Genetic markers in malignant progression of Barrett’s esophagus
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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 CytoVision 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. Hereto, 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 $\text{Her-2}$ locus: Cep17 $\geq 5:1$) correlated with strong Her-2 strong protein overexpression (+3), whereas low levels (ratio of $\text{Her-2}:\text{Cep17} > 2<5:1$) 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 $\text{Her-2}$ 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), $8q24$ ($c\text{-myc}$) 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 $c\text{-myc}$, $\text{EGFR}$ 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 an 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. Here, 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
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.