides were dried at room temperature, and then stored at -80°C.

Fluorescent in situ hybridization (FISH)
Dual color probe was used combining the chromosomal centromeric probe (CEP) for chromosome 17 SpectrumGreen™ with the locus specific probe (LSI) for Her-2 (17q11.2-q12) SpectrumOrange™ (Vysis, Downers Grove, IL). DNA-FISH was performed according to the manufacturer’s instructions provided by Vysis and as described previously.

Scoring of FISH signals
After the FISH procedure, 100 to 200 interphase nuclei of BE or adenocarcinoma cells were scored per slide by an experienced scorer (A.M Rygiel) using Olympus BX61 fluorescent microscope (Germany). The cases were evaluated without prior knowledge of histology findings. Damaged cells and cells with indistinct and blurry signals were excluded from the analysis. The categories of Her-2 locus abnormalities were determined by calculating the ratio of Her-2 locus signals (orange) to chromosome 17 centromeric signals (green) as described previously. The following categories were distinguished: ratio <2:1 were considered as having no amplification, ratio’s >2 <5:1 were considered as low amplification and ratio ≥ 5:1 was considered as high amplification. More then two green signals (CEP 17) accompanied by the same number of orange signals (Her-2 locus) was considered to be indicative of gain of chromosome 17 (ratio 1:1). Following these criteria the cases were classified as displaying a gain of chromosome 17 (cutoff ≥ 3 % of abnormal nuclei) and an amplification of Her-2 locus (cutoff ≥ 5 % of abnormal nuclei). The cutoff values were obtained from counts in the normal squamous epithelia taken from 20 BE patients without dysplasia and calculated as the mean percentage of squamous nuclei with signal gain plus 3xSD (standard deviation) as described previously.
Feulgen staining
The brush cytology samples of BE or EAC patients displaying gain of chromosome 17 and/or Her-2 amplification were included for Feulgen staining and subsequent DNA content analysis. Each brush cytology slide, apart of the cytospin spot with BE or adenocarcinoma cells, contained also a separate cytospin spot with normal squamous epithelial cells, which served as an internal diploid control. The slides were stained with Feulgen dye using ImagePath Blue Feulgen Stain kit (ImagePath System, Inc., Edwardsburg, Michigan), according to the manufacturer’s protocol. The staining involves treatment with concentrated hydrochloric acid that strips the cells of non-nuclear substances, and hydrolyses chromatin. The Feulgen dye then stoichiometrically binds to the nucleic acids, imparting a blue color. The intensity of the blue color is directly proportional to the amount of DNA content.

Image cytometry
After Feulgen staining, DNA ploidy analysis was performed using the Ariol system with software version 3.0 (Applied Imaging, New Castle, UK). The analysis was performed in a blinded manner with respect to histology and FISH findings. For each brush cytology slide, two separate areas of BE or adenocarcinoma and squamous cytospin spots were scanned (40 x magnification), digitalized and stored in the same file. Subsequently, only the nuclei of interest from BE or the adenocarcinoma (cylindrical or/and atypical cells) were marked for analysis and then quantified for DNA content and compared with the DNA content of normal squamous cells (internal control) from the same slides. The digitalized images of nuclei were converted into a series of pixels, which were quantified on the basis of the integrated optical density (IOD), reflecting the DNA content. Overlapping nuclei, nuclear debris and other artifacts that escaped auto-detection and removal by the system were deleted by the operator. At least 200 BE or adenocarcinoma cells and 100 control squamous cells of each case were quantified. DNA content histograms were automatically plotted in another window using the ploidy software. The mean integrated optical density (IOD) of control squamous cells was assigned as a DNA index (DI) of 1, which served as an internal diploid (2N) standard and reference for DI calculation of the BE cells. The DI values of 0.9-1.1 were considered to be within diploid range. Aneuploidy was defined when ≥ 10% of BE or adenocarcinoma cells had DI main peak ≥ 1.2. The cutoff of less then 10% of a coefficient of variation (CV) for diploid and aneuploid peaks was applied.

Statistical analysis
Differences in frequencies of abnormalities were tested using a chi-square test (2-sided) and statistical significance was set at a P value of <0.05. The statistical analyses were conducted using SPSS software (version 12.0; SPSS, Inc, Chicago, IL).
RESULTS

Patients characteristics
In this study, a total of 183 BE patients were investigated. The population consisted of 150 males and 33 females with a median age of 60 (range 26-84), and a median BE length of 3 cm (range 1-13 cm). The population included a subset of cases that were earlier analyzed for a larger panel of genetic abnormalities in one of our previous DNA-FISH studies. Our population included 118 patients with ND, 14 patients with IND, 12 patients with LGD, 22 patients with HGD and 17 patients with EAC as confirmed by histopathology. Fourteen out of 17 EAC patients were staged according to the Union International Control Center TNM system. The EAC population included 7 patients with T1/T2N0M0, 2 patients with T3N0M0 and 5 patients with T3N1M0 stage.

Evaluation of chromosome 17 and Her-2 locus copy number
Gain of chromosome 17 was detected in 3.5% (4/118) of ND, 21% (3/14) of IND, 16% (2/12) of LGD cases, and further increased to 68% (15/22) and 82% (14/17) in HGD and EAC cases, respectively (p<0.0001; Figure 1A). There was no amplification of the Her-2 locus in cases with ND, IND and LGD. Amplification, however, was detected in 45% (10/22) and 30% (5/17) of HGD and EAC patients, respectively (p=0.34; Figure 1A). Of the 15 cases with Her-2 locus amplification, 5 cases showed a low level and 10 cases a high level of the amplification (Table 1). There was no significant difference between HGD and EAC in the distribution of low and high Her-2 amplification levels (p=0.6, Table 1).

<table>
<thead>
<tr>
<th>Her-2 locus amplification</th>
<th>HGD</th>
<th>EAC</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>4 (40%)</td>
<td>1 (20%)</td>
<td>5 (34%)</td>
</tr>
<tr>
<td>High</td>
<td>6 (60%)</td>
<td>4 (80%)</td>
<td>10 (66%)</td>
</tr>
<tr>
<td>Total No.*</td>
<td>10</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1: Distribution of low and high level of Her-2 locus amplification between HGD and EAC cases. *The total number of cases with Her-2 locus amplification; HGD- high grade dysplasia, EAC- esophageal adenocarcinoma.

DNA ploidy analysis in cases with gain of chromosome 17 and/ or Her-2 amplification
DNA image cytometry measures the entire DNA content of the cells, and in this analysis any other formulation of chromosomes then 2N is considered to be aneuploid. To determine whether the chromosome 17 gains and/or Her-2 amplification are related to overall DNA content aneuploidy, we assessed the ploidy status in the abnormal cases using DNA image cytometry. In this analysis, the median DNA index (DI) value for control cells and normal BE cells (diploid peak) was 1.0 (range 0.9-1.1) with median coefficient of variation (CV) of 4.5
(range 3.17-6.34) and for abnormal cells (aneuploidy peak) the median DI value was 1.8 (range 1.4-2.0) with median CV of 6.3 (range 3.47-9.5).

In total, there were 40 BE patients displaying the chromosome 17/Her-2 abnormalities (Table 2). Of these cases 37 were available for DNA image cytometry analysis. None of the 4 cases with ND, and none of the 5 cases with IND/LGD displaying gain of chromosome 17 showed DNA content aneuploidy (Figure 1B). Aneuploidy was detected in 8 of the 15 cases (53%) of HGD and 8 of the 13 cases (61%) of EAC with gain of chromosome 17 and/or Her2 amplification (Figure 1B).

Figure 1: DNA-FISH and DNA image cytometry analysis in BE/EAC patients. (A) Frequency of chromosome 17 gain and Her-2 locus amplification in BE/EAC patients (n=183), including 118 ND (no dysplasia), 25 IND/LGD (indefinite for dysplasia/low grade dysplasia), 22 HGD (high grade dysplasia) and 17 EAC (esophageal adenocarcinoma) cases. Gain of chromosome 17 was detected in non-dysplasia and increased significantly through IND/LGD to HGD and EAC (p<0.0001). No Her-2 locus amplification was observed in ND, IND and LGD cases but the amplification was detected in comparable frequency in HGD and EAC cases (p=0.34). (B) Frequency of DNA content aneuploidy in the cases displaying gain of chromosome 17 and/or Her-2 amplification (n=37). The population included 4 cases of ND, 5 cases of IND/LGD, 15 cases of HGD and 13 cases of EAC. The DNA content aneuploidy was detected in HGD (53%) and EAC (61%) cases.
Analysis of subclones in the cases with Her-2 locus amplification
In order to gain more insight into the evolution of Her-2 locus amplification, we analyzed the 15 cases with Her-2 amplification for the existence of different subset of clones. We were specifically interested whether chromosome 17 gain and Her-2 amplification is the result of either aneuploidy, or is a ploidy unrelated specific biological event. Using FISH results and ploidy status as determined by DNA image cytometry, we were finally able to distinguish 4 different categories of clones within these 15 cases (Figure 2, Table 2).

Figure 2: Clonal diversity among the cases with Her-2 amplification. Using FISH results and ploidy status as determined by DNA image cytometry, we were able to distinguish 4 different categories of clones within 14 cases displaying Her-2 amplification (1 of 15 cases with Her-2 amplification was not available for image cytometry analysis). Category 1 and 2 were marked by the presence of Her-2 amplification in the clones with gain of chromosome 17 (> 2 copies) and category 3 and 4 by the presence of the amplification in the clones with two copies of chromosome 17. Category 1 and 3 showed overall diploid status (white graph area) while category 2 and 4 showed overall DNA aneuploidy (shaded graph area). There was 1 case (1/14, 7%) in category 1, 5 cases (5/14, 35%) in the category 2, 3 cases (3/14, 22%) in category 3 and 3 cases (3/14, 22%) in category 4. There were 2 cases (2/14, 14%) which were included in both categories 2 and 4 since they displayed Her-2 amplification in clones with two copies of chromosome 17 and in clones with gain of chromosome 17 in the same sample.

Nine of the 15 cases (60%) displayed the low or high level Her-2 amplification in clones with gain of chromosome 17 (> 2 copies of CEP17; Table 2). Image cytometry for overall DNA content analysis was performed in 8 of 9 of these cases. Of these 8 cases, DNA content analysis showed that only one was diploid (Figure 2: category 1; Table 2 case no. 211), while aneuploidy was found in the rest of them (Figure 2: category 2). Subclonal analysis showed that in 5 of these 9 cases, subclones with gains of chromosome 17 without Her-2 amplification coexisted with subclones with gains of chromosome 17 and low or high Her-2 amplification (Table 2: case no. 5, 124, 211, 167, 276).

We found that 6 of the 15 cases (40%) exclusively displayed low or high Her2/neu amplification in cells that had two copies of chromosome 17 (normal
<table>
<thead>
<tr>
<th>Case No.</th>
<th>Histology</th>
<th>Her-2 amplification</th>
<th>Gain CEP 17 (&gt;2 copies)</th>
<th>Aneuploidystatus DI, % of cells</th>
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<td>-</td>
<td>Gain (3)</td>
<td>-</td>
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<td>-</td>
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<td>IND</td>
<td>-</td>
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</tr>
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</tr>
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<td>-</td>
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</tr>
<tr>
<td>98</td>
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<td>-</td>
<td>Gain (5)</td>
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</tr>
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<td>-</td>
<td>Gain (14)</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
</tr>
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<td>HGD</td>
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</tr>
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<td>Low (5)</td>
<td>1.6 (60)</td>
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<td>Nd.</td>
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<td>-</td>
<td>Gain (20)</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>Nd.</td>
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<td>267</td>
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<td>-</td>
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<td>High (50)</td>
<td>Gain (13)</td>
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Table 2: Her-2/chromosome 17 and DNA content status in BE patients with the abnormalities. (-) indicates absence of certain abnormality, (low) indicates low level of Her-2 amplification, (high) indicates high level of Her-2 amplification, DI >1.1- aneuploidy, Nd.- undetermined, Gain- 3 to 6 signals of CEP 17 per cell, ND- no dysplasia, IND- indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia.
Chromosomal events and Her-2 amplification in a BE pattern). When looking at the overall ploidy level of these 6 cases, 3 of these were diploid (Figure 2: category 3; Table 2, case no: 170, 236, 200). The other three cases despite exhibiting the amplification in cells with two copies of chromosome 17, showed DNA-content aneuploidy (Figure 2: category 4; Table 2 case no:173, 193, 202)

Two of the 15 cases had both types of subclones: Her-2 locus amplifications in cells with two copies of chromosome 17 and with gain of chromosome 17, and both of the cases showed DNA content aneuploidy (Table 2, case no: 5 and 4). Therefore, these were classified into category 2 and 4 (Figure 2). Figure 3 shows Her-2 locus amplification displayed in clones with two copies of chromosome 17 and in clones with gain of chromosome 17.

Figure 3: Examples of different categories of BE clones with chromosome 17/Her-2 locus abnormalities. The images were captured by the CytoVision SPOT AX system (Applied Imaging, New Castle, UK). (A) Nuclei that exhibit gain of chromosome 17 (trisomy), including 3 copies of chromosome 17(green) and 3 copies of the Her-2 locus (17q11.2, orange). (B) Nuclei that exhibit low amplification of Her-2 locus in the clone with gain of chromosome 17 (trisomy) – ratio of CEP17 (green) to Her-2 locus (orange) copy number 1:3. C) Nuclei that exhibit high amplification of Her-2 locus in the clone with gain of chromosome 17 (pentasomy) - ratio of CEP 17 (green) to Her-2 locus (orange) copy number 1:≤5 (signal clusters). (D) Normal nuclei that exhibit two copies of chromosome 17 (green) and two copies of Her-2 locus (orange). (E) Nuclei that exhibit low amplification of Her-2 locus in a clone with two copies of chromosome 17 - ratio of CEP 17 (green) to Her-2 locus (orange) copy number - 1:2.5. (F) Nuclei that exhibit high amplification of Her-2 locus in the clone with two copies of chromosome 17 - ratio of CEP 17 (green) to Her-2 locus (orange) copy number- 1:≤5 (signal cluster). For color figure, see page 145.
Figure 4: The pathways leading to evolution of Her-2 locus amplification in a Barrett's esophagus.

The scheme is derived from the analysis of coexisting subclones in 15 BE/EAC cases with Her-2 locus amplification using FISH results and ploidy status as determined by DNA image cytometry. Three main clonal pathways leading to Her-2 locus amplification can be distinguished: (1) evolution of Her-2 amplification from clones with overall DNA content aneuploidy (2) evolution of Her-2 amplification from clones with selective gain of chromosome 17 with overall DNA content diploid (3) Her-2 locus amplification in diploid clones. The dotted arrow indicates that in time clones with selective gain of chromosome 17 may acquire overall aneuploidy.
DIscussion

The amplification of the proto-oncogene Her-2 is known to play a critical role in tumor progression\(^3\), therefore the understanding of sequential genetic events leading to this phenomenon is of major clinical importance. Here, we have evaluated sequential chromosomal events preceding Her-2 locus amplification in BE and EAC. We propose three evolutionary pathways that may lead to this amplification in BE/EAC. In two of these pathways, acquisition of Her-2 amplification is associated with precursor chromosomal abnormalities, i.e., aneuploidy or selective gains of chromosome 17, while in the third pathway the amplification may develop without any of these preceding chromosomal changes (Figure 4).

First important observation was that Her-2 locus amplification is absent in ND, IND/LGD cases but is detectable in comparable frequency in HGD (45\%) and EAC cases (30\%, \(p=0.34\)), which is in agreement with former DNA-FISH studies.\(^{20, 21}\) Further, low and high levels of Her-2 locus amplification were found in both HGD and EAC cases with the high-level amplification slightly more frequent in EAC cases. Previously, Walch et al. noted the heterogeneity of the Her-2 locus copy numbers, yet this was demonstrated only in EAC samples.\(^{29}\) Thus, our study confirmed that Her-2 locus amplification may be considered as a late marker in malignant progression of BE. However, we were mostly interested in the sequential chromosomal events that precede low and high level of Her-2 locus amplification and subsequent malignant progression in BE. In this sequence, gains of chromosome 17 seem to be an early event leading to Her-2 amplifications in BE. This is indicated by the fact that gains of chromosome 17 occur already in ND, increase significantly with dysplasia stage and have high frequency in EAC cases as shown here and in previous studies.\(^{25, 26}\) Interestingly, in this study we demonstrated that all cases of ND and IND/LGD displaying the gains of chromosome 17 were overall diploid by DNA image cytometry, and that these gains were also not associated with aneuploidy in 47\% of HGD and 39\% of EAC cases. This result in few cases could be explained by lower sensitivity of DNA image cytometry to detect small populations of aneuploid cells (less than 10\%). In most cases, however, the chromosome 17 gain was found in relatively large fraction of cells, indicating that gain of single chromosome can not be detect by DNA image cytometry. Indeed, previously it has been suggested that gains of single chromosomes results in too small DNA content change for detection by image cytometry.\(^{31}\) Thus, our results indicate that in the early stages of BE and in certain cases of HGD/EAC, gains of chromosome 17 can be an independent, selective genetic event rather than a random event related to aneuploidy and genetic instability. One, however, could still argue that in some cases these gains could be random events which balance against chromosomal losses in order to result in a net diploid score.
We further questioned whether the selective gain of chromosome 17 or DNA content aneuploidy are the precursor abnormalities of Her-2 locus amplification. Hereto, we analyzed the coexistence of different subclones in the cases with Her-2 amplification. From this clonal diversity, we propose three pathways that may have led to the Her-2 amplification in BE (Figure 4). The most frequent pathway of Her-2 amplification, as seen in 9 of 15 cases, is marked by clones with gain of chromosome 17 plus low or high amplification of the Her-2 locus, including 5 cases with co-existing subclones with gain of chromosome 17 alone. Most of these cases were proved to be aneuploid, thus suggesting that here the chromosome 17 gains was a random event caused by genetic instability, and that the Her-2 locus amplification evolved from an aneuploid background. (Figure 4, pathway 1). Unfortunately, the methodologies used did not allow measurement of the DNA content in exactly the same cells with the 17 gains and/or Her-2 amplifications. Therefore, it could also possible that the aneuploidy was present in distinct clones and even occurred after the chromosome 17 gains and/or Her-2 amplifications. Since we also detected some cases displaying Her-2 amplification in clones with two copies of chromosome 17 but with DNA content aneuploidy, we cannot rule out there were indeed coexisting aneuploid populations of cells without chromosome 17 and/or Her-2 abnormalities. On the other hand, the DNA content aneuploidy detected in cases with disomy of chromosome 17 may also be due to gains of other chromosomes, or aneuploidy followed by loss of extra copies of the chromosome 17 or its centromeres and retention of the Her-2 locus amplification. Aneuploidy is known to be associated with genomic instability. Interestingly, Maley at al demonstrated that expansion of clones with genetic instability, presented as aneuploid populations of cells, was a predictor for developing EAC. The authors suggested that genetic instability may increase the rate of generation of genetic variants (clonal diversity) and subsequent selection of clones with increased predisposition to cancer, which fits well with the first pathway showing that the Her-2 locus amplification evolves on the background of DNA content aneuploidy. However, we were able to distinguish two more pathways leading to Her-2 locus amplification that seem not to be related to overall aneuploidy. The second pathway is illustrated in one case of EAC displaying the Her-2 amplification in a clone with gains of chromosome 17 and with a DNA-content that is diploid, suggesting that in this case the amplification has evolved from a selective gain of chromosome 17 (Figure 4, pathway 2). This observation and the fact that we found gains of chromosome 17 in several cases of ND and IND/LGD without aneuploidy, suggest that selective gains of chromosome 17 may be followed by low and high Her-2 amplifications during BE progression. The third pathway is represented by the cases displaying low or high levels of Her-2 locus amplification in clones with only two copies of chromosome 17 and with diploid DNA content status. Thus, here the Her-2
Chromosomal events and Her-2 amplification in a BE

locus amplification was neither preceded by gains of chromosome 17 nor DNA content aneuploidy (Figure 4, pathway 3). In these cases, however, as well as in the case representative for pathway 2, the presence of genetic instability due to translocations, inactivating mutations, methylations or loss of heterozygosity of genes maintaining the genome stability can not be rule out.\textsuperscript{37}

The clonal evolution of Her-2 locus amplification is certainly very complex one. In this study, most of the clones with Her-2 amplification show either DNA-content aneuploidy or gains of chromosome 17 (Figure 2), suggesting that these abnormalities are precursor changes leading to Her-2 locus amplification in BE. It is important to realize, however, that in a subset of cases the Her-2 amplification may not be preceded by any of these chromosomal abnormalities. The mechanisms underlying the structural chromosomal changes are complex and still poorly understood.\textsuperscript{38} It is, however, known that amplifications of proto-oncogenes, similarly to deletions and translocations, result frequently from breakage-fusion-bridge (BFB) cycles involving the specific breakage of genomic DNA at fragile sites (FSs), leading to the intrachromosomal structural abnormalities.\textsuperscript{38-40} Interestingly, it was suggested, that aneuploid genomes, especially unbalanced aneuploidy (unpaired chromosomes), may be more vulnerable to chromosome breakage at FSs.\textsuperscript{33} Thus, we can speculate that clones with gains of chromosome 17, with or without background of overall aneuploidy, might be more susceptible for DNA breakage at FSs, which may trigger BFB cycles leading to the intrachromosomal amplifications. This hypothesis may be partly supported by a metaphase analysis of the breast cancer cell lines and primary cultures showing no extrachromosomal but only intrachromosomal localization of Her-2 locus amplification within chromosome 17 or other chromosomes as a result of translocation.\textsuperscript{41}

In summary, we identified three distinct evolutionary pathways that may lead to Her-2 amplifications in BE and EAC. Two of these pathways are associated with precursor chromosomal abnormalities, i.e., aneuploidy or selective chromosome 17 gains, while in the third pathway the amplification may develop without any of these preceding chromosomal changes. Future studies will show whether these three evolutionary subtypes may be associated with aggressiveness or different biological behavior of EAC. With respect to the chromosome 17 gains as seen in early stages of BE progression, future prospectively follow studies will demonstrate whether or not this abnormality can be used as an early marker and truly predict later Her-2 locus amplification and subsequent malignant progression of BE.
Chapter 4

REFERENCES


Chromosomal events and Her-2 amplification in a BE


Low level of *Her-2* locus amplification by Fluorescent in situ hybridization does not correlate with *Her-2* protein overexpression by Immunohistchemistry in Barrett’s esophagus

A. M. Rygiel, F. Milano, F. J. ten Kate, M. P. Peppelenbosch, J. J. Bergman, K. K. Krishnadath

Submitted
**ABSTRACT**

**Background & Aims:** Her-2 proto-oncogene alterations are associated with poor survival of patients with Barrett’s esophageal adenocarcinoma (EAC), however, studies correlating Her-2 amplification with Her-2 protein overexpression in Barrett’s esophagus (BE) are lacking. The aim was to correlate Her-2 locus (17q21) and chromosome 17 abnormalities with Her-2 protein overexpression in BE patients.

**Methods:** Thirty four BE cases showing Her-2 amplification and/or gains of chromosome 17 as assessed by fluorescent in situ hybridization (FISH) on brush cytology specimens, were examined by immunohistochemistry (IHC) for the Her-2 protein on biopsies taken at the same time of the brush cytology.

**Results:** Strong overexpression of Her-2 protein (+3) was found in 72% (5/7) of the cases with a high level of Her-2 amplification (Her-2: Cep17 ≥ 5:1), while moderate (+2) or faint (+1) overexpression was seen in 28% (2/7) of these cases. Of the 5 cases displaying a low level of Her-2 amplification (Her-2:Cep17 >2<5:1) one case (20%) showed moderate Her-2 overexpression (+2), while the rest of the cases were negative. Of the 22 cases with gain of chromosome 17, 19 cases (86%) were negative and 3 cases (14%) showed moderate Her-2 overexpression (2+).

**Conclusions:** Low levels of Her-2 locus amplification and gains of chromosome 17 as determined by FISH do not correlate with Her-2 protein overexpression as observed by IHC. FISH on brush cytology is a useful diagnostic tool that, at least in cases with low levels of Her-2 status changes, may be superior to IHC on biopsies to assess the Her-2 status of BE patients.
INTRODUCTION

Barrett’s esophagus (BE) is a pre-malignant condition of the distal esophagus that is associated with an increased risk of developing esophageal adenocarcinoma (EAC).\(^1\)\(^,\)\(^2\) In recent years, the incidence of BE and EAC has been increasing dramatically and death from EAC has become a major problem.\(^1\)\(^,\)\(^3\) Since long term survival of EAC patients is highly dependent on early diagnosis, detection of BE patients at high risk for malignant progression has become crucial.\(^4\) The present endoscopic and histopathologic surveillance of BE patients have been proven to be insufficient for effective identification of high-risk patients.\(^5\)\(^-\)\(^7\) Evaluation of objective, molecular markers may lead to a better rationale for surveillance programs, as well as, targeted therapeutic strategies.

Her-2\(^\text{(neu)}\) is a proto-oncogene localized on chromosome 17q, which encodes for a transmembrane tyrosine kinase growth factor receptor (Her-2/c-erbB-2).\(^8\) Amplification of the Her-2 gene and Her-2 protein overexpression has been studied extensively in many malignancies, especially in breast cancer and its precursor lesions.\(^9\) In breast cancer, Her-2 overexpression has been correlated with poor prognosis or a lack of response to chemotherapy.\(^10\)\(^,\)\(^11\) Interestingly, in the Her-2 positive breast cancers, the antibody-based (transtuzmab/Herceptin) therapeutic approach has been reported as an effective adjunctive treatment option.\(^12\)\(^,\)\(^13\) Importantly, Her-2 amplification and its protein overexpression have been reported in dysplasia and EAC associated with BE as well. Several immunohistochemical studies on BE, suggest that Her-2 protein overexpression is a frequent and early event\(^14\)\(^,\)\(^15\), whereas others indicate that it is much less common and occurs late during the process of BE progression into EAC.\(^16\)\(^,\)\(^17\) Most of the studies that investigated Her-2 gene amplification describe this as a rather late event in BE progression present in only HGD and EAC cases\(^18\)\(^,\)\(^19\), while others indicate that Her-2 amplification can already be seen in LGD.\(^20\) Thus, the association of the Her-2 gene amplification with Her-2 protein overexpression during BE progression is still unclear. The accurate detection of Her-2 alterations in BE and EAC may help to identify high-risk subpopulations and select patients who are the most likely to benefit from Her-2-targeted therapies.\(^21\)

The most widely used assays to determine Her-2 status is immunohistochemistry (IHC) for the detection of protein overexpression, and DNA fluorescent in situ hybridization (FISH) for assessing the gene copy number. Although the IHC staining is the predominant method utilized, it can be significantly affected by technical issues i.e tissue fixation, specificity of the antibody, and variation in quantification and interpretation of the intensity of Her-2 protein expression.\(^22\)\(^,\)\(^23\) DNA- FISH is not as widely available as IHC since it requires appropriate equipment and optimization for each tissue type, but, FISH is quantitatively accurate and very reproducible.\(^24\) This technique can be successfully applied
on both archival paraffin biopsies\textsuperscript{19, 20, 25} and on brush cytology samples.\textsuperscript{26-28} The important advantages of applying brush cytology to BE patients includes simplicity, lower cost and the potential to sample a larger area of the BE epithelium when compared with taking random biopsies. Cytology samples are also more reliable for the enumeration of FISH signals, since there is no artifact caused by nuclear truncation as is the case when using tissue sections.\textsuperscript{29}

Recently, we have evaluated the frequency of Her-2 locus amplification and the chromosome 17 copy numbers using DNA-FISH on brush cytology specimens of BE patients with different stages of dysplasia or EAC. The aim of the present study was to correlate previously detected Her-2 locus amplifications and chromosome 17 abnormalities with Her-2 protein overexpression as assessed by IHC on biopsy specimens in BE patients with various stages of dysplasia or EAC.

\section*{MATERIALS and METHODS}

\subsection*{Patients}
Patients with Her-2/chromosome 17 abnormalities were retrieved from our previous DNA-FISH study investigating the frequency of a variety of genetic abnormalities in BE brush cytology specimens.\textsuperscript{28} These BE patients underwent endoscopy at the Academic Medical Center in Amsterdam between 2002 and 2007. During endoscopy, biopsies for routine histological examination and brush cytology specimens for FISH analysis were taken of all BE patients. The biopsies were taken at least per 2 cm in 4 quadrants and of all suspected visible lesions using the protocol of Reid \textit{et al.}\textsuperscript{30} Only patients that had proven incomplete intestinal type of metaplasia in biopsies were enrolled. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material.

From the total of 183 previously examined BE patients, we retrieved 40 cases having Her-2 amplification or/and gain of chromosome 17. A total of 34 of these 40 cases had adequate biopsy material available that were taken at the same procedure when the brush cytology specimens for FISH analysis. Out of the 34 cases, 22 patients showed gain of chromosome 17, and 12 patients had gain of chromosome 17 and/or Her-2 locus amplification.

\subsection*{Brush Cytology}
Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S as described previously.\textsuperscript{28} From a cell suspension obtained from brushing a single layer of the cells on a glass slide was generated
using the Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA). The cytospin procedure was performed as described previously. After the procedure the cytospin slides were dried at RT, and then stored at -80°C.

**Fluorescent in situ hybridization (FISH)**

Dual color probe was used combining chromosomal centromeric probe (CEP) for chromosome 17 SpectrumGreen™ with the locus specific probe (LSI) for *Her-2* (17q11.2-q12) SpectrumOrange™ (Vysis, Downers Grove, IL). DNA-FISH was performed according to the manufacturer’s instructions provided by Vysis as described previously.

**Scoring of FISH signals**

As described previously, after the FISH procedure, 100 to 200 interphase nuclei of BE cells were scored per slide by an experienced scorer (A.M. Rygiel) using Olympus BX61 fluorescent microscope (Germany). The cases were evaluated without prior knowledge of histology findings. Damaged cells and cells with indistinct and blurry signals were excluded from the analysis. The categories of *Her-2* gene abnormalities were determined by calculating the ratio of *Her-2* locus signals (red) to chromosome 17 centromere signals (green) as described previously. The following categories were distinguished: a ratio <2 were considered as having no amplification, ratio’s >2 and <5 were considered as low amplification and ratio ≥ 5 was considered as high amplification. More then two green signals (CEP 17) accompanied by the same number of red signals (*Her-2* locus) was considered to be indicative of gain of chromosome 17 (ratio 1:1). Following these criteria the cases were classified as displaying a gain of chromosome 17 (cutoff ≥ 3 % of abnormal nuclei) and low or high level amplification of *Her2/neu* locus (cutoff ≥ 5 % of abnormal nuclei). The cutoff values were obtained from counts in the normal squamous epithelium taken from 20 BE patients without dysplasia and calculated as the mean percentage of squamous nuclei with signal gain plus 3xSD (standard deviation).

**Immunohistochemistry (IHC)**

Immunohistochemistry (IHC) was performed on archival material from paraffin embedded tissue obtained during the same endoscopy procedure as the brush cytology. IHC for *Her-2* protein (antibodies/*Her-2/neu* / c-erbB-2 Ab-17 clone e2-4001+ 3 B5, mouse monoclonal, Neomarkers™, Stratech Scientific Ltd, Cambridgeshire, UK) was performed according to a standard IHC protocol. Briefly, paraffin sections were deparaffinised and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 20 minutes and then washed (3 x 5 mins in PBS). Antigen retrieval was performed by boiling slides for 10 min in 0.01M Sodium Citrate Ph 6.0.
Non-specific binding sites were blocked with 5% goat serum in PBS for 10 mins, and then washed (3x 5 mins in PBS). Slides were incubated with the primary antibodies diluted 1: 2000 in Normal Antibody Diluent (Scytek, Logan, Utah, USA) for 60 minutes. After washing (3 x 5 mins in PBS), post-antibody blocking solution (Immunologic, Duiven, The Netherlands) diluted 1:2 in PBS was applied for 15 minutes. After washing (3 x 5 mins in PBS), slides were then incubated with biotinylated secondary antibodies diluted 1:2 in PBS (Poly-HRP-Goat anti Mouse IgG, Immunologic) at room temperature for 30 minutes. Slides were washed (3 x 5 mins in PBS), and then the peroxidise activity was detected with ‘Fast DAB’ (3,3’-diaminobenzidine, Sigma, St Louis, MO) with 0.05% hydrogen peroxide. Finally, sections were counterstained with Mayer haematoxylin, dehydrated and mounted with Pertex under cover slips. Her-2 protein expression was evaluated by an experienced pathologist (F. ten Kate) according to the scoring system recommended by the DACO HercepTest. No staining at all or membrane staining in <10% of the epithelial cells was considered negative (score 0). Faint or barely perceptible, incomplete membrane staining in > 10% of the epithelial cell was scored 1+. Weak to moderate staining of the entire membrane in > 10% of the epithelial cells was scored 2+, and strong staining the entire membrane in > 10% of the epithelial cells resulted in a score 3+.

RESULTS

Patients
Of the 34 cases included in this study, there were 31 male and 3 female with a median age of 60 (range 26-84), and median BE length of 6 cm (range 1-13 cm). These cases included 4 patients with ND, 5 patients with IND or LGD, 13 patients with HGD and 12 patients with EAC as determined by histopathology. Of these patients, there were 22 patients (4 ND, 5 IND/LGD, 4 HGD and 9 EAC) with gain of chromosome 17 and 12 patients (9 HGD and 3 EAC) with Her-2 locus amplification. Of the 12 cases with the Her-2 locus amplification 5 patients (41%) showed low level, and 7 patients (58%) a high level of the Her-2 locus amplification.

Correlation between gain of chromosome 17, Her-2 locus amplification and Her-2 overexpression
The comparison between chromosome 17 status, the levels of Her-2 locus amplification and Her-2 protein expression is presented in table 1 and 2. Of the 7 cases with a high level of Her-2 amplification, 5 (72%) showed strong overexpression of the protein (3+), including 3 HGD and 2 EAC patients, whereas two cases with HGD (28%) showed moderate or faint Her-2 overexpression (1-2+).
Her-2 amplification and overexpression in a BE

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Table 1: FISH, IHC and histology results. (-) indicates absence of certain abnormality; gain - ratio of 1:1 of Her-2:Cep17, the number of signals in different cases varied from 3 to 6 signals per cell; low amplification - ratio of >2<5:1 of Her-2:Cep17; high amplification - ratio of ≥5:1 of Her-2:Cep17; IHC score 0 - no Her-2 staining, IHC score +1 - faint Her-2 staining, IHC score +2 - moderate Her-2 staining, IHC score +3 - strong Her-2 staining; ND- no dysplasia; IND- indefinite for dysplasia; LGD- low grade dysplasia; HGD- high grade dysplasia
Figure 1: Her-2 status as determined by IHC on BE biopsy specimens and FISH on BE brush cytology specimens. (A) strong overexpression of Her-2 protein (3+) in HGD (B) moderate overexpression of Her-2 protein (2+) in EAC, (C) no overexpression of Her-2 protein (score 0) in EAC; (D) high level of Her-2 locus amplification (clusters) in cytology sample of HGD case- CEP 17 (green) and Her-2 locus (red), (E) gains of chromosome 17 in cytology sample of EAC case- CEP 17 (green) and Her-2 locus (red), (F) low level of Her-2 amplification (ratio of >2<5:1 of Her-2, red :Cep17 :green) in cytology sample of EAC case, CEP 17 (green) and Her-2 locus (red). For color figure, see page 146
Of the 5 patients displaying a low level of Her-2 amplification only one case (20%, HGD) showed moderate Her-2 overexpression (+2) while 4 other cases (80%), including 1 EAC and 3 HGD cases, were negative for protein overexpression (0-1+). Of the 22 cases with gain of chromosome 17, 3 cases showed moderate Her-2 overexpression (2+), including 1 case of HGD and 2 cases of EAC, whereas the rest showed faint or no staining of the Her-2 protein (0-1+). Figure 1 shows examples of different Her-2 status as determined by IHC and FISH in biopsy and brush cytology specimens of BE patients, respectively.

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<th>FISH</th>
<th>IHC</th>
<th>No. (%)</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>Cep17 Gain</td>
<td>14 (63)</td>
<td>5 (23)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Her-2 low amplification</td>
<td>3 (60)</td>
<td>1 (20)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Her-2 high amplification</td>
<td>0</td>
<td>1 (14)</td>
<td>1 (14)</td>
</tr>
</tbody>
</table>

Table 2: Correlation of Her-2 locus amplification and gain of chromosome 17 with Her-2 protein status.

**DISCUSSION**

It is generally thought that Her-2 gene amplification is always coupled with Her-2 protein overexpression. Here, we correlated the Her-2 locus and chromosome 17 abnormal status with Her-2 protein overexpression in the histological sequence of no-dysplasia-dysplasia-adenocarcinoma in BE. We found important differences in the association between the level of Her-2 locus amplification and Her-2 protein overexpression. We demonstrated that, independently of the BE stage, only a high level of Her-2 amplification (ratio of Her-2:Cep17 ≥ 5:1), correlates with strong Her-2 protein overexpression (3+), whereas low levels of Her-2 amplification (ratio of Her-2:Cep17 > 2<5:1) and chromosome 17 gain (ratio of Her-2:Cep17 1:1) is associated with moderate or no Her-2 overexpression.

In general, the majority of our cases (72%) with high level of the Her-2 locus amplification showed strong protein overexpression (3+ score), which is in agreement with literature data showing a high correlation between IHC 3+ staining and amplifications detected by FISH. In EAC cases, high levels of Her-2 amplification were uniformly associated with strong protein overexpression, whereas this association was found in 60% (3/5) of the HGD cases. Two HGD cases with high level of Her-2 amplification showed faint or moderate Her-2 overexpression. This discordance may be due to heterogeneity of the lesion or to the subjective interpretation of the staining intensity. Further, we demonstrated...
that the low level of Her-2 amplification locus does not correlate with strong Her-2 overexpression. Only one case (HGD case) with low Her-2 amplification (20%) showed moderate Her-2 staining, whereas the rest of them showed no or faint Her-2 staining (80%). In several of these cases, the gene amplification as found by FISH was confined to a relative low percentage of cells (<10%), which may partly explain the discordance between IHC and FISH. Another reason of this finding may be due to our methodology. We applied DNA-FISH on brush cytology samples and compared these results to IHC on biopsy specimens. In case of tumor heterogeneity, random biopsies may have missed areas with the Her-2 amplification due to sampling errors, while brush cytology potentially samples the whole or majority of BE surface and may better represents the different cellular clones that may coexist in BE. However, since we did not compare FISH on brush cytology with FISH on biopsies, a definite conclusion on this issue can not be drawn. Nevertheless, a similar disconcordance between low Her-2 amplification levels and the protein overexpression was also found in ovarian tumors comparing IHC with FISH on biopsy samples. Moreover, some studies on breast cancer comparing IHC with FISH demonstrated that only a minority of cases (3-7%) with low levels of Her-2 amplification shows protein overexpression. This indicates that in general DNA-FISH seems to be a more sensitive technique then IHC to detect low levels of Her-2 status changes. This is also supported by the fact that the majority of our cases with gain of chromosome 17 (86%) showed no Her-2 protein overexpression, and only three cases (14%) showed moderate overexpression, including 2 EAC cases and 1 case with HGD. These observations are consistent with the studies evaluating Her-2 status by IHC and FISH on BE/EAC tissue sections, showing the association of chromosome 17 gain with moderate (2+) Her-2 protein overexpression in some cases but no association with strong overexpression (3+). In general, our results indicate that low levels of Her-2 locus as a result of either low Her-2 locus amplification or gain of chromosome 17 determined by FISH does not correlate with Her-2 protein overexpression by IHC. This is probably because FISH, as demonstrated in breast cancer studies, is quantitatively accurate and very reproducible. The other explanation is that particularly in cases with low-level Her-2 amplification, gene transcription and posttranscriptional or posttranslational events could be down-regulated or abnormal, ultimately leading to low Her-2 protein levels or abnormal epitope production. Alternatively, tissue preservation could have been insufficient, leading to protein degradation.

There is no consensus with regard to the optimal test for Her-2 assessment so far. Although, IHC staining is the predominant method utilized, it can be significantly affected by technical issues, especially in archival fixed paraffin tissues, resulting frequently in false positive result since the scores are based on the staining intensity. Therefore, in breast cancers it is strongly recommended
Her-2 amplification and overexpression in a BE

that IHC 2+ cases are confirmed by FISH to more appropriately select the patients for targeted therapy, for instance with the anti Her-2 monoclonal antibody Herceptin. In our study, we examined a selected group of BE patients displaying Her-2 amplification and gain of chromosome 17, therefore, we excluded the possibility for false positive results by IHC. However, due to this selection we were able to show that IHC may give false negative results since it does not correlate with low levels of Her-2 amplification (ratio of >2<5:1 of Her-2:Cep17), which in breast cancer is regarded as an indication for immunotherapy with Herceptin.

Our results suggest that DNA-FISH on brush cytology samples is a representative and useful diagnostic tool, which at least in cases with low level of the Her-2 amplification, is superior to IHC. In the near future, the Her-2 amplification status in BE patients will be highly relevant for proper selection of patients that are eligible for treatment with Hercepitin (Transtuzmab) or other Her-2 targeted molecular therapies.
REFERENCES


Her-2 amplification and overexpression in a BE


Gains and amplifications of *c-myc*, *EGFR* and 20.q13 loci in the no dysplasia - dysplasia - adenocarcinoma sequence of Barrett’s esophagus

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Submitted
ABSTRACT

Background & Aims: Barrett’s esophagus (BE) is a condition associated with an increased risk of developing esophageal adenocarcinoma (EAC). The progression of BE to cancer is characterized by increasing dysplasia and the accumulation of genetic abnormalities. The goal of this study was to evaluate copy number alterations of several oncogene loci including 7p12 (EGFR), 8q24 (c-myc) and 20q13 in the sequence of no dysplasia- dysplasia - adenocarcinoma of BE.

Methods: Multi-color FISH using a panel of DNA probes for the centromeric region of chromosome 7 and for the locus specific regions of 7p12 (EGFR), 8q24 (c-myc) and 20q13 was applied on 99 brush cytology specimens of BE patients with different stages of dysplasia or EAC.

Results: Gains (3-4 copies) of chromosome 17, 8q24 (c-myc) and 20q13 loci were found in the low frequencies in non-dysplatic BE. Their combined frequency significantly increased with the stage of dysplasia and reached a high incidence in EAC (p<0.0001). Amplification (>4 copies) of at least one of the loci was observed in 14% of high grade dysplasia (HGD) and increased to 50% in EAC cases (p=0.015). The most frequently amplified locus was c-myc (18%) followed by 20q13 (13%) and EGFR (11%) in the HGD/EAC cases. High- amplification levels (>10 copies) of the loci were significantly more frequent in EAC (72%) compared to HGD (20%, p=0.049).

Conclusions: Amplifications of the c-myc, EGFR and 20q12 loci may serve as diagnostic markers to identify BE patients with HGD or EAC. Gains of the loci might be of value as prognostic markers since they are already present in ND and LGD cases and may precede the later event of the amplification as observed in HGD and EAC.
INTRODUCTION

Barrett’s esophagus (BE) is a condition, which involves replacement of normal squamous epithelium with a columnar type of mucosa.\textsuperscript{1,2} Compared to the general population, patients with BE have an increased risk to develop esophageal adenocarcinoma (EAC).\textsuperscript{3-5} The transformation of BE to invasive EAC may follow a predictable progression of histological changes, known as dysplasia, but adenocarcinoma develops in only a minority of patients with BE.\textsuperscript{5} Since long term survival of EAC patients is highly dependent on early diagnosis, identification of BE patients at high risk for malignant progression is important.\textsuperscript{6-8} The present endoscopic and histopathologic evaluations of BE do not allow effective identification of high-risk patients at an early stage.\textsuperscript{9,10} Therefore, it is of importance to search for specific markers that can identify BE patients at a higher risk for EAC and serve as a supplement to the histopathologic staging of dysplasia.\textsuperscript{11,12}

Numerous studies have demonstrated the accumulation of genetic abnormalities from BE cells to invasive malignant cells.\textsuperscript{11,13-15} Among the most important genetic changes contributing to BE progression are mutations and allelic losses involving \textit{p16} and \textit{p53} tumor suppressor genes and DNA ploidy changes.\textsuperscript{13,16-18} In addition to chromosomal deletions, amplification of oncogenes and growth factors also may play an important role in promoting neoplastic progression.\textsuperscript{19-21} A variety of gains and amplifications of chromosomal regions have been detected in EAC specimens and adjacent mucosa by Q-PCR, traditional cytogenetic and metaphase-based comparative genomic hybridization (CGH) techniques.\textsuperscript{19,22-24} Among the genomic amplifications suggested to play a role in EAC are \textit{c-myc} and \textit{EGFR}, and amplification of the 20q13.2 locus.\textsuperscript{19,23,25}

One of the methods to detect the cytogenetic abnormalities is fluorescent in situ hybridization (FISH), which uses fluorescently labeled DNA probes to pericentromeric chromosomal regions or unique chromosomal loci. The DNA-FISH technique gives an accurate quantification of the signals and has advantages over CGH methodologies, particularly in analyzing the alteration of specific genes. DNA-FISH allows also simultaneous evaluation of genetic abnormalities with cellular morphology and can be successfully applied to both tissue section\textsuperscript{14,23,26} and brush cytology specimens of BE.\textsuperscript{27-29} DNA-FISH on brush cytology specimens 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 with taking random biopsies.\textsuperscript{30,31} Therefore, the purpose of the present study was to assess the frequency of \textit{c-myc}, \textit{EGFR}, and 20q13 locus gains and amplifications in the sequence of no dysplasia-dysplasia-adenocarcinoma in BE patients using DNA FISH on brush cytology specimens. DNA probes for the centromeric region of chromosome (CEP) 7 and probes for the locus specific regions (LSI) of 7p12 (\textit{EGFR}), 8q24.12 (\textit{c-myc}), 20q13.2 were
applied on 99 brush cytology specimens of BE patients with different stages of dysplasia or EAC. Hereupon, FISH outcomes were compared to histopathology.

MATERIALS AND METHODS

Patients
In this prospective study, a cohort of 99 BE and BE associated adenocarcinoma patients, who underwent endoscopy at the Academic Medical Center in Amsterdam between 2002 and 2007, were included. The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material. Only patients that had proven (incomplete) intestinal type of metaplasia in biopsies, taken during endoscopy were included. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. During endoscopy, the brush cytology specimens were taken prior to biopsies. The brushes of the normal squamous epithelium were taken from patients without dysplasia at least 3 cm above the BE segment, and were used for control purposes. Biopsies for routine histological examination were taken at least per 2 cm in 4 quadrants and of all suspected visible lesions using the protocol of Reid et al. 9

Brush Cytology
Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S. Directly before brushing, the mucosal surface was sprayed with acetylcysteine (50mg/ml) for dissolving the mucus layer. After the procedure the brushes were inserted in a vial with 20ml of 5% acetylcysteine in 0.9% of NaCl, mixed gently to obtain a homogeneous cell suspension. The cell suspension from the brush was poured into a 50 ml conical tube and centrifuged at 2100 rpm for 10 minutes at 4 ºC. The majority of supernatant was discarded leaving the pellet in 5ml of solution that was subsequently agitation to generate a cell suspension. A Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to generate a single layer of the cells on a glass slide. First, 50 µl of phosphate-buffered saline (PBS) was loaded to the cytospin chambers and centrifuged for 1 min at 550 rpm at room temperature (RT). Subsequently, up to 150 µl of cells suspension was loaded into the cytospin chambers and centrifuged 2 min at 550 rpm at RT. The cytospin slides were dried overnight at RT, and then stored at -80ºC until FISH was performed.

Fluorescent in situ hybridization (FISH)
We used directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 7 and the locus specific probes (LSI) for regions of 7p12 (EGFR),
Amplification of proto-oncogenes in a BE

8q24.12-q24.13 (c-myc) and 20q13.2, obtained from Vysis (Downers Grove, IL). Dual color probes were used: CEP 7 SpectrumGreen with LSI EGFR (7p12) SpectrumOrange and LSI 20q13.2 SpectrumOrange with LSI c-myc (8q24.12-q13) SpectrumGreen. DNA-FISH was performed according to the manufacturer’s instructions provided by Vysis as described previously.²⁹

Criteria for determining FISH abnormalities
After the FISH procedure, 100 to 200 interphase nuclei of BE cells were scored per slide by an experienced scorer (A.M Rygiel) using Olympus BX61 fluorescent microscope (Germany). The cases were evaluated without prior knowledge of histology findings. Damaged cells and cells with indistinct and blurry signals were excluded from the analysis. The following categories of abnormalities were distinguished: 3-4 signals of CEP 7 (green) accompanied by the same number of signals for EGFR locus (7p12, orange) was considered as a gain (polysomy) of chromosome 7, >4< 10 signals of EGFR locus corrected for chromosome 17 copy numbers (green) were considered as low amplification and ≥10 EGFR locus signals corrected for chromosome 17 copy numbers or a tight cluster were considered as high amplification. Similarly, for the 8q24 (c-myc) and 20q13.2 locus 3 or 4 signals were considered as gain, >4 <10 signals were considered as low amplification and number of signals ≥ 10 or a tight cluster was considered as high amplification. The cutoff value for gains and amplifications of the loci was ≥ 4% of abnormal nuclei. The cutoff values were obtained from counts in the normal squamous epithelium (100 nuclei evaluated) of 20 BE patients without dysplasia and calculated as the mean percentage of squamous nuclei with signal gain plus 3xSD (standard deviation).

Statistical analysis
Differences in frequencies of abnormalities were compared using Fisher exact test and statistical significance was set at a P value of <0.05. The statistical analyses were conducted using SPSS software (version 12.0; SPSS, Inc, Chicago, IL).

RESULTS

Patients and histopathology
Of 99 BE patients 80 were men and 19 women, median age was 62 (range 31-87), median length of BE segment was 4 cm (range 1-13cm). The study included 36 patients with no dysplasia (ND), 11 patients with low grade dysplasia (LGD), 14 patients with indefinite for dysplasia (IND), 22 patients with high grade dysplasia (HGD) and 16 patients with EAC. The EAC patient population included 5 patients with T1/T2N0M0, 3 patients with T3N0M0 and 4 patients with T3N1M0 stage. The TNM classification data were not available for 4 of 16 EAC patients.
**Frequency of the amplification of the oncogenic loci in BE**

Detailed FISH results together with histology diagnosis are presented in Table 1. Gains of chromosome 17, c-myc and 20q.13 loci were detected already in non-dysplastic cases in a low frequency of 8%, 14% and 8%, respectively (Figure 1). Their combined frequencies were 22% (8/36) in ND, 24% (6/25) in IND/LGD cases and significantly increased to 72% (16/22) in HGD and 94% (15/16) in EAC cases (p<0.0001). Amplification of any of the loci was found in none of ND and IND/LGD patients but it was detected in 14% (3/22) of HGD and 50% (8/16) of EAC patients (p=0.015). In HGD patients, the amplification mostly concerned the c-myc locus, seen in 14% (3/22) of the cases, and EGFR and 20q13 locus amplification was detected in a lower frequency of 5% (1/22 each, Figure 1). In EAC cases, amplifications of c-myc, 20q13 loci and EGFR locus were observed in the frequency of 25%, 25% (4/16) and 19% (3/16), respectively (Figure 1). Although the frequencies of the amplification of c-myc, 20q13 and EGFR locus were higher in EAC compared to HGD (25% vs. 14%, 25% vs. 5% and 19% vs. 5%, respectively), these differences did not reach statistical significance (p=0.38, p=0.06, and p=0.15 respectively).

![Figure 1](image)

**Figure 1:** The frequencies of gains and amplifications of the oncogenic loci in the sequence of no-dysplasia-dysplasia-adenocarcinoma in BE. The analysis induced 36 ND cases, 25 IND/LGD cases, 22 HGD cases and 16 EAC cases. ND- no dysplasia, IND- indefinite for dysplasia, LGD- low grade dysplasia, HGD - high grade dysplasia, EAC- esophageal adenocarcinoma.
In total, we detected 16 amplification events of c-myc, EGFR or 20q13 in 11 cases (3 cases of HGD and 8 cases of EAC), including low and high levels of amplification of these loci (Table 1). High amplification events had significantly lower frequency in HGD cases (20%, 1/5) compared to EAC cases (73%, 8/11; p=0.049). Figure 2 depicts examples of BE nuclei with the oncogenic loci copy number changes.

**Figure 2: Examples of BE nuclei with the oncogenic loci copy number change.** The images were captured by the CytoVision SPOT AX system (Applied Imaging, New Castle, UK) (A) Nuclei that exhibit gain of chromosome 7 (trisomy)- 3 copies of chromosome 17 (green) and 3 copies of the EGFR locus (7p12, orange). (B) Nuclei that exhibit low amplification of EGFR - 7 copies of EGFR locus (orange) and 2 copies of CEP7 (green). (C) Nuclei that exhibit high amplification of EGFR locus and gain of chromosome 17- signal cluster of EGFR locus (orange) and 4 signals of CEP 7 (green) (D) Nuclei that exhibit gain of c-myc locus (8q24,) and low amplification of 20q13 locus - .4 copies of c-myc locus (green) and 8 copies of 20q13 locus (red). (E) Nuclei that exhibit high amplification of c-myc locus (8q24) - signal cluster of c-myc locus (green) and two copies of 20q (red) (F) Nuclei that exhibit high amplification of 20q13 locus- signal cluster of 20q13 locus (red) and two copies of c-myc locus (green). For color figure, see page 147.
### Table 1: Detailed FISH results and histology diagnosis of BE cohort with abnormalities.

(gain): indicates gain of chromosome 7 (Cep 7) or locus specific probe; (low): indicates low level of the locus amplification; (high): indicates high level of the locus amplification; ND- no dysplasia, IND- indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia, EAC- esophageal adenocarcinoma.
DISCUSSION

In the present study, we assessed the frequencies of \( c\text{-}myc \), \( EGFR \), and 20q.13 locus gains and amplifications in the sequence of no dysplasia- dysplasia-adenocarcinoma of BE. DNA-FISH on brush cytology samples revealed that gains, which presumably are the precursor changes before true amplifications of the loci occur, appear early in non-dysplastic BE. We found that low and high-level amplifications of the investigated loci occur late, only in HGD and EAC cases.

We showed that gains (3-4 copies) of chromosome 17, \( c\text{-}myc \) and 20q.13 loci can already be seen, at low frequencies of 8-14%, in non-dysplastic BE. Their combined frequencies significantly increase with increasing stage of dysplasia and have high incidence in EAC. These findings correspond well with CGH and FISH studies examining the same chromosomal regions. 23, 28, 32 The detected gains in our study may reflect aneuploidy. Aneuploidy is indicative for BE malignant progression as shown by DNA flow cytometry studies.33-35 We further observed that amplification of at least one of the loci (\( c\text{-}myc \), \( EGFR \), and 20q.13) is seen in 14% of HGD and increases significantly to 50% in EAC patients. This is concordant with other reports showing gene amplification as a late event with higher frequencies in EAC compared to HGD cases. 25, 36 In addition, we found low levels (>4<10 copies) and high levels (>10 copies or clusters) of the loci amplifications in both HGD and EAC, indicating extensive genetic heterogeneity of these cases. We also showed that high amplification events have significantly higher frequency in EAC cases (72%) compared to HGD cases (20%). This finding corresponds well with the study by Miller et al using Q-PCR which demonstrated much higher levels of oncogene loci amplifications in adenocarcinoma tissues compared to HGD. 19 To date, there is a very limited number of DNA-FISH studies investigating different levels of the oncogene loci amplification in BE. Previously, different levels of \( Her\text{-}2 \) oncogene amplifications have been described in EAC cases and adjacent HGD samples by Walch et al. 23, 36 In general, our results suggest that the low and high levels of oncogenic loci amplification initiate in HGD and impel further malignant transformation and invasive growth. Therefore, low and high levels of these loci amplification may be considered as late markers for BE malignant progression. Importantly, our results indicate the \( EGFR \), \( c\text{-}myc \) and 20q.13 amplification may be preceded by early gains of these loci, which are detectable already in non-dysplastic cases, and therefore may be good candidates for early markers of BE malignant progression.

The \( c\text{-}myc \) proto-oncogene encodes a transcriptional factor involved in the regulation of normal cellular proliferation, differentiation and apoptosis. 37 \( C\text{-}myc \) amplification and overexpression of the protein is found in a variety of human tumors. 38 In this study, \( c\text{-}myc \) locus (8q24) amplification was the most frequent one, detected at comparable frequency in HGD (14%) and EAC (25%) cases. Previously, \( c\text{-}myc \) locus amplification has been found in 14-25% of esophageal
cancers, which corresponds well with our results.\textsuperscript{21} The \textit{c-myc} amplification has been also documented in BE patients with HGD.\textsuperscript{19,20,36}

It has been shown that an increased copy number of 20q is associated with cellular immortalization and amplification of 20q13.2 has been correlated with genomic instability.\textsuperscript{39,40} Interestingly, many human tumor types e.g., breast cancer\textsuperscript{41}, ovarian cancer\textsuperscript{42} and head-and-neck cancer\textsuperscript{43}, display gain or amplification of this region suggesting that the genes on 20q play an important role in carcinogenesis. In this study, we found the 20q13 loci amplification in a much lower frequency in HGD (5\%) compared to EAC (25\%). The frequency of 20q13 amplification in the EAC cases is, however, lower then that detected in the study by Falk et al.\textsuperscript{27} who demonstrated this abnormality in 62\% of EAC cases (5 out of 8 patients). In that study, however, a smaller number of patients were analyzed and there was no discrimination between gains and amplification, which may explain this discrepancy. It is important to note that the 20q13.2 probe used in the present study lies at the center of the region within 20q. Several candidate genes have been proposed as potential target genes in this region, e.g. \textit{NABCI}, \textit{BTAK}, \textit{ZNF217}, \textit{BCASI} and it is likely that more then one putative oncogene is involved in the overrepresentation of 20q in BE.\textsuperscript{44-46}

The epidermal growth factor receptor (\textit{EGFR}), another proto-oncogene investigated in this study, plays an important role in tumor cell survival and proliferation. \textit{EGFR} is amplified and overexpressed in many epithelial cancers, including lung non-small-cell carcinoma and colorectal adenocarcinoma.\textsuperscript{47,48} Here, we found \textit{EGFR} locus amplification in 19\% of EAC which fits with the 8-30\% range of the locus amplification reported in studies using PCR techniques.\textsuperscript{19,49} In these studies, the \textit{EGFR} amplification was not detected in HGD cases. In contrast, we clearly found low amplification of the \textit{EGFR} locus in one of the HGD case. In a recent FISH study, gains of the \textit{EGFR} locus were reported but amplification was not described in HGD cases.\textsuperscript{28}

To our knowledge, this is the first study discriminating between gains, low and high levels of amplification of the \textit{c-myc}, \textit{EGFR} and the 20q13.2 loci in the sequence of metaplasia-dysplasia-adenocarcinoma of BE using FISH on brush cytology specimens. Our results indicate that the amplification of these loci, observed in EAC and to a lesser extend in HGD, initiates in HGD and impel subsequent malignant growth. Detection of these amplifications may help to identify HGD patients at higher risk for developing cancer. In addition, this study shows that gains of the investigated chromosomal loci, which are highly prevalent in EAC and HGD, occur already at the stage of ND. These gains may be a result of early polyploidization, preceding the later genetic event of amplification as observed in HGD and EAC. Prospective follow-up of the BE cohort displaying these gains will determine whether any of them are predictive as early markers for progression to malignancy.
REFERENCES


Amplification of proto-oncogenes in a BE


Association between Y-chromosomal haplotypes and susceptibility to Barrett’s esophagus


Manuscript in preparation
ABSTRACT

Background & Aims: Barrett’s esophagus (BE) is a metaplastic condition of the distal esophagus, which predisposes for esophageal adenocarcinoma (EAC). It is believed that BE results from mucosal damage caused by gastro-esophageal-reflux disease (GERD), however, only 5-20% of patients with GERD develop BE. Of interest is that BE and EAC are most prevalent in Caucasian populations, and occur 3-7 times more frequently in males than in females. Since the Y chromosome is the most obvious genetic determinant of sexual dimorphism, we hypothesized that Y chromosomal haplotypes are associated with a susceptibility to BE in Caucasian males.

Methods: The frequency of seven Y chromosomal haplotypes (AB-C, DE, F(xJ,xK), K(xP), J, P(xR1a) and R1a) was determined in 463 men with BE or BE associated EAC, 183 age-matched controls with GERD symptoms but without BE, and 256 Dutch Caucasian males from the general population.

Results: Y haplotypes DE and J were significantly associated with a lower risk of BE presence in GERD patients with odd ratios of 0.34 (0.16-0.73; 95%CI) and 0.44 (0.2-0.98; 95%CI), respectively. Comparison of the Y haplotype frequencies between patients with BE/EAC and Dutch Caucasians from general the population did not show any significant difference.

Conclusions: This study links for the first time the Y-haplotypes DE and J with a lower susceptibility for BE in Dutch Caucasian men with GERD. We suspect that these haplotypes are linked to genetic variants that protect against BE development.
INTRODUCTION

Barrett’s esophagus (BE) is a metaplastic change from squamous epithelium to columnar epithelium in the distal esophagus. Individuals with BE have a 30- to 125-fold increased risk of developing esophageal adenocarcinoma (EAC) compared to the general population.\(^1\)\(^-\)\(^3\) It is commonly believed that the occurrence of BE results from mucosal damage caused by gastro-esophageal-reflux disease (GERD). However, only 5-20% of patients with GERD develop BE, and not all patients with BE have a history of GERD.\(^4\)\(^-\)\(^6\) The precise reason why certain individuals with GERD do not and others do develop BE is unclear. Besides environmental factors, there is substantial evidence for a genetic predisposition for developing BE.\(^7\) Identification of genetic factors associated with BE may allow for a more rational BE patient identification.

It is known that BE and EAC are most prevalent among Caucasians and occur 3-7 times more often in males than in females.\(^8\)\(^-\)\(^10\) Interestingly, loss of the Y chromosome, is one of the most consistent genetic changes that has been observed in BE. Several studies have shown that Y chromosome loss occurs already in the stage of metaplasia and has a high frequency in dysplasia and EAC.\(^11\)\(^-\)\(^15\) Importantly, the Y chromosome loss and rearrangements have been associated also with some types of cancers, suggesting that both tumor suppressors and oncogenes exists on this chromosome and may act at different points in the multistep process of carcinogenesis.\(^16\)

The relevant genetic features of the Y chromosome include the haploid status, and absence of recombination over most of its length (male specific region- MSY). Therefore, the MSY region of the Y chromosome is transmitted as a block from father to the male offspring, with all functional variants and polymorphisms being linked and preserved over generations.\(^17\)\(^,\)\(^18\) Genotyping for Y-chromosomal polymorphic markers allows both, distinguishing individual Y chromosomes, and the robust definition of Y haplotypes, which has been mainly used for investigation of human evolution and for forensic purposes or paternity analysis.\(^19\)\(^-\)\(^22\) Interestingly, definition of Y haplotypes can be also applied as an indirect approach to look for an association between genes on the Y chromosome and possible Y-linked phenotypes or diseases. An increased or decreased frequency of a particular Y haplotype in an affected population may unmask the presence of a functional variant, which is in linkage with a polymorphism defining the haplotype.\(^16\) Indeed, several correlations have already been reported between Y chromosome variants and diverse phenotypes, such as protection against Y chromosome transfer to the X chromosome leading to XX-Y\(^+\) maleness,\(^23\) high blood pressure,\(^24\) alcoholism,\(^25\) reduced sperm concentration in Japanese and Danish populations,\(^26\),\(^27\) and testicular cancer.\(^28\)
In the current study, we hypothesized that certain Y chromosome haplotypes are associated with the susceptibility for BE and/or EAC. To test our hypothesis, we used a set of six Y chromosome linked polymorphisms to define seven major Y chromosome haplotypes in Dutch Caucasian males with BE or BE associated EAC (n=463). Their Y-chromosome haplotype frequencies were subsequently compared with age-matched Dutch Caucasian males with GERD symptoms who at endoscopy had no BE (n=183), and with a general Dutch Caucasian male population (n=256).

**MATERIALS and METHODS**

**Study population**
This study was approved by the local ethics committee and all participants signed informed consent for the use of their samples. A total of 646 patients that underwent upper gastrointestinal (GI) endoscopy between 2002 and 2006 at the Academic Medical Center (AMC), the VU University Medical Center in Amsterdam, and at the Erasmus MC-University Center Rotterdam in the Netherlands were included. Upper GI endoscopy was performed for the following reasons: upper typical GI-complaints related to Gastro-esophageal reflux disease (GERD), routine surveillance of BE patients and staging or endoscopic treatment in case patients were suspected for EAC. For histological confirmation of BE, biopsies were taken above the z-line in the esophagus from the typical pink appearing mucosa that endoscopically is recognized as BE. In case of EAC, biopsies were taken from the tumor mass and adjacent to the mass to confirm BE associated EAC. In case of active reflux esophagitis, this was endoscopically classified according to the standard Los Angeles (LA) classification. To obtain genomic DNA for this study, a blood sample was drawn, or biopsies were taken from the stomach cardia or normal squamous esophageal mucosa. Data on drug use, particularly on Proton Pump Inhibitors and risk factors such as smoking, drinking and BMI was obtained from patient files and/or by questionnaires. To test whether the case and control populations are representative for the general Dutch Caucasian population, a control group of 256 Dutch Caucasian men recruited at the center for Reproductive Medicine at the AMC were included. Some of these men suffered from spermatogenic failure and presented at the Center for Reproductive Medicine for unfulfilled child wish.

Data on ethnic origin for all individuals were obtained from patient files and/or by questionnaires. The Dutch Caucasian ethnic origin of the different populations was confirmed to the third in 542 out of 902 (60%) of the cases, and to the second generation in 360 out of 902 (40%).
Y-chromosomal haplotypes and BE

Genomic DNA of each patient was extracted from normal esophageal and cardia tissues (Invitrogen) or from a 5 ml of whole blood sample by standard salt-out procedure. Six Y-linked binary markers, which are known to be polymorphic in the European population, were chosen to genotype all individuals: M9, SRY10831, M89, DYS257, Yap, p12f. (Underhill PA, 1997,996; Underhill 2001, 43). Genotyping was performed by polymerase chain reaction restriction (PCR) and restriction fragment length polymorphism (RFLP) analysis. PCR reactions were carried out in a 25µl volume containing approximately 50ng of genomic DNA, 1x Ready PCR Master Mix (Applied Biosystem) and 100ng of Forward and Reverse primer. PCR primer sequences and annealing temperature for each of the used polymorphic markers is given in Table 1. PCR products (5 µl) of M9, SRY10831, M89, DYS257 markers were then digested with 1 unit of appropriate restriction enzyme (New England Biolabs, UK) in a volume of 10 µl as described in the manufacture’s guidelines (Table 2). Digests were visualized on a 3% agarose gel with ethidium bromide (10 µg/ µl). Subsequently, the Y chromosomal haplotype profile of each patient was defined as summarized in Table 3.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYS257</td>
<td>GAACTTGTGCGGAGGCAAT</td>
<td>TGATACACTCCTCTTTAGTGCGG</td>
<td>61ºC</td>
</tr>
<tr>
<td>SRY10831</td>
<td>AAAATAGCAAAAAATGACACAGGC</td>
<td>TCCTTAGCAAACATATACTCTTGGG</td>
<td>61ºC</td>
</tr>
<tr>
<td>M9</td>
<td>GCAACATATAAACACTTTTCAGG</td>
<td>AAAAACTACTTTGCTCAAGC</td>
<td>61ºC</td>
</tr>
<tr>
<td>M89</td>
<td>CTCTTTCAAGTTATGACAAAAAGCT</td>
<td>TCCTAGGAGATATCCCCTCA</td>
<td>60ºC</td>
</tr>
<tr>
<td>Yap</td>
<td>CAGGGGAAGATAAAGAAAAATA</td>
<td>ACTGCTAAAGGGGGATGGAT</td>
<td>55ºC</td>
</tr>
<tr>
<td>P12F2</td>
<td>CAAATTGTAAGACAAGGACATGC</td>
<td>GCACGTGTGACATGTACTC</td>
<td>61ºC</td>
</tr>
</tbody>
</table>

Table 1: Primer sequences for the different Y- polymorphisms

### Y chromosome haplotyping

Genomic DNA of each patient was extracted from normal esophageal and cardia tissues (Invitrogen) or from a 5 ml of whole blood sample by standard salt-out procedure. Six Y-linked binary markers, which are known to be polymorphic in the European population, were chosen to genotype all individuals: M9, SRY10831, M89, DYS257, Yap, p12f. (Underhill PA, 1997,996; Underhill 2001, 43). Genotyping was performed by polymerase chain reaction restriction (PCR) and restriction fragment length polymorphism (RFLP) analysis. PCR reactions were carried out in a 25µl volume containing approximately 50ng of genomic DNA, 1x Ready PCR Master Mix (Applied Biosystem) and 100ng of Forward and Reverse primer. PCR primer sequences and annealing temperature for each of the used polymorphic markers is given in Table 1. PCR products (5 µl) of M9, SRY10831, M89, DYS257 markers were then digested with 1 unit of appropriate restriction enzyme (New England Biolabs, UK) in a volume of 10 µl as described in the manufacture’s guidelines (Table 2). Digests were visualized on a 3% agarose gel with ethidium bromide (10 µg/ µl). Subsequently, the Y chromosomal haplotype profile of each patient was defined as summarized in Table 3.

### Statistical analysis

Differences in the frequency of Y-chromosomal haplotypes, as well as differences in body mass index (BMI), drinking and smoking habits between the different populations, were determined with Pearson chi-square test (2-
sided). To assess the predictive power of Y haplotypes on group allocation (odds ratios), a binary logistic regression was performed in which the Y haplotypes were converted into dichotomous variables (one investigated haplotype versus all the others). Then, a multiple logistic regression model was applied to investigate if the risk factors (age, BMI, drinking and smoking) significantly influenced these odds ratios. Statistical significance was set at a \( P \) value of <0.05. The statistical analyses were conducted using SPSS software (version 12.0; SPSS, Inc, Chicago, IL).

### Table 3: Polymorphisms defining the different Y-haplotypes

<table>
<thead>
<tr>
<th>Y-haplotypes</th>
<th>Ysp</th>
<th>M89</th>
<th>P12f</th>
<th>M9</th>
<th>DYS257</th>
<th>SRY10831</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-B or C</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>DE</td>
<td>+</td>
<td>C</td>
<td>+</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>F(xJ,xK)</td>
<td>-</td>
<td>T</td>
<td>+</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>J</td>
<td>-</td>
<td>T</td>
<td>-</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>K (xP)</td>
<td>-</td>
<td>T</td>
<td>+</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>P(xR1a)</td>
<td>-</td>
<td>T</td>
<td>+</td>
<td>G</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>R1a</td>
<td>-</td>
<td>T</td>
<td>+</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

### RESULTS

#### Patients

The endoscopically controlled population consisted of 463 Dutch Caucasian males with histologically proven BE (mean age 60, SD± 12.2) and 183 Dutch Caucasian male controls without BE but with GERD symptoms (mean age 54, SD± 13.9). In the GERD group, 125 out of 183 (68%) had grade A or B

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>GERD No.(%)</th>
<th>BE</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>17 (25)</td>
<td>35 (17)</td>
<td>( p=0.15 )</td>
</tr>
<tr>
<td>Ever</td>
<td>51 (74)</td>
<td>168 (84)</td>
<td></td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>16 (23)</td>
<td>50 (25)</td>
<td>( p=1 )</td>
</tr>
<tr>
<td>Ever</td>
<td>53 (77)</td>
<td>154 (77)</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>31 (45)</td>
<td>89 (44)</td>
<td>( p=0.44 )</td>
</tr>
<tr>
<td>Overweight</td>
<td>25 (36)</td>
<td>87 (43)</td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>13 (19)</td>
<td>25 (13)</td>
<td></td>
</tr>
<tr>
<td>Total no.</td>
<td>69</td>
<td>201</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4: Risk factors in GERD and BE groups.
esophagitis (erosive reflux esophagitis; RE), while 58 out of 183 men (32%) had no esophagitis but had typical reflux symptoms, and were classified as non erosive reflux disease, grade 0. In the BE group, 118 out of 463 patients had BE associated EAC that was confirmed by histology. Thirty nine percent (71/183) of the GERD patients were on proton pomp inhibitor (PPI) therapy. Risk factors assessment was obtained from 43 % (201/463) and 38% (69/183) of BE and GERD patients, respectively. There were no significant differences in Body Mass Index (BMI), smoking and drinking habits between the BE and the GERD population (Table 4). The mean age of the 256 Dutch Caucasian males from general population was 39 (SD± 6.1).

The frequencies of Y chromosomal haplotypes between the groups

To address whether the selected GERD and BE/EAC groups were representative for the general Dutch Caucasians, we compared the Y-haplotypes frequencies between these groups and that of Dutch Caucasian males from the general population (Table 5). The overall distribution of the Y-haplotypes of the BE/EAC and GERD population did not differ significantly from that of Dutch Caucasian males from the general population (p=0.93 and p=0.069, respectively), indicating that ethnically homogeneous groups were compared (Table 7).

<table>
<thead>
<tr>
<th>Y-haplotype</th>
<th>Dutch Caucasians No. (%)</th>
<th>GERD</th>
<th>BE/EAC</th>
<th>BE</th>
<th>EAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB or C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DE</td>
<td>8 (3)</td>
<td>15 (8)</td>
<td>14 (3)</td>
<td>12 (3.5)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>F (xJ,xK)</td>
<td>80 (31)</td>
<td>42 (23)</td>
<td>139 (30)</td>
<td>100 (29)</td>
<td>39 (33)</td>
</tr>
<tr>
<td>J</td>
<td>9 (4)</td>
<td>12 (6.6)</td>
<td>14 (3)</td>
<td>10 (3)</td>
<td>4 (3.4)</td>
</tr>
<tr>
<td>K(xP)</td>
<td>6 (2)</td>
<td>5 (2.7)</td>
<td>10 (2)</td>
<td>7 (1.2)</td>
<td>3 (2.5)</td>
</tr>
<tr>
<td>Px(R1a)</td>
<td>144 (56)</td>
<td>101 (56)</td>
<td>272 (59)</td>
<td>207 (60)</td>
<td>67 (57)</td>
</tr>
<tr>
<td>R1a</td>
<td>9 (4)</td>
<td>8 (4.4)</td>
<td>14 (3)</td>
<td>12 (3.5)</td>
<td>3 (2.5)</td>
</tr>
<tr>
<td>Total no.</td>
<td>256</td>
<td>183</td>
<td>463</td>
<td>345</td>
<td>118</td>
</tr>
</tbody>
</table>

Table 5: Y-haplotype distribution in BE/EAC, GERD patients and a Dutch Caucasian male population.

Interestingly, a significant difference was observed in overall distribution of Y haplotypes between GERD (n=183) and BE/EAC (n=463) population (p=0.007, Table 7). To find the specific Y haplotypes associated with BE susceptibility, we compared the frequency of each of the Y haplotypes between these groups (Table 6). This analysis showed that the DE and J haplotypes were significantly underrepresented in BE/EAC as compared to the GERD group (3% vs. 8%, p=0.023 and 4% vs. 7%, p=0.047, respectively). The DE and J haplotypes were significantly associated with a lower risk for BE presence in the GERD population as indicated by low odds ratio (OR) of 0.34 (0.16-0.73 95%CI) and 0.44 (0.2-0.98
95% CI), respectively. To adjust for potential confounding risk factors, age, BMI, smoking and drinking habits were entered in a multiple logistic regression model. This analysis showed that none of these risk factors had a significant influence on the odds ratios (data not shown).

**Comparison of the subgroups of BE with and without EAC**

We addressed whether or not the DE and J Y haplotypes were associated with a lower risk for BE independently of the presence of associated EAC, and whether these haplotypes are associated with the risk for EAC. Hereto, we divided BE/EAC group (n=463) into two subgroups: BE without (n=345) and with the associated EAC (n=118) and then compared their Y haplotypes frequencies with those of the GERD group and to each other (Table 5 and Table 6). The comparison of the Y haplotype distribution between BE without EAC (n=345) and EAC (n=118) group showed no significant differences (Table 6). Further analysis showed that haplotype DE was still significantly underrepresented in BE without EAC group as compared with the GERD group (OR= 0.40, 0.18-0.88 95% CI, p=0.023), whereas there was a trend for the J haplotype (OR=0.42, 0.18-1 95% CI, p=0.069). Similarly, the frequency of DE but not J haplotype was still significantly different between EAC and the GERD group (OR=0.19; 0.043-0.86 95% CI, p=0.02 and OR=0.5; 0.15-1.5 95% CI, p=0.29, respectively).

<table>
<thead>
<tr>
<th>Y haplotype</th>
<th>GERD vs. BE/EAC</th>
<th>GERD vs. BE</th>
<th>GERD vs. EAC</th>
<th>BE vs. EAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB or C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DE</td>
<td>0.34 (0.16-0.73); 0.004</td>
<td>0.40 (0.18-0.88); 0.023</td>
<td>0.19 (0.043-0.86); 0.02</td>
<td>0.47 (0.10-2.1); 0.53</td>
</tr>
<tr>
<td>F (xJ,xK)</td>
<td>1.4 (0.96-2.14); 0.071</td>
<td>1.37 (0.90-2.07); 0.149</td>
<td>1.65 (0.99-2.77); 0.063</td>
<td>1.2 (0.77-1.89); 0.41</td>
</tr>
<tr>
<td>J</td>
<td>0.44 (0.2-0.98); 0.039</td>
<td>0.42 (0.18-1); 0.065</td>
<td>0.5 (0.15-1.59); 0.29</td>
<td>1.17 (0.36-3.82); 0.76</td>
</tr>
<tr>
<td>K (xP)</td>
<td>0.54 (0.17-1.74); 0.33</td>
<td>0.41 (0.11-1.57); 0.28</td>
<td>0.92 (0.21-3.96); 1</td>
<td>2.2 (0.49-10); 0.37</td>
</tr>
<tr>
<td>Px(R1a)</td>
<td>1.17 (0.83-1.6); 0.35</td>
<td>1.2 (0.84-1.75); 0.30</td>
<td>1.04 (0.65-1.66); 0.90</td>
<td>0.87 (0.57-1.33); 0.58</td>
</tr>
<tr>
<td>R1a</td>
<td>0.73 (0.30-1.75); 0.48</td>
<td>0.78 (0.31-1.96); 0.64</td>
<td>0.56 (0.14-2.1); 0.53</td>
<td>0.72 (0.20-2.6); 0.77</td>
</tr>
<tr>
<td>Total no.</td>
<td>183 vs. 463</td>
<td>345 vs. 183</td>
<td>118 vs. 183</td>
<td>345 vs. 118</td>
</tr>
</tbody>
</table>

**Table 6: The relative risk of different Y chromosomal haplotypes for the presence of BE and EAC**
Y-chromosomal haplotypes and BE

DISCUSSION

Gastro-esophageal reflux disease (GERD) is a major risk factor for developing BE and EAC, however only small proportion of people exposed to esophageal reflux indeed develop BE or EAC, suggesting that a genetic predisposition may be involved in this process. Here, we performed a genetic association study to investigate the impact of Y-chromosomal haplotypes on susceptibility to BE and EAC. We found that Y-haplotypes DE and J are associated with a lower risk of BE and EAC in a Dutch Caucasian population with GERD. Therefore, we speculate that there is a protective effect of the DE and J haplotype against BE and EAC development in this population.

Here, we determined Y chromosomal haplotypes distribution in BE/EAC patients and control group of GERD patients as well as in general population of Dutch Caucasian males included here as an ethnic reference. The most frequently observed Y-haplotype (56-60%) in all groups was haplotype P(xR1a). Indeed, this haplotype is the most common in Europe. Haplotype F(xK,J) was the second most frequent Y haplotype seen (23-31%), which is also in agreement with literature data. Haplotypes DE, J, K(xP) and R1a were observed in the lowest frequencies from 2-8%. In this study, we did not observe any AB or C haplotypes, which is not surprising since the incidence of these haplotypes is mostly found in Africans. Interestingly, we found a significant difference in the overall distribution of the Y haplotypes between the GERD and BE/EAC group (p=0.007). More detailed analysis revealed that haplotypes DE and J are significantly underrepresented in BE/EAC as compared to the GERD group (3% vs. 8% and 3% vs.7%, respectively) and are associated with an approximately 60% lower risk for the presence of BE/EAC as evidenced by odds ratios (OR) of 0.34 (0.16-0.73 95%CI) and 0.44 (0.2-0.98 95%CI), respectively. The association of DE haplotype with lower risk for BE was still significant when BE group without EAC was compared to GERD controls (OR=0.40, 0.18-0.88, p=0.023), in contrast to the J haplotype that only showed a trend towards lower risk for BE (OR=0.42, 0.18-1, p=0.065). Similar results were obtained when comparing the DE and J Y-haplotypes of the EAC and GERD groups. The lack of a significant association of J haplotype with lower susceptibility to BE and EAC may be due to lower statistical power caused by the reduced sample sizes in the subgroup analysis. Further, we did not observe significant associations of the DE and J haplotypes with susceptibility to EAC in BE patients (OR=0.47, 0.10-2.1 95%CI and OR=1.17, 0.36-3.82 95%CI, respectively). Because of the wide confidence intervals it is, however, not excluded that the association may exist but is hidden in our study. Studies with higher sample size are needed to address this issue.

There are some limitations of our study. First is lack of follow up data of the GERD population meaning that we do not know how many cases could develop BE or EAC in the future. Another possible confounder is the use of proton pomp

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inhibitors drugs (PPI), and the absence of erosive esophagitis in a subset of GERD cases, which may have prevented BE development in susceptible GERD cases. However, to include an untreated GERD population is difficult, since most of patients with typical reflux complaints that are referred for upper GI endoscopy will already be on PPI treatment. We were also not able to obtain all data on BMI, drinking and smoking habits to adjust the odds ratios. The available data, however, suggests that these risk factors did not differ significantly between the investigated groups and as such may have not influenced the outcome. The possibility of false-positive results because of the geographical stratification and ethnical distribution of Y-haplotypes must always be considered in Y haplotype-phenotype association studies. To avoid this, the appropriate choice of the control population (non-blood relatives from the same geographical area as a disease group) is crucial. The ethnicity of our controls and the BE/EAC population was verified to the third or at least to the second generation. All our control populations were non-related Dutch Caucasians males, thus ethnically matched with the BE/EAC group. Therefore, the DE and J Y-haplotypes association with a lower susceptibility to BE and EAC found in our study is unlikely to be due to bias caused by different ethnical background. This is also supported by the fact that the distribution of the Y haplotypes of the GERD and BE/EAC groups was similar to that of a general Dutch Caucasian population, included here as an ethnical reference.

Literature data indicate that haplotype DE, although it can be found in low percentages in Northern Europe, is generally more frequent in Africa and also has a high incidence in Asia and Japan, while the J haplotype is found in similar frequencies in both Europeans and Asians (Indus Valley). The association of the DE and J haplotypes with a lower susceptibility to BE/EAC is highly interesting considering that in general in Africans and Asians BE and EAC is less common compared to white European Caucasians. The reason for the low prevalence of BE and EAC in these countries is unclear. There might be different environmental factors or a lower prevalence of GERD, but sufficient evidence to support this theory is lacking. Moreover, recent studies suggest that the prevalence of GERD in Asian countries like Japan, for instance, has been underestimated due to a lack of awareness of the condition and proper diagnostic techniques. Therefore, it is likely that the difference in susceptibility for BE and EAC development between oriental countries, Africa, and Europe is at least partly due to a distinct genetic background. In this light it seems striking that the DE and J haplotype are commonly seen in African and Asians populations, which might have a protective effect against BE or EAC development. It would be highly interesting to study the frequency of the Y haplotypes with respect to BE and EAC in different ethnic groups. This is also of interest since screening of Caucasian populations for markers, like Y-haplotypes that are related to a non Caucasian population might be of help to identify individuals with a lower susceptibility to BE and EAC.
The mechanisms how the Y chromosomal haplotypes influence the susceptibility to BE and EAC is not clear. We know, however, that Y-chromosomes from distinct Y-haplotypes show a considerable amount of structural variation. Interestingly, it was shown that the Y chromosome belonging to haplotype J was associated with protection against microdeletions in AZFc (azoospermia factor) gene on Y chromosome in north Italian population. It was suggested that this is because the haplotype J does not contain the L1PA4 element that is supposed to facilitate the homologous intrachromosomal recombination leading to AZF deletions. Thus, it might be that Y chromosomes defined by haplotypes DE and J are associated with protection against occurrence of certain Y chromosomal rearrangements (deletions or amplifications) influencing expression of genes important in BE and EAC development. The candidate genes on Y chromosome involved in this process may be those that are expressed ubiquitously in the body, including, for instance, SRY, ZFY and SMCY transcription factors, PRKY protein kinase or EIF1AY translational inaction factor. It is, however, not excluded that Y chromosomal haplotypes are associated with functional variants of genes on autosomal chromosomes. Such an association, for instance, was found between a Y chromosome variant and the polymorphism on autosomal gene of aldosterone synthase in European men with high blood pressure. In the future, linkage disequilibrium studies may reveal which genetic variants on Y chromosome itself or autosomal chromosomes are linked to the DE and J haplotypes and susceptibility to BE and EAC.

In summary, this study is the first to report an association of the DE and J Y chromosomal haplotypes with a lower susceptibility to BE/EAC in a GERD population of Dutch Caucasian males. How these Y chromosomal haplotypes influence this susceptibility is not known. We could, however, speculate that the DE and J Y-haplotype might be linked to genetic variants protecting against BE/EAC development. The identification of such genetic variants in the future may improve our understanding of the pathogenesis of BE and EAC.
REFERENCES

Y-chromosomal haplotypes and BE


Chapter 7


Chapter 8

Summary and General discussion


SUMMARY

Barrett’s esophagus (BE) is a metaplastic condition of the distal esophagus, which is a result of long term gastroesophageal reflux disease (GERD). BE is a premalignant condition associated with an increased risk for developing esophageal adenocarcinoma (EAC). In Western countries the incidence of EAC is the most rapidly increasing of all malignancies. EAC is a highly malignant disease, with overall 5 yrs survival of <20%, while long term survival may improve up to 95%, in case detection of patients is in early stage of disease, i.e., high grade dysplasia (HGD) or early cancer. Since progression of BE into EAC is characterized by histopathological changes of low grade dysplasia (LGD) and HGD before invasive disease occurs, routine surveillance programs of BE patients aim on screening for these early changes. A major problem is that the endoscopic and histopathological evaluation of BE are unable to identify those patients that particularly in the long run will progress to malignancy. For optimal risk stratification, better and more efficient screening protocols need to be developed. It is anticipated that molecular changes that accumulate during BE progression may be more reliable in identifying BE patients at higher risk for malignant progression.

FISH applied on brush cytology is regarded as a promising method to screen BE patients for genetic abnormalities associated with dysplasia and/or malignancy. Automated assessment of FISH markers on brush cytology may enhance the clinical applicability of this methodology. The first part of this thesis (Chapter 2) describes the validation of a novel automated CytoVison SPOT AX system for efficient assessment of FISH results in BE brush cytology specimens, using DNA probes for chromosome 9, 17, Y and the locus specific regions of 9p21 (p16), 17p13 (p53) and 17q11 (Her2/neu). In this study, a high concordance of 98% between the automated and manual method was observed. Moreover, good to excellent agreement for each of the used FISH probes was shown. Further, by using the automated FISH analysis, the cytogenetic abnormalities were prospectively evaluated in a cohort of 151 BE surveillance patients. This analysis showed that loss of the 17p13.1 (p53), Y chromosome loss and gains of chromosome 17 and 9 can be detected in low frequencies in non-dysplasia and correlate with increasing grade of dysplasia, suggesting their potential value as prognostic markers of BE progression. The 17q11.2 (Her2/neu) amplification, as seen in high frequency and exclusively in HGD cases, may be a useful diagnostic marker to detect HGD in BE. In the future, the use of the automated FISH analysis may enhance the clinical applicability of these FISH markers as a surveillance tool for BE.

Previously, abnormal ploidy status has been indicated as a prognostic marker for malignant progression of BE. Chapter 3 of this thesis compares DNA ploidy status as assessed by image cytometry (ICDA) and FISH analysis for chromosomal gains (chromosome 7 and 17) on BE brush cytology specimens. Additionally, this study
describes the value of the detected abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC. Gains of chromosome 7 and/or 17 were detectable already in ND stage and further increased with BE progression while DNA content aneuploidy as determined by ICDA were detectable predominantly in late stage of HGD and EAC. In general, FISH detected abnormalities in a higher number of BE cases as compared to ICDA (41% vs. 22%, respectively). The analysis of discordant cases showed that this is because FISH is more sensitive to detect chromosomal changes in low numbers of cells as well as gains of only single chromosomes, which probably results in a too small DNA content change to be detected by ICDA. Moreover, it was shown that assessment of chromosomal gains by FISH on brush cytology specimens is superior to conventional cytology in detection of HGD or EAC as well as represents a valuable adjunct to the conventional cytology to identify IND or LGD.

Chapter 4 focuses on the understanding of the sequential chromosomal events leading to Her-2 (17q11.2) locus amplification during BE progression. Hereeto, the frequency of chromosome 17 gains and the level of the Her-2 locus amplification with respect to overall DNA-content ploidy status were studied in BE cases with different stages of dysplasia or EAC. Then, subclonal analysis of the cases with Her-2 amplifications was performed to gain more insight in the clonal evolution of the Her-2 amplification. This study confirmed that gains of chromosome 17 occur early in non dysplastic BE cases and showed that low and high levels of Her-2 locus amplification occurs in high frequency and exclusively in HGD and EAC cases. Most importantly, the analysis of co-existing subclones in Her-2 amplified cases showed that gains of chromosome 17, either due to selection or as a result of overall DNA content aneuploidy, precede Her-2 amplification in BE with HGD or EAC. However, it was also noted that in certain cases Her-2 amplifications may as well occur without prior chromosome 17 gains or DNA content aneuploidy. On important conclusion from this study is that it would be interesting to prospectively follow the BE cases with gains of chromosome 17 in order to find whether or not this abnormality can be used as an early marker and truly predict later Her-2 locus amplification and subsequent malignant progression of BE.

The evaluation of Her-2 gene amplification and protein overexpression has therapeutic and prognostic implications in breast cancer and other carcinomas. There is, however, no consensus so far with regard to the optimal test for Her-2 assessment. In chapter 5, the correlation between Her-2 (17q11.2) locus amplification as assessed by FISH on brush cytology and Her-2 protein overexpression as determined by immunohistochemistry (IHC) on biopsies of BE patients was investigated. Here, important differences in the association between the level of Her-2 locus amplification and Her-2 protein overexpression were found. Independently of the BE stage, only high levels of Her-2 amplification
(ratio of Her-2 locus: Cep17 ≥ 5:1) correlated with strong Her-2 strong protein overexpression (+3), whereas low levels (ratio of Her-2:Cep17 >2<5:1) and chromosome17 gains were associated with moderate or no staining for the Her-2 protein. These results suggest that DNA-FISH on brush cytology samples is a useful diagnostic tool that, at least in BE cases with low levels of Her-2 status changes, is superior to IHC on biopsies. In the near future, the assessment of Her-2 amplification status in BE patients will be highly relevant for proper selection of patients that are eligible for treatment with Hercepitin (Transtuzmab) or other Her-2 targeted molecular therapies.

In chapter 6 of this thesis heterogeneity of copy number changes of several other oncogene loci including 7p12 (EGFR), 8q24 (c-myc) and 20q13 BE was investigated in BE patients with various stages of dysplasia or EAC. FISH analysis on BE brush cytology samples revealed that that gains of these loci (3-4 copies) appear early in non-dysplastic BE and further their incidence significantly increases with stage of dysplasia and is high in EAC. The low (>4<10 copies) and high-level (>10 copies) amplifications of the investigated loci occur only in HGD and EAC cases. Interestingly, high- amplification levels of the loci were significantly more frequent in EAC compared to HGD, suggesting that high-level amplification may be a sign of malignancy. The results obtained in this study suggest that amplifications of the c-myc, EGFR and 20q13 loci may serve as diagnostic markers to identify BE patients with HGD or EAC. Gains of the loci might be of value as prognostic markers since these are already present in non-dysplasia cases and may precede the later event of the amplifications as observed in HGD and EAC.

Of interest is that BE and EAC are most prevalent in Caucasian populations, and occur 3-7 times more frequently in males than in females. In chapter 7 it was hypothesized that certain Y chromosomal haplotypes may be associated with susceptibility to BE/EAC in male Caucasian populations. To test this hypothesis, genotyping for six Y chromosome linked polymorphisms to define Y chromosome haplotypes in Dutch Caucasian males with BE or BE associated EAC was performed. Their Y-chromosome haplotypes frequencies were subsequently compared with age-matched Dutch Caucasian males with GERD who at endoscopy had no BE and with a general Dutch Caucasian male population, included here as a ethnical reference. Interestingly, Y-chromosomal haplotypes J and DE were found to be associated with a lower risk of BE/EAC in a Dutch population with GERD. How these Y chromosomal haplotypes may influence this susceptibility is not clear. The Y haplotypes DE and J might be linked to genetic variants protecting against BE/EAC development. The identification of such genetic variants, for instance by linkage disequilibrium studies, in the future may improve our understanding of the pathogenesis of BE and EAC.
GENERAL DISCUSSION

Previous studies have demonstrated numerous molecular changes occurring during the progression of Barrett’s esophagus (BE). Only some of these changes are likely to play causal roles in the carcinogenesis of esophageal adenocarcinoma that is related to BE (EAC). Other molecular abnormalities may be random events or epiphenomena of other causally important alterations. Although these changes may not drive neoplastic transformation, they may still serve as useful clinical markers, in case there is an association with malignant progression of BE.

In this thesis, a panel of genetic markers have been evaluated by DNA-FISH on BE brush cytology specimens in the non dysplasia-dysplasia-adenocarcinoma sequence. It was first demonstrated that DNA-FISH abnormalities in BE brush cytology specimens can be efficiently and objectively assessed by an automated FISH analysis system. In the future, the high throughput analysis of prognostic FISH markers using the automated FISH analysis may improve efficacy of surveillance programs (prognosis) of BE patients.

From studies described in this book, two specific genetic markers sets are emerging. The first marker set seems to be potentially *prognostic* for development of dysplasia or EAC, while the second is potentially *diagnostic* for finding HGD or EAC.

The potentially *prognostic* genetic marker set consists of those markers that were found in low frequency in patients with no dysplasia and then significantly increased in frequency in LGD, HGD/EAC group. Hereto, gains of chromosome 7, 9 and 17, loss of chromosome Y and loss of locus specific region of 17p13 (p53) can be included. Interestingly, one of the observations made in this thesis is that gains of single chromosomes determined by FISH result in too small DNA content change to be detected by image cytology DNA analysis (ICDA). Since single chromosomal gains (chromosome 7 or 17) seem to be present already in non-dysplastic BE and early stages of dysplasia, FISH with appropriate centromeric probes may be a more sensitive method than ICDA to assess early DNA content changes in these patients. The assessment of chromosomal gains by FISH alone or with combination with cytology diagnosis might improve detection of dysplasia in surveillance programs of BE patients in the future. One emerging question is whether frequently observed chromosomal gains can actually predict development of the gross chromosomal changes (overall DNA content aneuploidy) in BE. This is of interest since some prospective studies have shown that patients with DNA content aneuploidy have an increased risk of developing HGD or EAC. The results from prospective follow up study of our BE cohort displaying chromosomal gains will enlighten us on this matter. The other interesting observation made in this thesis is that gains of chromosome 17 and the low and high level of *Her-2* locus amplification status seem to be subsequent
Chapter 8

events correlating with the increasing stages of dysplasia in BE. The provided data suggest existence of three distinct evolutionary pathways that may lead to low and high levels of Her-2 amplifications in BE, and the associated EAC. Two of these pathways are associated with precursor chromosomal changes, i.e., DNA content aneuploidy and/or selective chromosome 17 gains, while in the third pathway the amplifications may develop without any of these preceding chromosomal abnormalities. Future studies will show whether these three evolutionary subtypes are associated with different biological behavior of the cancer and whether the gains of chromosome 17 can be used as an early marker and truly predict later Her-2 locus amplification and progression of BE.

The diagnostic set of markers as evaluated in this thesis consists of those found in high frequency or specifically in HGD and EAC. This includes low and high level of amplifications of oncogenic loci including 17q11.2 (Her-2), 8q24.12.13 (c-myc), 7p12 (EGFR) and 20q13 region as well as gross chromosomal changes (DNA content aneuploidy). These abnormalities may be diagnostic for the presence of HGD or in situ adenocarcinoma or indicative for progression of HGD towards malignancy on short term. As such they may have additive value to histology diagnosis.

Additionally, the work described in this thesis indicate for the first time that Y-chromosomal haplotypes DE and J are associated with lower risk of having BE/EAC in a GERD population of Dutch males and therefore might be of value to identify low risk groups for BE/EAC development in this population. The mechanistic link between the DE and J Y-haplotype and the susceptibility to BE/EAC remains to be elucidated. These Y-haplotypes might be linked to genetic variants protecting against BE/EAC development. In the future, identification of such genetic variants, for instance by linkage disequilibrium studies, may improve our understanding of BE and EAC pathogenesis.

In conclusion, this work evaluated a number of genetic events which are promising prognostic or diagnostic markers for BE malignant progression. However, the true value of these markers can only be establish after long term follow up where their frequency will be compared to histological changes and patient outcome. Probably no single ‘universal’ genetic marker will be sufficient to enable us to predict which patient will and which will not develop cancer. Most likely combinations of markers will lead to a further stratification of risk for progression with presymptomatic intervention and individualized treatment as the ultimate goals.
Appendices
Samenvatting

Barrett’s slokdarm (Barrett’s esophagus in de Angelsaksische taal oftewel BE) is een metaplastisch fenomeen in de distale slokdarm (i.e. ver van de mond en dicht bij de maag) die het gevolg is van het oprispen van zuur uit de maag (gastroesophageal reflux disease, GERD). Belangrijkerwijze, BE is een protomaligine toestand welke een nauwe associatie vertoont met het ontstaan van slokdarm kanker. Belangrijkerwijze, neemt in Westerse samenlevingen de sterfte door slokdarmkanker steeds verder toe, wat onderzoek naar ontstaan, preventie en behandeling van deze meestal fatale ziekte urgent maakt.

In het bovenliggende proefschrift heb ik mij met name geconcentreerd op het gebruik van de zogenaamde Fish techniek als een methode om mogelijke maligne verandering van het proto-maligne BE epitheel in echte kanker te detecteren. Hiertoe heb ik in eerste instantie een geautomatiseerd systeem opgesteld om de genetische integriteit van materiaal verkregen via slokdarm veegproeven te beoordelen. De resultaten van deze studie zijn beschreven in hoofdstuk 2 en geven aan dat dergelijke automatische analyse zeker levensvatbaar is en wellicht superieur aan de momenteel gebruikelijke bewerkelijke handmatige methodiek.

De kracht van Fish analyse werd verder aangetoond in hoofdstuk 3, waar “image cytometry” (ICDA) en Fish analyse werden vergeleken met betrekking tot de detectie van aanwezigheid van aanmerkelijk hoge kopienummers van chromosoom 7 en 17. Over het algemeen was Fish meer efficiënt in het detecteren van dergelijke chromosomale abberaties (41% versus 22%). De conclusie van dit werk dat Fish erg veelbelovend is als een stuk gereedschap m.b.t. de detectie van genetische abnoramliteiten in veegmateriaal verkregen uit de slokdarm van BE patiënten.

In hoofdstuk 4 heb ik meer specifiek gekeken naar de zich opeenvolgende cascade van chromosomale veranderingen die uiteindelijk leiden tot Her-2 vermenigvuldiging gedurende de transformatie van BE tot volledige slokdarmkanker. Deze studie bevestigde dat de regio waar Her-2 in het genoom is ondergebracht al vroeg in deze transformatie optreedt. Nochtans blijft het vooral onduidelijk of een dergelijke vermenigvuldiging van de Her-2 locus daadwerkelijk voorspellende waarde heeft m.b.t. het ontstaan van volwaardige slokdarmkanker.

Momenteel is er geen consensus met betrekking tot de optimale wijze om Her-2 status te bepalen in BE alsmede de implicaties van een dergelijke status op het verloop der ziekte. In hoofdstuk 5, vergeleken we zowel de correlatie tussen vermenigvuldiging van Her-2 in het genoom zoals gedetecteerd met Fish op veegmateriaal met een dergelijke analyse middels immunohistochemie. Het bleek dat Fish de superieure techniek was en mogelijk van belang kan worden bij selectie van die patiënten die hoogste kans hebben baat te hebben bij Hercepitin (Transtuzmab behandeling (een therapie gericht juist tegen Her-2 positieve kankers).
De moleculaire evolutie van BE werd verder onderzocht in hoofdstuk 6. Wederom gebruikmakend van Fish kon ik vaststellen dat genetische vermenigvuldiging van met name c-myc, EGFR en 20q12 loci mogelijk prognostische markers kunnen zijn voor verdere maligne ontwikkeling.

In het laatste experimentele hoofdstuk 7, tenslotte, gaan we in op de opvallende observatie dat BE met name prevaleert is in Indo-europese groep en dan nog met name in mannen en niet in vrouwen. Daar met name het Y chromosoom behouden in deze groep onderzochten we haplotypes op dit chromosoom voor hun associatie met BE. We vonden dat in Nederlanders met name de Y-chromosomale haplotypes J en DE beschermend waren m.b.t. to BE, zelfs in de aanwezigheid van zzure reflux. Vervolg onderzoek zal de mechanismen die deze bescherming veroorzaken blootleggen.
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Agnieszka
List of publications


Appendices

Curriculum Vitae

Agnieszka Rygiel was born on 1st of May 1977 in Elk, Poland. From 1992 to 1996, she attended Stefan Zeromski High School in Elk. In 1997, she started the studies at the Faculty of Biology at Warsaw University in Warsaw. As a student she was a member of a Student Molecular Biology Society and was engaged in organizing scientific workshops “Gen cloning” during 4th (2000) and 5th (2001) Science Festival in Warsaw as well as international workshops on “Scientific Presentation Techniques” (2001). From 2000 to 2002, she conducted a research for her master thesis in the Department of Applied Microbiology focused on the mechanisms of \textit{Yersinia enterocolitica} O9 pathogenesis. In 2002, Agnieszka graduated Cum Laude and in the next year she started a PhD project at the Laboratory of Experimental Internal Medicine in the Academic Medical Center in Amsterdam. Under the supervision of Dr. K.K Krishnadath and Prof. M.P Peppelenbosch she conducted a PhD research focused on evaluation of a variety of genetic abnormalities during progression of Barrett’s esophagus. This thesis describes the results of her research.
Color figures

Figure 1, chapter 2
Figure 1, chapter 3
Figure 3, chapter 4
Figure 1, chapter 5
Figure 2, chapter 6