A search for molecular biomarkers in gastro-intestinal cancer
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chapter 6

SUMMARY AND GENERAL DISCUSSION

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6.1 OUR BIOMARKER SEARCH

Barrett’s esophagus (BE) is a pre-malignant metaplastic lesion as a consequence of chronic gastro esophageal reflux, that can develop into esophageal adenocarcinoma (EAC) (1,2). The yearly incidence for this cancer in patients with BE is rather low at around 0.1 to 0.6%. BE patients however still have a 11 fold higher risk of developing EAC compared to the general population (2). As indicated in the introduction the main preventive treatment in this disease is endoscopic surveillance coupled with random biopsies and histo-pathological staging. However this approach is not cost-effective due to the low cancer incidence rates and its validity has been questioned as the staging process itself can have significant inter- and intra-observer variation (3-6). To reduce the number of unnecessary invasive procedures, psychological strain and quality of life reduction, it is necessary to more accurately assess which Barrett’s patients actually have the highest significant risk of developing cancer. This histologically well-defined pre-malignant state in EAC pathogenesis opens up the ability for bio molecules to function as molecular biomarkers in a novel approach to improve the risk stratification and treatment of Barrett’s patients. Further, these evaluated candidate biomarkers also have the ability to potentially serve as diagnostic aids as well as therapeutic targets for amelioration of disease. In line with these wishes there is a sizeable search for biomarkers in Barrett’s esophagus ongoing.

Sporadic colorectal cancer (CRC) is a well-researched cancer, which has been shown to be genetically quite heterogeneous. In sporadic CRC some biomarkers, derived from the knowledge of the aforementioned genetic heterogeneity, have already been shown to be of value, especially in a predictive setting (7-9). In this disease, effective biomarkers, also for other aspects such as prognostic and diagnostic applications, are of interest.

The research as collected in this thesis is intended to add to and aid in the biomarker search for these two types of gastro-intestinal cancers.

6.2 MATRIX METALLOPROTEASES AS INFLAMMATION RELATED BIOMARKERS

Gastroesophageal reflux disease (GERD), one of the major risk factors for BE, leads to chronic inflammation of the esophagus, i.e. esophagitis (10-12). This chronic inflammation is still present upon the development of BE and is characterized by the presence of inflammatory cells and the production of a number of chemokines (13-17). There is also an intimate link between inflammation and a group of proteases called the matrix metalloproteases (MMPs), which can also be produced, by inflammatory cells (18). As such we investigated the ability of the MMPs to function as biomarkers in BE. Additionally we desired to take advantage of the innate enzymatic ability of these proteins to broaden their biomarker potential through a potential real time application. This was achieved through the use of the MMP activatable near infrared fluorescent probe MMPsense680. Our initial experiments showed that direct MMP activity, as measured by the MMPsense680 probe, gradually increased in esophageal cell lines and tissues of the Barrett’s pathogenic sequence, from low probe activation in normal squamous esophageal cells and tissues to a significantly higher activation in the aberrant cells and tissues. Interestingly, a switch in MMP subtype expression was observed in the BE-EAC tissue sequence. The expression
of MMP13 was higher in non-dysplastic Barrett tissues compared to the EAC samples, while MMP9 was mainly present in the cancer samples. Also, in patient tissues the MMP activity as defined by the activation signals mostly co-localized with CD45 positive inflammatory cells. This was largely validated in our surgical mouse model of chronic esophageal bile reflux, where as early as the esophagitis stage, stromal localization of MMP activity could be seen at the regions with epithelial hyperplasia. This protease activity was also maintained in the metaplastic mouse tissues.

The results from our study could be employed in the future in a number of ways. Firstly, the presence of MMP activity and the MMP subtype specific pattern in the aberrant tissues of the Barrett’s sequence could be used to diagnose the more malignant dysplastic and cancerous tissue types. During standard white light endoscopy these lesions could be missed. But by applying MMP activatable probes one could in real time track these lesions during fluorescence imaging adjusted endoscopy. The MMP subtype switch during the Barrett’s pathogenic sequence as observed by us, will be important in this respect. The probes will have to be specifically tailored for the tissue types of interest. In fact, ongoing studies are being performed to develop activatable probes geared towards specific proteases, including towards specific MMPs (19,20). Ryu et al for instance developed an MMP13-specific probe through computer modeling for the detection of this protease believed to play a prominent role in osteoarthritis (21). This near-infrared protease activatable probe was shown to be more specifically targeted by this MMP than other MMP family proteases. The use of fluorescently labelled bio molecules during endoscopy is something that is already being investigated. For instance, Bird-Lieberman et al discovered changes in glycosylation patterns within the esophageal mucosa during Barrett’s pathogenesis (22). Based on this observation the researchers attempted to use specific glycan binding proteins, the lectins, as biomarkers for the detection of dysplasia in BE. In this study, binding of the fluorescently labelled lectin WGA, significantly correlated with the degree of dysplasia ($p = 0.0002$) as measured ex vivo in four esophageal resection specimens. In the study, areas with HGD actually showed lower fluorescent WGA binding (22).

The data we collected on the predominantly stromal localization of MMP activity during the development of Barrett’s disease also offers important mechanistic insight. In a recent article it was shown that solely the overexpression of the pro-inflammatory cytokine IL-1β in the esophagus and fore stomach of mice leads to the development of columnar metaplasia at the age of 12-15 months (21). IL-1β expression had prior been shown to correlate with the presence of Barrett’s tissues and increasing BE tissue aberrancy (14,17). This cytokine lies upstream of the IL-6 cytokine, which has also been shown to be more highly expressed in Barrett’s tissues compared to squamous esophageal tissues. IL-6 expression is believed to be involved in disease progression in BE by contributing to the activation of anti-apoptotic genes in the diseased tissues (13,23). Interestingly, IL-1β has been shown to be activated and degraded in vitro by a number of MMPs, such as MMP2, -3 and -9 (24,25). Conversely, IL-1β can also lead to the expression of MMPs (26,27). Our data showed an early rise in MMP activity during the process of Barrett’s pathogenesis. In our mouse model we could already spot MMP activity at the early stage of esophagitis and epithelial hyperplasia as a result of the bile influx into the esophagus, preceding the development of columnar metaplasia. MMPs could thus possibly contribute to
the transformation process through the activation of IL-1β and/or might be activated early on by this cytokine and contribute to the pathogenic process through the activation of other soluble factors (18,28,29). In this respect the search for the best MMP biomarker could thus also reveal important and possibly useful early targets for diverting from developing Barrett’s metaplasia in patients with GERD.

With respect to the improving of the current experimental set-up it would be important to address the different expression patterns of the specific MMP subtypes. So a panel of different MMPs should first be tested within the different Barrett tissues to more accurately determine their protein levels. Immunohistochemistry stainings could serve well in this respect.

6.3 P53 AS A PROGNOSTIC BIOMARKER IN BARRETT’S PATHOGENESIS

Biomarker research in BE has mostly focused on molecules that could serve as prognostic biomarkers for cancer progression in BE patients. A well-known biomarker that has been extensively studied in BE risk stratification is p53. Aberrations of this molecule can be detected through a variety of techniques, such as immunohistochemistry (IHC), loss of heterozygosity analysis (LOH) and fluorescent in situ hybridization (FISH). P53 mutations that result in aberrant protein expression can be detected by IHC. FISH can detect other abnormalities such as large chromosomal abnormalities or locus loss. Some studies have reported a significant correlation between p53 abnormalities as detected by these techniques and an increased risk for progression to cancer (6,30,31). These studies however showed relatively low prognostic sensitivities (6), had small number of patients (30) or made use of techniques that are time-intensive and difficult and might thus not be easily translated into general clinical practice (31). We decided to evaluate whether two quick and easily applicable methods, IHC and FISH, could complement one another in detecting p53 abnormalities and whether they could be used as prognostic tools in the predicting of the development of high grade dysplasia (HGD) and esophageal adenocarcinoma (EAC) in Barrett’s patients. For FISH we used a centromeric (CEP17) and locus specific probe (LSIp53) to assess P53 aberrancy in two ways. Here gain of CEP17 combined with a CEP17/LSIp53 signal ratio larger than one was defined as relative loss and diploid CEP17 with a loss of a LSIp53 signal was termed as absolute loss. For IHC, nuclear P53 accumulation as defined by very dark staining and a complete lack of nuclear staining in aberrant tissues was defined as indicative of an aberrancy of the molecule.

We applied these two techniques in a cohort of 116 patients and found that an increase in p53 abnormalities correlated with increased histological aberrancy. This is in line with the literature (6,32). Combination of both techniques led to an increase in the frequency of detected p53 abnormalities of up to 100% for both HGD and EAC. This additive effect of the two techniques upon one another was further observed when the prospective follow-up cohort was used to investigate the actual prognostic power of p53 abnormalities in BE. Our follow-up data indicated that both techniques were independent predictors of progression and combining the two techniques led to an increase of the p53 IHC and FISH sensitivity for progression prediction. The combination of techniques led to a sensitivity and specificity of respectively
A study by Kastelein et al of 635 BE patients showed a sensitivity and specificity of respectively 49 and 86% for P53 aberrations as solely detected by IHC in HGD/EAC progression prediction (6). A smaller study of 16 patients, only investigating progression from the higher risk low grade dysplasia (LGD), showed a sensitivity and specificity of respectively 88 and 75% for P53 IHC aberrations in HGD/EAC progression prediction (30). The test statistic values of our p53 IHC and FISH combination thus seems comparable and at times slightly improved with respect to the literature.

An important finding of our study is the complementary effect of IHC and FISH with respect to the detection of prognostic p53 aberrations. Results from the cell lines and the patient studies seem to indicate that whereas P53 mutations occur mainly in the early stages of Barrett’s carcinogenesis, the larger P53 allelic losses occur later in this sequence. As indicated in the literature, some of these early mutations can result in a truncated P53 protein, which can be missed by P53 IHC (33,34). The genetic instability that results from these P53 mutations in turn may lead to a loss of P53 alleles that can be detected by FISH. Inversely, P53 mutations detected by IHC can be missed by FISH if these mutations do not lead to the gross chromosomal aberrancies detected by FISH. These results reaffirm the idea that different techniques and markers will need to be combined to develop a clinically applicable BE prognostic test that covers as many of the high risk patients as possible. Our investigated techniques are relatively easy to apply and can quickly deliver results, which make them very practical for potential future clinical use. P53 IHC is a relatively cheap technique that is already used by numerous laboratories and pathology departments and has been amply investigated and validated. FISH as applied in our “two pronged” way with two probes, is able to detect the disease outcome predictive relative loss category that would have been considered as “normal” and missed if only the P53 locus would have been assessed. These two approaches thus form good candidates for a future multi-marker panel for BE prognosis. Ongoing experiments are currently being performed in our lab in which other tumor suppressor and oncogene molecules, such as p16, are being investigated and validated as additional biomarkers to be added to this putative multi-marker panel.

6.4 PHOSPHORYLATION ACTIVITY AS A BIOMARKER AND POSSIBLE TREATMENT TARGET IN ESOPHAGEAL ADENOCARCINOMA

In spite of improvements in combinatorial therapy for BE related EAC, the outcomes of this cancer still remain poor (35). The 5-year survival has been shown to still be around 15-20% (36,37). As described earlier, biomarkers can be developed into molecular targets for therapeutic intervention and as a consequence also predictors of therapeutic response. One group of molecules that have been assessed and developed in this manner are the kinases. For instance, the targeting of the HER2 receptor tyrosine kinase with the humanized immunoglobulin trastuzumab has been shown to be of benefit in breast cancer treatment (38,39). With respect to esophageal adenocarcinomas, a recent trial showed improvement in survival of patients with gastric and gastro-esophageal junction cancer upon combining
chemotherapy with trastuzumab (40). We thus believed phosphorylation activity and kinases might be an interesting source of predictive biomarkers.

We decided on a bifurcated approach to investigate phosphorylation activity in EAC. Firstly we used a tyrosine kinase chip, consisting of 144 peptides derived from kinase target proteins, which indicated that total phosphorylation activity is higher in EAC compared to normal squamous esophageal tissues. Around 86% of the analysed peptides were more highly phosphorylated in tumor compared to normal squamous tissues. Of interest was that a large portion of these peptides are involved in immunity. This likely reflects the important stimulating role of chronic inflammation in Barrett’s disease (41-43). Looking at players of pathways related to inflammation could thus yield some useful biomarkers and potential targets. In our second step, we decided to focus on the Rb-p16 pathway as it has been shown to be involved in carcinogenesis and p16 is often aberrantly regulated in BE. We found that Rb is more highly phosphorylated in EAC cells and tissues, than in normal squamous esophageal cells and tissues. There was specifically a significantly higher phosphorylation of Rb on the S795 amino acid in cancer tissues. Upon looking at phosphorylation on different Rb groups in serial EAC tissue sections, we could see that the nuclear phosphorylation pattern for the S795 and T356 aminoacids did not completely overlap. These results seem to indicate the presence of specific Rb phosphorylation within EAC tissues.

The application of the assessed phosphorylated molecules, i.e. Rb, as predictive biomarkers necessitates the development of appropriate treatments. Luckily, the literature shows research into specific molecules that affect Rb phosphorylation in cancer. Song et al examined the green tea extract mixture of polyphenon E in Barrett’s pathogenesis and found that in cell lines of BE and EAC it leads to an inhibition of proliferation and induction of apoptosis (44). This seemed to occur through regulation of cyclin-D1, an upstream phosphorylating regulator of Rb. In another experiment flavopiridol, a pan-inhibitor of CDKs, led to a significantly lower prevalence of BE and EAC in an esophago-jejunostomy induced reflux, p27 knockout mouse model of Barrett’s pathogenesis (45). For the further development of Rb phosphorylation as a predictive biomarker it will be important to first correlate this process, as measured potentially through immunohistochemical stainings, with patient characteristics and actual disease outcome. Another interesting point will be to identify whether the disease indicating function of Rb phosphorylation differs, depending on which residue of this protein is phosphorylated. More antibodies for the large number of Rb phospho-residues, which are commercially available, should thus be tested. Correlating these results with the large number of proteins that interact with Rb will also be important and might even lead to additional biomarkers (46).

### 6.5 SATB1 as a Colorectal Biomarker

Our look into the potential function of SATB1 as a biomarker in colorectal cancer was inspired by the putative ability of this single protein to have a large effect on gene expression. This single protein through its genome re-organizing function seemed to regulate a very large list of genetic targets, which led to a number of detrimental effects in breast cancer cells, such as increased metastatic potential (47). SATB1 could thus possibly function as a prognostic, ‘umbrella’ biomarker indicative of a large slew of genetic changes that might lead to more
aggressive cancer. Furthermore, this protein could potentially also be developed into an ideal target for therapeutic intervention. Instead of targeting separate molecular targets one could actually modulate a single, central upstream protein.

A look into the SATB1 in cancer literature already showed however that the role of this protein might not be quite as straightforward as the SATB1 in breast cancer results would indicate. For instance, in certain types of lung cancer the loss of the protein actually seemed to be detrimental. In an article by Selinger et al, the authors actually discovered that there was a significant decrease of SATB1 nuclear expression in a number of lung cancer types. Furthermore, in 87 samples of laryngeal squamous cell carcinoma (SCC) loss of SATB1 expression was found to be an independent adverse prognostic factor for disease specific survival (hazard ratio = 2.06, 95% CI: 1.2–3.7, p = 0.016) (48). Also, this protein, believed to be mainly involved in the development of T-lymphocytes (49), has been consistently shown to be expressed in normal cells of a variety of organs, including colon, albeit at a lower frequency than in the oncogenic cells (50-53). In our colorectal samples we saw expression of the protein both in cancerous and normal tissues. Interestingly, in our normal tissues there was a higher frequency of SATB1 expression than the cancerous tissues according to our scoring system. This is likely to be due to our choice of including a broad range of staining intensities and number of stained nuclei as indicative of SATB1 positivity. As there is no general scoring system for this protein yet, we selected this approach to not miss any potential biomarker capability of SATB1. In our tissues there was also a marked difference in the heterogeneity of the strength of expression between normal and cancerous tissues. The cancer tissues had a higher frequency of strong SATB1 protein expression while the normal tissues had a much higher frequency of light to moderate expression. The choice of scoring system is thus important for the interpretation of SATB1 expression studies and must be taken into account. Using our chosen method of assessment, we found no correlation of SATB1 with a number of disease characteristics. However, upon stratification for patients with non-metastasized disease, we could see a correlation of increased disease relapse with SATB1 protein expression positivity in our colorectal cancer patients. This alludes to an earlier role for SATB1 in CRC development in contrast to its metastasis and invasion-stimulating role in breast cancer. In another article on the role of SATB1 in colorectal cancer it was also upon stratification, in this case for the absence of the similar protein SATB2, that the researchers discovered a significant correlation with a poorer disease outcome (52).

Combining our results with the literature on this subject we can conclude that SATB1 might function as a prognostic biomarker in colorectal cancer with respect to a poor disease outcome. The role of this protein is however likely dependent on the presence and activity of other co-factors as reflected by its prognostic power reaching significance only upon stratification for co-factors (SATB2) and/or stage of disease. The biomarker ability of SATB1 might therefore differ, depending on its tissue localization and moment of expression. Initial experiments should thus first look at correlation of SATB1 expression with other more researched prognostic factors in CRC such as KRAS aberrations and microsatellite instability status. Larger cohorts with more extensive patient data, with respect to for instance genetic signature, will be needed for these experiments.
6.6 CONCLUSION

With the designing of better and faster techniques for bio-molecular assessments the door has been opened in medical oncology for personalized treatments. It is becoming more and more apparent that cancer is a genetically heterogeneous disease that desires different treatment approaches for different (genetic) sub-groups of patients. Biomarkers are likely to play an important role in this respect. However, we are still at the dawn of clinical applicability of biomarkers. An important emerging paradigm is the multi-marker approach towards diagnostic, prognostic and predictive biomarkers. Due to the great heterogeneity of cancer it appears that more than one molecular or biological marker as well as a variety of techniques should be assessed to clearly identify different groups with specific characteristics with respect to disease outcome or treatment options. Our studies support this notion. For instance, the tested MMP-activatable probe upon adjustment could be used as a diagnostic marker to detect those small, higher risk dysplastic lesions in Barrett’s esophagus that might be missed by standard white-light endoscopy coupled with random biopsies. However, it’s upon combination with these histological lesions, that MMP activity in Barrett’s would then truly show its worth, as a prognostic biomarker (Chapter 2). The prognostic value of the p53 molecule with respect to Barrett’s outcome prediction was improved upon combining two techniques, which separately assessed p53 mutant protein and gross chromosomal aberrations (Chapter 3). The specific and varying phosphorylation of Rb in EAC tissues (Chapter 4) seems to indicate that different Rb interacting proteins might be involved in these tissues. However, a true correlation of Rb phosphorylation with disease outcome will need to be determined first. Afterwards it will likely be important to take along Rb interacting partners in further assessments of the predictive and prognostic capabilities of Rb phosphorylation. As for the multi genetic loci affecting SATB1 in CRC, we found that only upon combination with another marker of disease (i.e disease stage), it achieved prognostic significance with respect to disease-free survival (Chapter 5). Therefore, the way ahead in gastro-intestinal cancer treatments might likely have to follow the multi-marker approach.

In this multi-marker approach it will, however, not only be important to select the correct collection of bio molecules. As important will be the selection of which techniques to apply in the assessment of these bio molecules. This aspect of biomarker research was reflected in the complementary effect of the two techniques of FISH and IHC in the detection of the different variants of the p53 molecule (Chapter 3). The importance of the scoring system in the SATB1 measurements with respect to defining biomarker positivity (Chapter 5) also underlines the value of choosing the right biomarker assessment tool. For future clinical application, the cost-effectivity and ease of use of these techniques will also be factors that will have to be taken into account.
**REFERENCE LIST**


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