A search for molecular biomarkers in gastro-intestinal cancer
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Citation for published version (APA):

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chapter 1

GENERAL INTRODUCTION

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Partly adapted for a review under construction
1.1 INTRODUCTION

Cancer is a very broad disease that has manifested itself as one of the most prominent medical problems in the world. Data from the World Health Organization (WHO) showed that in 2008 all forms of this disease together accounted for over 13% of the yearly mortalities, making it the third largest killer behind infectious diseases and cardiovascular diseases (1). Looking at 2010 data for the Netherlands, we find that cancer is actually the largest reason of death as summarized over all ages, accounting for 32% of all mortalities (2). Research into this disease has a very long history going back centuries. Yet as indicated by the mortality rates, there is still room left for the improvement and designing of preventive and curative measures. In this thesis we will outline the investigation of one of the newer molecular approaches to cancer treatment with a focus on cancers of the gastrointestinal tract and in particular esophageal adenocarcinoma (EAC) with a small sidestep to sporadic colorectal cancer (CRC).

1.2 ESOPHAGEAL ADENOCARCINOMA AND BARRETT’S ESOPHAGUS

Esophageal adenocarcinoma (EAC) is becoming a health problem of high relevance as indicated by an increasing incidence rate (3-6). A complicating factor is the fact that the clinical outcome of EAC is rather poor. Even after surgical resection, the overall 5 years survival rate of patients is no more than 20% and adjuvant treatments such as chemo- and radiotherapy only slightly improve patient outcome (7-10). EAC has a strong association with Barrett’s esophagus (BE), a metaplastic premalignant transformation of the esophageal epithelium associated with gastro-esophageal reflux disease (GERD) (11-14). Interestingly BE, is also showing an increase in incidence (6,15).

The diagnosis of GERD indicates that there is a chronic influx of gastric and duodenal contents, such as acid and bile into the distal part of the esophagus. This reflux can cause the stepwise transformation of the squamous cells of the esophagus into intestinal-type columnar cells (4). This type of esophageal tissue, known as intestinal-type metaplasia (IM) can in time become inflamed and damaged, progressing through the histopatological stages of low grade dysplasia (LGD) and high grade dysplasia (HGD) ultimately into EAC. As patients progress through this sequence, there is an increase in not only morphological but also genetic abnormalities which ultimately might lead to the neoplastic lesion (16-18). The amount of patients with BE that actually progresses to EAC is rather small, with an incidence of approximately 0.12 to 0.6 % per year (5,19). Notwithstanding this seemingly minute percentage, people suffering from BE still have a higher risk of developing EAC compared to the general population, with the risk being as much as a 11-fold higher (19). Interestingly, the risk to progress to HGD or EAC increases once LGD has developed and varies between 0.4 to as high as 13.4 % (19-21). And finally, upon separating the two most advanced histological stages the literature shows incidence rates for EAC in non-treated HGD patients that vary between 5.6 to 14.4% (22,23). The appearance of the successive dysplastic stages of BE progression are thus indicative of EAC development. These observations have been translated into the current BE endoscopic surveillance protocol as defined by the American College of Gastroenterology. According to these guidelines, biopsies
should be taken in four quadrants every two centimetres of the part of the esophagus covered in intestinal-type epithelium (24, 25). Upon diagnosis of ‘no dysplasia’ follow-up surveillance should be performed within 3 years. A diagnosis of LGD should lead to follow-up surveillance after one year. A diagnosis of HGD should lead to follow-up surveillance after 3 months or endoscopic intervention depending on the case (25). These guidelines are similar to those established by the British Society of Gastroenterology (26). Histopathological staging of BE has become the reigning gold standard when it comes to the prognosis of development of EAC in BE (27). Combined with endoscopic surveillance it is the main preventive strategy to reduce the risk for EAC in BE (28). This surveillance strategy is contested however, as histological grading has proven to be subject to significant intra- and inter-observer variation (29). Moreover, the malignant progression from non-dysplastic BE to EAC does not necessarily follow the assumed sequence of histopathological events (30). Furthermore even with careful and prudent sampling, small or buried lesions might still be missed during standard endoscopic surveillance. There is thus room for improvement of the endoscopic surveillance strategies employed in BE for prognostic purposes with respect to a poor disease outcome.

1.3 BIOMARKERS IN THE PATHOGENESIS OF ESOPHAGEAL ADENOCARCINOMA

One way to improve disease management strategies in BE is through the usage of biomarkers. Biomarkers are biological molecules such as specific genes, mRNA and protein products measured in humans, that are usually differentially expressed in normal compared to aberrant tissues and other biological samples. The expression or appearance of these bio molecules is assumed to be an indication of the presence or initiation of the (malignant) process of interest. The Early Detection Research Network (EDRN), which commits itself to aiding the realization of molecular diagnostics and biomarkers as clinically applicable strategies against cancer, has suggested five developmental phases to guide (prognostic) biomarker research (31). These phases consist of phase 1, which is explorative research into a bio molecule of interest. Phase 2, which mostly focusses on the assessment of a clinically applicable method to detect the earlier discovered bio molecule of interest and the diagnostic power of this molecule with respect to the disease of interest. Phase 3 concerns itself with a retrospective study of the prognostic power of the molecule of interest in stored, well-defined samples. Phase 4 contains a true prospective look at the prognostic power of the molecule of interest, while phase 5 finally investigates the practical effects of a screening method based on the molecule of interest with respect to the reduction of cancer deaths and economical costs. A number of prognostic biomarkers have and are currently being assessed in BE. These assessments are usually in the early phases (phase 1-3 with respect to the EDRN guidelines) and none of them have yet been found to have been adequately studied and validated, to be clinically applicable yet.

DNA content abnormalities. Some of the well-known biomarkers which have been tested in BE disease prognosis are cellular DNA content abnormalities in the form of aneuploidy and abnormal 4N fractions. These abnormalities are usually analysed by flow cytometry.
Experiments have shown an increase of these abnormalities with increasing dysplasia (32). Also in predicting the development of EAC from less advanced Barrett’s disease aneuploidy and a raised 4N fraction have shown prognostic power. In one study the development of cancer from the combined IM, indefinite for dysplasia and LGD stages showed a 19 times increased risk in patients with either aneuploidy or a raised 4N fraction versus neither abnormality (33). The results of DNA content abnormalities though promising show that not all patients with these abnormalities progress to cancer, while some patients without the abnormalities progress to cancer (32, 33).

Epigenetic abnormalities. The epigenetic changes in esophageal tissues during the process of Barrett’s pathogenesis are another set of biological occurrences being looked at as a source of potential prognostic biomarkers. Aberrant methylation is a process that has been detected already early on in the process of Barrett’s pathogenesis (34,35). Research has indicated a wide array of loci that are hypermethylated within Barrett’s disease (36). Of even more interest, Schullmann et al found in a cohort of 107 patients that promoter hypermethylation of p16, HPP1 and RUNX3 genes was actually an independently significant predictor of progression from IM and LGD to HGD/EAC (30). Whole genome methylation analysis actually showed that even with cell free DNA as collected in the serum of BE patients it was possible to distinguish BE and EAC patients from healthy patients (37).

P53. A biomarker which has been often investigated with respect to disease outcome prediction in BE is the P53 molecule. This transcription factor has a well-known role as a tumor suppressor in cancer. Also in Barrett’s disease it has shown potential as a biomarker. Many different techniques have been used to assess the biomarker function of p53 in BE. One approach which early on yielded very promising results was the detection of loss of heterozygosity (LOH) of the P53 allele. In a study by Reid et al 37% of Barrett’s patients with intestinal metaplasia who had P53 LOH progressed to cancer, while only 3% of patients with wildtype allele status developed cancer (38). This led to a relative risk of 16 (95% CI = 6.2-39; p < 0.001) for BE patients with P53 LOH to develop cancer (38).

Our search. As earlier inidicated none of these potential biomarkers have been shown to be applicable for general clinical applications as described by phase 4 and 5 of the EDRN guidelines for biomarker research (31). However a paradigm that has emerged from all of these strands of research is that likely a single biomarker will be insufficient to serve in the identification and prediction of disease outcome in BE (39). A number of separate biomarkers will likely have to be combined to truly cover all those patients with pre-malignant disease at risk. Most biomarker research in EAC has focussed on the prognostic potential of the biomolecules, partly due to the advantage of the clearly defined pre-malignant state of BE that can be assessed. But biomarkers can also be used for the diagnosis of disease, i.e. the detection of malignancy. These biomarkers could play a role in the detection of the small BE lesions that could be missed during endoscopic surveillance. Also, another advantage of biomarkers is that the discovery of bio molecules that are differentially regulated in aberrant and normal tissues can give potential targets for molecular targeted treatment modalities. Furthermore, biomarkers developed in
this sense can become predictive biomarkers. Upon development of treatment modalities aimed at their molecules, they can possibly be employed as an indication of response to the aforementioned treatment. As the survival rate of EAC patients is still relatively low even with the current improved surgical and chemo-radiotherapy approaches this is also a necessary avenue of research (10). All these factors leave room for the discovery, validation and development of biomarkers in EAC. In this manuscript we will primarily describe the first stages of investigation into the separate, potential biomarkers. In this respect the biomarkers have mostly not yet been separated according to their prognostic, diagnostic or predictive abilities. Also oftentimes the bio molecules investigated as candidate biomarkers are capable of functioning within multiple biomarker roles. Their capabilities are thus not so clearly demarked. However, per chapter we will indicate which of the different biomarker facets most interested us with respect to the molecules of interest. For instance, in chapter 2 we looked into Matrix metalloproteases aided by a protease activatable probe, with a special interest in their use as potential diagnostic biomarker aids. In chapter 3 the tumor suppressor p53 as assessed by two different techniques was investigated as a prognostic factor in BE disease outcome prediction. In chapter 4, kinase activity and the specific phosphorylation of the Retinoblastoma protein were assessed in EAC as potential starting points for the development of therapeutic modalities. They were thus viewed as candidate predictive markers. Following we will elaborate on these different candidate BE and EAC biomarkers.

**1.4 ACTIVE PROTEASES AS BIOMARKERS IN BE**

An important risk factor for BE is the occurrence of GERD (40,41). It has been shown that when independently considering GERD as a risk factor, there is already an increased risk for developing EAC. In a meta-analysis the odds ratio, as a measure for relative risk of EAC, for individuals with GERD with daily symptoms compared to individuals with no or few symptoms was determined as 7.40 (95% CI = 4.94 - 11.1) (42). In comparison the standardized incidence ratio as a measure for relative risk of EAC in BE patients was given as 11.3 (95% CI, 8.8 to 14.4) in a recent report (19). GERD leads to the chronic inflammation of the esophagus defined as esophagitis (43-45). Chronic inflammation is increasingly being viewed as one of the hallmarks of cancer and also in BE it is believed that inflammatory pathways contribute to malignant oncogenic transformation (46-48). The chronic inflammation of esophagitis, still present upon the development of BE, corresponds with the recruitment of inflammatory cells and the production of a number of chemokines and cytokines such as IL-6, IL-8, IL-1β and NF-κB by stromal as well as epithelial cells (49-53). Fitzgerald et al observed that even patients that receive treatment with proton pump inhibitors (PPIs) to reduce the inflammation causing reflux, still show microscopic evidence of inflammation in their esophageal tissues (50). Interestingly they also indicated that there appears to be an inflammatory gradient in BE with the more proximal squamous and metaplastic tissues showing higher pro-inflammatory IL-1β, while the distal metaplastic tissues showed higher anti-inflammatory IL-10 (50). Another recent article investigating the development of Barrett’s pathogenesis in a genetically modified mouse model also showed a close link between inflammation and development of Barrett’s disease (48). In 10 animals, overexpression of the pro-inflammatory cytokine IL-1β in the esophagus led to inflammation,
which ultimately led to the development of metaplasia in 90% and even dysplasia in 20% of the animals. Even though these animals were kept on acidified water (pH ≤ 2.0), age-matched wild type mice kept on the same water did not show any of the histo-pathological changes seen in the mutant animals (48). This indicated that solely chronic inflammation is already able to initiate the process of BE pathogenesis.

A group of enzymes with quite some potential for the role of biomarkers in cancer are the proteases. Through cleavage of peptide bonds in proteins these enzymes modulate a large number of biological pathways. In the field of cancer research these molecules have been targets of interest for some time. Proteases were originally thought to be mostly important as modulators of the oncologic processes of invasion and metastasis, through the cleavage of the extracellular matrix (ECM) (54-58). It was envisioned that they would ‘eat away’ the ECM opening a route for those tumor cells with the ability to invade, to exit the primary tumor and enter surrounding tissues and ultimately the lymph or blood vessels, initiating the process of distant metastasis. However, research has also shown a role for these enzymes beyond the classical scope of invasion and metastasis, in general tumor progression and growth (44-48) and these enzymes can also be found in early pre-neoplastic lesions (49,50). Importantly there is also a strong correlation between certain proteases and inflammation as they are produced by inflammatory cells and their production is up regulated under inflammatory conditions (54,56,58-61). With respect to proteases, cancer and inflammation, the matrix metalloproteinases (MMPs) form an interesting group of enzymes. The MMPs are either secreted or membrane bound in normal, healthy tissues (58). Concordantly, most of their targets are extracellular proteins, protein-complexes and also membrane-bound molecules (58). Their cleavage of the extracellular-matrix (ECM) causes the release of a number of cytokines and other bio molecules that contribute to cell signalling processes, a fact that is likely essential for their putative role in cancer. During normal development and under homeostatic conditions this family of proteases produced by epithelial and stromal cells, play important and varied roles in tissue remodelling and inflammation related processes (62,63). In oncogenesis these enzymes perform again a variety of functions ranging from apoptosis resistance to angiogenesis to metastasis (54,58,62,64). There is also some tentative evidence for a role for the MMPs in BE and related EAC, with Salmela et al showing a large number of EAC biopsies ubiquitously expressing MMP7 mRNA. The intestinal metaplasia samples also showed expression of MMP7 mRNA while in contrast the normal esophagus was negative for this molecule (60). In another experiment, Herszenyi et al showed a gradual increase in MMP9 protein expression as detected by immunohistochemistry along the pathogenic cascade of esophagitis to Barrett’s metaplasia up to EAC (65).

We decided to combine the intimate link between inflammation, MMPs and the oncogenic process as collected in Barrett’s esophagus in an effort to discover possible prognostic and diagnostic biomarkers. For a future real time application of a protease biomarker we decided to employ the MMP activated fluorescent probe MMPsense680 in this search. This probe is activated by a number of MMPs and is visualized by near infrared light. Visualizing in this part of the spectrum should lead to deeper tissue penetration and less background fluorescence from abundant tissue molecules such as water and haemoglobin.
1.5 P53 AS A BIOMARKER IN BE

As shortly alluded to earlier, one of the most prominent candidates for the biomarker role in cancer has been the P53 gene and its associated protein product. The P53 protein functions as a tumor suppressor through the stimulation of apoptosis and growth arrest upon experiencing certain cellular stresses such as DNA damage. In cancers, P53 activity is often down regulated or aberrantly regulated (66,67). This can occur through various pathways. One of the P53 alleles might for instance contain an inactivating mutation. Consequent genetic insults can result in more mutations and general chromosomal instability leading to the loss of the other P53 gene, a process termed loss of heterozygosity and detected through an eponymous technique (38). On the other hand mutations can at times lead to stabilization of the P53 protein resulting in a longer-lived molecule that accumulates in the cell. This protein accumulation has been correlated with neoplastic development for a variety of tumors (68). Interestingly, in BE it has been shown that there is an increase in the amount of patients showing this P53 accumulation for the consecutive stages of the IM-dysplasia-EAC cascade (32,68-71). Research has also shown a correlation between the rate of malignant progression of BE patients with LGD and p53 accumulation positivity of their esophageal tissues (72). In a small prospective study of 48 patients, those subjects whose tissues had P53 accumulation had a higher percentage of progression towards HGD and cancer than those with P53 negativity, namely 30 versus 5% (72). And in a recent large prospective cohort study of 635 patients, P53 protein accumulation as detected by immunohistochemistry outperformed the current golden standard of histopathological staging, showcasing a better sensitivity and specificity in the prediction of the development of HGD and EAC in BE (73). P53 protein accumulation had a sensitivity and specificity of respectively, 49 and 86%, while a diagnosis of LGD had a sensitivity and specificity of respectively, 44 and 78%.

Immunohistochemistry (IHC) is the most commonly used method to detect aberrant p53 accumulation. At the Amsterdam Medical Center P53 IHC is used by pathologist as an aid for the staging of BE tissues. However, the weakness of this technique as a potential prognostic test in BE, comes from the fact that the P53 accumulation as detected through staining does not necessarily indicate a mutation of the gene. Theoretically, increased wild type P53 expression as a consequence of cellular stresses can also be detected with IHC. Furthermore not all P53 mutations lead to protein stabilization, thus leaving the possibility of some cases potentially being missed. Notwithstanding some promising results, the test statistics of P53 IHC with respect to outcome prediction still need to be improved on.

Another way to detect P53 gene abnormalities is through the use of fluorescence in situ hybridization (FISH). In contrast to IHC which indirectly detects mutations leading to P53 accumulation, this technique directly detects gross chromosomal abnormalities such as gene loci or whole chromosomal losses or gains (16,17,74). These gross genetic abnormalities are the result of genetic instability and are thought to occur at later stages during the pathogenic progression of BE as opposed to the initial (single) mutations that are detected by IHC (17). Research on gains and amplifications of a number of oncogenesis related genes, such as c-myc and EGFR, has shown that chromosomal abnormalities as detected by FISH may be of diagnostic and potentially also prognostic value in BE patients (74). However, the true prognostic value of
this relatively novel approach has not been conclusively proven as of yet. The fact that FISH could potentially detect a subset of cases with p53 aberrancies that are missed by IHC, has led to us contemplating the possibility of the combination of both techniques in the detection of p53 abnormalities. The two techniques might supplement one another and compensate each other’s deficiencies in BE with respect to progression prediction. Our aim was thus to see how p53 IHC and FISH relate to one another as potential diagnostic and prognostic techniques in BE and whether a combination might improve upon the golden standard of histopathological staging.

1.6 PHOSPHORYLATION ACTIVITY AS A TARGET IN EAC

As indicated earlier, biomarkers can also lead to a broader understanding of the bio molecular changes underlying the aberrancies of diseases, such as the EAC related pathogenesis. This deeper insight coupled with the measured molecular changes can also ultimately offer up potential therapeutic targets for disease modulation. Potential routes to this deeper molecular insight are kinases and their enzymatic activities. Through addition of a phosphate group to specific targets, this group of enzymes activates large and far-reaching signalling cascades, which are pivotal in maintaining homeostasis in living organisms. However, this pivotal role also means that the precarious biological balance they help sustain can be easily broken, through the mutation or aberrant expression of these bio-molecules. The elucidation of phosphorylation activity with respect to specific processes, such as cell proliferation, has been shown to be an attractive avenue of research as molecular strategies developed around inhibitors of the involved kinases might serve as anticancer therapeutics. This line of research has led to kinase targeting drugs such as trastuzumab. The specific targeting of the HER2 receptor tyrosine kinase with this humanized antibody has been shown to be of benefit in breast cancer treatment (75-77). The HERA trial for instance showed that one year of trastuzumab admission to HER2-positive breast cancer patients after conventional surgical and adjuvant chemo- radiotherapy treatment led to an approximately 50% improvement of the disease-free survival rate (75). In EAC amplification of the HER2 gene has also been noticed in up to 21% of samples (78). Miller et al also found gene amplification through qPCR of the ERBB2 receptor tyrosine kinase in around 21% of 87 esophageal adenocarcinoma samples. This was the highest percentage of cancer-related amplification they found out of a list of oncogenes they were testing for (79). A recent trial has shown improvement in survival of patients with gastric and gastro-esophageal junction cancer upon combining chemotherapy with trastuzumab (80). In this ToGA trial the patients treated with chemotherapy plus trastuzumab had a median overall survival of 13.8 (95%CI = 12 – 16) months versus 11.1 (95%CI = 10 – 13) in patients solely on chemotherapy (80).

These results with modulation of HER2 in cancer indicate that identifying novel aberrancies of other phosphorylation related molecules involved in cell proliferation, could be useful for ameliorating the effects of cancers such as EAC. A potential source for these molecules is the p16-Rb pathway, which is frequently affected in cancer (81). This well-examined pathway contains a large number of kinases and important instances of phosphorylation (81). One of its prime constituents, the p16/INK4A protein, inhibits the cyclinD-CDK4/6 complexes that modulate progression through the G1/S-phase checkpoint of the cell cycle through hyper-phosphorylation of the RB-E2F complex. Release of the E2F transcription factors consequently
leads to DNA replication (81). Aberrancies of p16, such as mutations, methylations and deletions, are some of the earliest events in cancer (82). These events are also some of the earliest events in Barrett’s pathogenesis with a number of alterations such as LOH, mutations and importantly, methylation being present in the early stages of BE (83-86). In general, these p16 aberrations seem to increase with increasing tissue aberrancy (83,84,86). As such, this gene and its related protein are being investigated for insight into the mechanisms driving the initial steps of carcinogenesis. It is very likely that some of the other kinases or targets of phosphorylation within this pathway could also serve as molecules of interest in this respect. And in actuality in a number of cancers it has been shown that aberrations of p16 affect phosphorylation status of Rb (87,88). We believed that it would be of interest to investigate whether a similar bio molecular effect could be seen in EAC.

As such we’ve applied a bifurcated approach to study phosphorylation activity as a driver of EAC pathogenesis and thus a potential source for molecular insight and targets. Firstly we’ve used a high-throughput kinase chip approach to look at general phosphorylation activity in EAC. The kinase chip we employed contains over 140 peptides derived from a selected group of known tumor-related kinases. Secondly, we examined the p16-Rb pathway in EAC to investigate whether phosphorylation of Rb might play a role in EAC pathogenesis.

1.7 SPORADIC COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth most common cause of cancer related deaths worldwide (89). This type of cancer consists mostly for around 75% of the non-heritable, sporadic type in contrast to hereditary types of colorectal cancer such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (90). There are a variety of risk factors for CRC, with advanced age being an important factor (90). CRC is a heterogeneous disease believed to develop through an accumulation of genetic and epigenetic changes, which gradually change normal colonic mucosa into invasive cancer (90). A large amount of research has been devoted into the unravelling of the mechanisms behind this disease. This led to the discovery of different molecular pathways leading to different CRC phenotypes. The most common pathway in sporadic CRC is believed to be the chromosomal instability (CIN) pathway. In around 70% of sporadic CRCs, aberrant chromosomal separation and defects in DNA damage repair mechanisms lead to aneuploidy and gross gains and losses of chromosomes and alleles thusly affecting pathways essential for colorectal carcinogenesis (90). Aberrations of the p53 and APC genes are characteristic for this pathway. The other pathways are the microsatellite instability and promoter hypermethylation pathways. These pathways, as currently defined, are however not mutually exclusive and can overlap. This great variety of molecular pathways and specific molecules active in sporadic CRC opened up the possibility of biomarker application in sporadic CRC. These biomarkers are envisioned to be used for the detection of early or established disease and also to potentially predict disease outcome. A large number of these candidate biomarkers still need to be fully validated for clinical applicability, however (91,92). Because of the heterogenic nature of CRC it is likely that more than one biomarker will be needed to be able to detect and risk stratify all sporadic CRC patients. In CRC, biomarkers are also sought to stratify which patients would be most suitable for some of the new anti-cancer
adjuvant molecular modalities developed such as the monoclonal antibodies cetuximab, which targets the receptor tyrosine kinase EGFR and bevacuzimab, which targets the vascular endothelial growth factor, VEGF-A (91,92). For cetuximab, mutations in the KRAS gene seem to be a good indication of a poor response. For bevacuzimab no valid predictive biomarker has been found yet with respect to predicting treatment response. There is thus still room for biomarker discovery and refinement in sporadic CRC also for this particular use. Below we will shortly touch upon some of the researched and reported on biomarkers in sporadic CRC.

1.8 Biomarkers in Sporadic CRC

**KRAS and BRAF.** The KRAS protein and one of its downstream effectors the BRAF protein play roles in numerous important cellular functions as part of the RAS signalling pathways, such as cell survival and proliferation (93). These proteins have come into focus in sporadic CRC as candidates for predictive biomarkers with respect to treatment with the EGFR targeting monoclonal antibody cetuximab (91,92). For instance, Lièvre et al investigated the effect of KRAS mutation, as assessed by sequencing of exon 1 of the gene, in 30 metastatic colorectal cancer patients treated with cetuximab in a combined therapy setting (94). They found that none of the responding patient tumors contained a KRAS mutation compared to 13 of the 19 non-responders, leading to a significant association of the mutation with an absence of cetuximab response (p = 0.0003) (94). These results were validated in a number of other studies (91,92), which has led to KRAS mutations being advised in some cases as predictive negative biomarkers in case of treatment of metastatic CRC with anti-EGFR therapies. Also for BRAF there is some evidence for non-response to anti-EGFR therapies upon mutation. In a retrospective study of 113 patients with metastatic CRC who had received treatment regimens that included cetuximab, it was discovered that 0% of the tumors containing the BRAF V600E mutation showed response, compared to 32% of the wildtype BRAF tumors (95). Apart from the predictive capabilities of these two biomolecules these studies employing anti-EGFR therapies have also shown a prognostic ability for KRAS and BRAF. Patients with mutated KRAS or BRAF showed on average a poorer progression-free and overall survival rate (94,95). There is however also some evidence for a prognostic effect of KRAS and BRAF independent of anti-EGFR therapies such as cetuximab. In a study by Richman et al 711 patients with advanced CRC randomized for 5-FU/irinotecan or oxaliplatin combinatorial treatment were assessed for KRAS and BRAF mutations followed by a determination of overall and progression free survival (96). This study showed that while KRAS and BRAF mutations showed no correlation with progression free survival, there was a clear correlation with a poorer overall survival (respectively hazard ratio: 1.24; 95% CI = 1.06 - 1.46, p = 0.008 and hazard ratio: 1.82; 95% CI = 1.36 - 2.43; p < 0.0001). Also, upon combining patients with mutations in either gene there was some evidence for a poorer progression free survival in the adjusted analyses (hazard ratio: 1.20; 95% CI = 1.01 to 1.41; p = 0.03).

**MSI.** Microsatellites are stretches of nucleotide repeats found spread out within the genome. Microsatellite instability (MSI) is defined as a change of length of a microsatellite due to insertion or deletion of repeating units. This instability is believed to be a consequence of faulty DNA base mismatch repair. MSI was first discovered in one of the hereditary forms of
CRC, namely HNPCC and correlates with mutations in a number of mismatch repair genes \((MLH1, MSH2, MSH6)\) and a lifetime risk of developing CRC of around 80% \((97)\). Around 15% of sporadic CRC is also characterised by MSI. In sporadic CRC, MSI is believed to be mostly the consequence of allelic methylation of \(MLH1\) \((97)\). According to consensus, MSI is currently determined by a panel of 5 markers; three di-nucleotide \((D2S123, D5S346, D17S250)\) and two mononucleotide markers \((BAT25, BAT 26)\) \((97,98)\). If changes are observed upon comparing tumor to normal tissues in \(\geq 30\%\) of the markers this is considered MSI high \((MSI-H)\). Changes in less than 30% of the markers is considered low level MSI \((MSI-L)\) and if no changes are observed this is considered microsatellite stable \((MSS)\). There is still some debate however on whether \(MSI-L\) and \(MSS\) are truly separate entities. Apart from aiding in the diagnosing of HNPCC, MSI has been of interest in sporadic CRC as it seems that MSI-H tumors have distinct bio-molecular and histo-pathological characteristics. These tumors are often diploid, poorly differentiated and contain marked lymphocytic infiltration \((97)\). More importantly MSI is believed to be a prognostic marker for disease outcome in CRC. In a meta-analysis, Guastadisegni et al, looked into 31 studies, for a total of 12782 patients, at the effect of MSI on CRC survival. Upon pooling the suitable data they found an odds ratio for overall survival and thus mortality for MSI-H compared to MSS of 0.60 \((95\%\) CI = 0.53–0.69, \(p < 0.0001)\) for all stages of CRC. For disease free survival this odds ratio was 0.58 \((95\%\) CI = 0.47–0.72, \(p < 0.0001)\). These results indicate that MSI could be a prognostic marker for an improved overall and disease free survival \((99)\). They also found upon more closely investigating 7 studies that a clear survival improving effect of 5-FU chemotherapy on MSS tumors \((treated vs un-treated MSS, odds ratio of 0.52, 95\%\) CI 0.4–0.6, \(p < 0.0001)\) was less apparent for MSI-H tumors \((99)\). Potentially reflecting the DNA damage repair impaired characteristics of MSI-H tumors, MSI could thus also function as a predictive biomarker with respect to the choice of adjuvant therapy applied in CRC.

**CIMP.** CpG islands are regions in the genome containing a large amount of CpG di-nucleotide repeats. A large percentage of genes contain these islands in their 5’ promoter-containing region \((100)\). Research has shown that in certain CRCs, hypermethylation of several loci containing these promoter CpG islands takes place, while the normal colon mucosa lacks this methylation \((100)\). These tumors are defined as having a CpG island methylator phenotype \((CIMP)\) and are associated with such patient characteristics as female sex, older age and MSI.

The discovery of this specific sub-group of CRCs naturally prompted a look into whether CIMP could function as a biomarker. The results in this respect have been varied however, likely due to the fact that CIMP affects and thus silences a wide variety of genes involved in a wide variety of cellular pathways. In contrast to MSI there is also less of a consensus of how to determine CIMP positivity with respect to which and how many markers to assess. The most widely adopted definition for CIMP positivity consists of methylation in three or more of the five loci \(CACNA1G, IGF2, NEUROG1, RUNX3\) and \(SOCS1\). In one specific study the researchers found a significant effect of CIMP-high positivity on colon cancer specific mortality upon adjusting for other patient characteristics \((multivariate hazard ratio: 0.44, 95\%\) CI = 0.22–0.88) \((101)\). CIMP-high in this case was defined as methylation in \(\geq 6\) of the CIMP-specific markers \(CACNA1G, CDKN2A, CRABPI, IGF2, MLH1, NEUROG1, RUNX3\) and \(SOCS1\). Interestingly, upon testing
different panels of CIMP-specific markers they found differences in the hazard ratio for colon cancer specific mortality with respect to CIMP positivity (101). Contrary to this, in a prior study the same researchers had shown a negative effect of CIMP-high on survival in 30 metastatic CRC patients receiving combinatorial chemotherapy (102). CIMP-high patients, defined by methylation in more than 9 of the CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A, CRABP1, MLH1, MINT1, MINT31, IGFBP3, MGMT and WRN markers, had a 1 year overall survival of 33% compared to 85% for the CIMP-low/-0 patients (102). Individual markers such as CACNA1G (p < 0.0001), IGF2 (p = 0.0004) and MLH1 (p < 0.0001) also showed this significantly poorer survival upon methylation. With respect to chemotherapy response, no significant correlation could be found with CIMP status in this study (102). The researchers took care however to indicate that caution should be applied with respect to these results due to the low number of patients and CIMP-high tumors (n= 3, according to the most stringent definition). Reflecting the predictive potential of CIMP an older study on 206 stage III CRC patients indicated that CIMP positive patients had a significantly better disease specific survival when treated with 5-FU additional to surgery compared to surgery alone (103). There was no significant difference in survival for the CIMP negative group. In this study CIMP positivity was defined as methylation at ≥2 of the P16 promoter, MINT-2 clone and the MDR1 promoter site.

Our approach. There is thus a wealth of biomarkers being tested and investigated for CRC, with some of these even already being advised for clinical application, such as KRAS. Paradigms such as MSI and CIMP indicate that as in Barrett’s associated EAC, also in sporadic CRC the idea of application of multiple bio molecules as a marker seems to be supported. A ‘blanket’ marker that could affect a large number of pathways and thus encapsulate the multiple markers approach in one neat molecule still seems like an enticing (and cost-effective) prospect however. In this light we decided to look at SATB1 as a potential prognostic and predictive sporadic CRC biomarker.

1.9 SATB1 AS A BIOMARKER IN SPORADIC COLON CARCINOMA

A potential biomarker for sporadic colorectal carcinoma is the genome organizer, SATB1. SATB1 has originally been described and analyzed in immunological research, where this protein has been shown to be vital in T-cell development and activation (104,105). This protein functions in an intriguing way, as it controls a multitude of other proteins through genome reorganization and consequent gene expression changes (104-106). To this effect, SATB1 functions as a scaffolding of sorts for genome reorganizers such as histone deacetylases, resulting in specific gene expression patterns (104-106). This protein showed of its potential as an oncologic target in the 2008 paper by Han et al (107). In this article these investigators showed that SATB1 expression correlates with a poor prognosis in breast cancer. Furthermore, functional experiments with cell lines and mice showed that abrogation of the protein’s activity reduced the invasive and metastatic qualities of breast cancer cells (107). Changes in gene expression as a result of presence or absence of this protein were also investigated. This lead to a large list of cancer-related genes, with the interesting presence of a large number of genes involved in invasion and metastasis (107).
Since then, the results of this ‘flag-bearer’ article have become somewhat disputed. Iorns et al for instance broadly repeated the research done by Han, but were unable to replicate the results, seeing no correlation between SATB1 and a poor prognosis nor any metastatic effect of the protein in breast cancer (108). However, in this research mRNA was used as a read-out for SATB1 expression in contrast to the protein levels measured by Han et al. Pattani et al on the other hand found that SATB1 gene expression did correlate with poor prognostic parameters such as tumor grade in breast cancer. However, they couldn’t find any correlation with survival (109). The potential oncogenic activities of the protein have prompted people to also look at other cancers. In laryngeal squamous cell carcinoma for instance some explorative research found higher expression of SATB1 mRNA and protein in tumor compared to normal tissue. The researchers also found expression of the gene to positively correlate with cancer progression and invasion (110). Interestingly, Selinger et al discovered that there was a significant decrease of SATB1 nuclear expression in a number of lung cancer types. In laryngeal squamous cell carcinoma they actually found loss of SATB1 expression to be an independent adverse prognostic factor (111). All in all, in various cancer types it seems that the SATB1 protein does play a role, although there are some contradicting results. Also, the exact nature of the role of the protein seems to vary.

In colorectal cancer there have been a small number of reports recently published on a putative role for SATB1 in this specific type of cancer. Meng et al found that SATB1 protein expression in tumor samples from rectal cancer patients correlated with increasing tumor stage and tumor depth of invasion (112). This finding was validated by Zhang et al who further found that down regulation of SATB1 in a CRC cell line lead to decreased proliferation, increased apoptosis and reduced invasion (113). Contradictory to these findings, Nodin et al found no correlation with clinico-pathological characteristics, but did observe a correlation of SATB1 expression with poorer cancer related survival within a specific CRC sub-group (114). Since the protein’s exact role in colorectal cancer and its potential as a prognostic and potentially predictive biomarker is as yet unclear, we decided to further investigate this subject.

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General introduction


