Somatic-genetic aberrations, specific protein levels and their prognostic value in colon cancer
Westra, Jantine Lolkje

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CHAPTER 1

GENERAL INTRODUCTION

Modified version of

Genetic alterations in locally advanced stage II and III colon cancer: a search for prognostic markers.
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*Clinical Colorectal Cancer (2004) in press*
Colorectal cancer (CRC) is the second most common cause of cancer-related deaths in developed countries. The overall 5-year survival rate is around 45%. Several factors including depth of tumour invasion into the bowel wall (stage II) and presence of node metastasis (stage III) determine the prognosis. The 5-year survival rate rapidly drops from ≥90% in stage I to 75% and 50% in stages II and III, respectively. Adjuvant chemotherapy significantly reduces the recurrence rate, improving the disease-specific survival (Moertel et al., 1995; IMPACT, 1995; IMPACT B2, 1999). Although disease-free chemotherapy has improved during the last decades, about half of the patients will still develop recurrent disease, usually presented as distant metastases to the liver (Gatta et al., 1998). Molecular biological tumour markers are thought to be helpful in discriminating long-term and short-term survivors and might predict chemosensitivity of these tumours making a more tailored adjuvant treatment possible (molecular stratification) (Wright et al., 2000; Goel et al., 2003).

CLINICAL ASPECTS

Treatment
Standard treatment consists of a wide surgical resection of the primary tumour with adjacent structures, including all lymph nodes along the vessels of the resected specimen. Patients with stage III colon cancer are treated adjuvantly with chemotherapy, usually consisting of 5-fluorouracil (5-FU) with leucovorin (LV). Recently, the addition of oxaliplatin or irinotecan (CPT-11) to this combination has resulted in longer survival in advanced disease (Douillard et al., 1999; De Gramount et al., 2000). Patients with stage II colon cancer are treated more and more with adjuvant chemotherapy mainly because 20-40% of these patients will die of metastatic disease. Some studies in patients with stage II colon cancer do show an improved survival after adjuvant treatment compared to patients with surgery alone. However, this treatment is not yet evidence-based and results from randomised studies are pending (O'Connell et al., 1998; Mamounas et al., 1999; Wein et al., 2000; Mamounas et al., 2000). Selection of high-risk patients in stage II colon cancer with poor prognosis may be useful, to consider whether adjuvant therapy should be given (Chaplin et al., 1998; Merkel et al., 2001; Burdy et al., 2001; Newland et al., 1995). Adjuvant chemotherapy administered to patients with stage III colon cancer is a standard issue and increases survival rates with about 15% (Moertel et al., 1995; IMPACT, 1995). However, it is still not possible to predict in which patient adjuvant chemotherapy will be beneficial. Furthermore, it should be noted that stage III CRC represents a heterogeneous group of patients with variable survival rates and even the lately revised pTNM staging system is not specific enough to serve as clear guidance for individual treatment and prognosis.

Clinical prognostic markers
After curative resection, the clinical prognosis is mainly determined on pathologic tumour stage based on depth of tumour invasion (T category) and nodal involvement (N category).
(Newland et al., 1994; Newland et al., 1995). The number of positive regional lymph nodes is the most strongly predictive factor in the outcome after surgical resection. Patients with more than three involved nodes have a worse survival compared to patients with less than four positive lymph nodes (Merkel et al., 2001; Burdy et al., 2001; Cserni, 2003; Chapuis et al., 1985). The presence of signet-ring cells and the existence of mucin content of more than 50% in the tumour are also associated with a poor prognosis (Kanemitsu et al., 2003; Psathakis et al., 1999). Other less important factors, which are associated with an increased risk of lymph node metastasis, are the small-cell neuroendocrine differentiation and the presence of lymphatic and vascular invasion (Granowski et al., 2001; Compton et al., 2003).

MOLECULAR ASPECTS OF CRC

General

The development of malignant tumours is a multistep process in which genetic alterations play a crucial role (Fearon and Vogelstein, 1990; Vogelstein et al., 1988). These genetic alterations accumulate in time and are associated with tumour progression. This process of tumour development evolves through selection on a background of genomic instability eventually leading to clonal expansion. A normal cell acquires a mutation resulting in loss or gain of a protein crucial for normal cell development. This may lead to a slight growth advantage over its neighbouring cells. A malignant tumour can develop when a specific combination of mutations in a cell provides additional growth advantages that lead to clonal expansion. The alterations on a genomic level may be point mutations or insertions or deletions, but also gains or losses of substantial parts of chromosomes. They lead directly or indirectly to changes in protein expression and functioning. Genes or proteins that are altered in the tumour compared to normal tissue may be called molecular markers.

Colon carcinogenesis

In 1990 Vogelstein and co-workers postulated that a minimal requirement to transform a cell from normal to malignant would be a mutation in one of the alleles of an oncogene (\textit{KRAS}) in combination with inactivation of both alleles of three tumour suppressor genes (\textit{APC, DCC} and \textit{TP53}) (Fearon and Vogelstein, 1990; Vogelstein et al., 1988). This carcinogenesis process would be initiated by inactivation of the tumour suppressor gene \textit{APC}, after which mutations are found in the oncogene \textit{KRAS}; and in the tumour suppressor genes \textit{DCC} and \textit{TP53}. Since, not in every tumour all these events are noted, it is thought that other unknown genetic events are required. In the "adenoma-carcinoma sequence" model, mutations or overexpression of different genes, including oncogenes and tumour suppressor genes, are crucial for colorectal tumourigenesis. The accumulation of genetic alterations is initiated by genomic instability. Two major independent molecular mechanisms have been suggested for genomic instability leading to the development of colon cancer, namely chromosomal instability (CIN) and DNA microsatellite instability (MIN) (Lengauer et al., 1998).
**Genomic instability**

Chromosomal instability (CIN), is found in the majority of colon cancers and is characterised by multiple chromosomal rearrangements, leading to multiple loss of heterozygosity (LOH) events and an aneuploid DNA content (Table 1) (Fearon and Vogelstein, 1990; Vogelstein et al., 1988; Lengauer et al., 1998; Calin et al., 2000). Besides these chromosomal abnormalities, an accumulation of somatic mutations occurs in key oncogenes as *KRAS* and in several tumour suppressor genes, including the cell cycle checkpoints, *APC* (in the chromosomal region 5q21-22), *TP53* (17p13), *DCC*, *SMAD2* and *SMAD4* (18q21).

Microsatellite instability (MIN) is characterised by a defective DNA mismatch repair (MMR), which is found in approximately 15% of all sporadic CRC and in most hereditary CRC (Wu et al., 1999; Jass et al., 1998; Brasnett et al., 1996; Boland et al., 1998). Tumours are considered high frequency instable (called MSI-H) when over 30% of the investigated microsatellite loci demonstrate instability (Boland et al., 1998). Mismatch repair defects result in an accumulation of unrepaired mutations mainly in repetitive sequences in both non-coding and coding sequences. As a cause of MIN, alterations have been found in MMR genes *MLH1*, *MSH2*, *PMS1*, *PMS2*, *MSH6*, *EXO1* and *MLH3* (Ward et al., 2001; Fujiwara et al., 1998; Huang et al., 1996; Young et al., 2001; Ionov et al., 1993; Thibodeau et al., 1993; Nash et al., 2003; Loeb et al., 1994; Salahshor et al., 1999; Bubb et al., 1996; Feeley et al., 1999). Microsatellite instability in sporadic colon cancer is mainly caused by the loss of the mismatch repair gene *MLH1* due to promoter hypermethylation of this gene (Young et al., 2001; Yamamoto et al., 2002; Jass et al., 2003; Kane et al., 1997; Kim et al., 1994). Genes known to be a target for mutations in MSI-H tumours, because they contain oligonucleotide repeats in their coding sequence include *TGFβRII*, *IGFIR*, *BAX*, *TCF4*, *MSH3*, *MSH6*, *CASP5*, *GRB14*, *RHAMM*, *RAD50*, *RIZ*, *E2F4*, *PTEN*, *MBD4*, *CHK1*, *TNFRSF6*, *APAF1*, *BCL10* (see glossary as appendix; Markowitz et al., 1995; Souza et al., 1996; Malkhosyan et al., 1996; Duval et al., 2001; Bader et al, 1999; Riccio et al, 1999; Bertoni et al., 1999; Chadwick et al., 2000; Piao et al., 2000; Gunati et al., 2000; Schwartz et al., 1999; Yamamoto et al., 2000; Duval et al., 1999; Rampino et al, 1997).

A summary of all associated characteristics according to the CIN and MIN phenotype is given in Table 1. The mechanisms by which CIN and MIN give rise to genetic alterations are clearly different. Many reports also suggest a clear difference between the genetic alteration caused by the different instability phenotypes.

For instance many studies have suggested that *TP53* mutations are associated with CIN (Livingstone et al., 1992, Yin et al., 1992, Agapova et al., 1996, Leslie et al., 2003) and aneuploidy (Carder et al., 1993, Fukasawa et al., 1996, Clausen et al., 1998, Campomenosi et al., 1998). Even an inverse relationship between microsatellite instability and *TP53* has been suggested (Salahshor et al., 1999, Elsaleh et al., 2001 Samowitz et al., 2001b) making presence of a *TP53* mutation an indicator for CIN.

Whether this really is the case is questionable as some degree of overlap has been found (Goel et al., 2003; Li et al., 2003; Westra et al., 2003; Plukker et al., 2003). A substantial degree of overlap has been found between the CIN characteristics like LOH or *TP53* and/or *KRAS* mutations on one hand and the MIN characteristics on the other (Goel et al., 2003; Li et al., 2003; Westra et al., 2003; Plukker et al., 2003). This raises the question whether the
effects of CIN and MIN, in terms of genetic alterations, are really different, i.e. whether these phenotypes are fully independent or do have overlaps.

Table 1: CIN and MIN tumour characteristics

<table>
<thead>
<tr>
<th></th>
<th>CIN</th>
<th>MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenotype</td>
<td>suppressor</td>
<td>mutator</td>
</tr>
<tr>
<td>DNA ploidy:</td>
<td>aneuploidy</td>
<td>diploidy</td>
</tr>
<tr>
<td>LOH</td>
<td>no LOH</td>
<td></td>
</tr>
<tr>
<td>MSS</td>
<td></td>
<td>MSI-H</td>
</tr>
<tr>
<td>TGFβ pathway:</td>
<td>SMAD2 SMAD4</td>
<td>Mutations in target genes</td>
</tr>
<tr>
<td></td>
<td>TP53 APC KRAS</td>
<td>few mutations TP53 APC KRAS</td>
</tr>
<tr>
<td>KRAS mutations</td>
<td>BRAF mutations</td>
<td></td>
</tr>
<tr>
<td>Wnt pathway:</td>
<td>APC</td>
<td>beta-catenin</td>
</tr>
<tr>
<td>p53 pathway:</td>
<td>TP53 mutations</td>
<td>BAX mutations</td>
</tr>
<tr>
<td>(Promoter) hyper-methylation of CpG islands</td>
<td>CIMP negative</td>
<td>CIMP+ (MLH1 p14 p16?)</td>
</tr>
<tr>
<td>Localisation:</td>
<td>more distal</td>
<td>more proximal</td>
</tr>
<tr>
<td>Prognosis:</td>
<td>worse</td>
<td>better in general</td>
</tr>
<tr>
<td>Histologic type:</td>
<td>non mucinous</td>
<td>mucinous tumours</td>
</tr>
<tr>
<td>Histologic grade:</td>
<td>low; (well) differentiated</td>
<td>high; poorly differentiated</td>
</tr>
</tbody>
</table>

Inherited and somatic mutations

Approximately 85-90% of the CRC tumours are sporadic, because they seem to develop independent of germline mutations. In the other cases CRCs seems to present a familial clustering (Vasen et al., 1991; Lynch et al., 1997; Peel et al., 2000). A rather small part of these patients belongs to two hereditary colon cancer syndromes referred to as the Hereditary Non-Polyposis Colorectal Cancer (HNPCC), in about 2-3% and Familial Adenomatous Polyposis (FAP), in less than 1% (Lynch et al., 1997; Peel et al., 2000). To emphasise the genetic heterogeneity with allelic variation in both the CIN and MIN genomic instability, these two most common inherited colon-cancer syndromes (HNPCC and FAP) are briefly reviewed here.

HNPCC

HNPCC is caused by germline mutations in mismatch repair (MMR) genes, mostly in MLH1 and MSH2 (over 80% of mutations are found in the two genes) (Fishel et al., 1993; Leach et al., 1993) Mutations in PMS1 and MSH6 are found as well but considered minor players in HNPCC as they account for 1% and 10% of all mutations found respectively (Malkhosyan et al., 1996; Nicolaides et al., 1994; Horii et al., 1994; Jass et al., 1993). These mutations result in functional loss of the protein product for which the mutant allele encodes and thus in a diminished repair capacity (Ionov et al., 1993; Peltomaki et al., 1997; Liu et al., 1996; Lynch et al., 1998). When the second allele encoding that specific MMR protein is also inactivated by a somatic mutation, the cell loses its ability to repair mutations that arise during DNA replication, resulting in an accumulation of mutations in coding and non-coding microsatellites. HNPCC tumours therefore belong to the MIN group of tumours.
FAP
Familial adenomatous polyposis, which is characterised by the presence of numerous adenomatous polyps, is caused by germline mutations of the APC gene. These proliferating polyps inevitably lead to colorectal cancer when left untreated. The APC protein is involved in homeostasis maintenance of proliferating cells, regulation of Wnt-signaling, cell adhesion via beta-catenin and cell migration and is present in the kinetochore and most likely involved in inaccurate chromosomal division resulting in aneuploidy (Kinzler and Vogelstein, 1996; Fodde et al., 2001; Kaplan et al., 2001). FAP tumours are examples of the CIN phenotype.

PREDICTION OF CLINICAL OUTCOME

Prediction of clinical outcome in MIN colon cancers
The development of sporadic MSI-H colon cancer shows similar features as tumours in the hereditary colon cancer syndromes. Histologically, these sporadic tumours are not easily distinguishable from tumours in HNPCC. Usually they arise at an early age of onset, have a more proximal location and show a frequent occurrence of synchronous and metachronous tumours (Grady et al., 2002; Eshleman et al., 1998; Wilmink et al., 1997). In contrast with HNPCC tumours, these sporadic MSI-H cancers have a greater preponderance among females, with a nearly exclusive involvement of MLH1 and a lower frequency of mutations in APC and KRAS (Breivik et al., 1997; Olschwang et al., 1997). These early-onset MSI-H tumours are usually larger and more frequently proximal of the splenic flexure. These tumours also are more likely of mucinous histology and poor differentiation (Thibodeau et al., 1993; Kane et al., 1997; Gafa et al., 2000; Lothe et al., 1993). Although often poorly differentiated, MSI-H tumours have been correlated with a less aggressive clinical course and with a longer survival after surgical treatment compared to the MSS tumours (Thibodeau et al., 1993; Lothe et al., 1993; Hemminki et al., 2000; Gryfe et al., 2000; Carethers et al., 1998; Watanabe et al., 2001). Several authors found MSI-H to be even an independent prognostic factor (Table 2) (Wright et al., 2000; Hemminki et al., 2000; Elsaleh et al., 2001; Halling et al., 1999; Elsaleh et al., 2000a; Elsaleh et al., 2000b; Samowitz et al., 2001a; Ribic et al., 2003).
Table 2: Relation of the MSI-H tumour phenotype with survival of patients with stage II/III CRC

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of patients</th>
<th>Stage</th>
<th>Site</th>
<th>Chemotherapy*</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halling et al., 1999</td>
<td>508</td>
<td>II/III</td>
<td>CR</td>
<td>treated?</td>
<td>longer survival</td>
</tr>
<tr>
<td>Hemminki et al., 2000</td>
<td>327</td>
<td>III</td>
<td>CR</td>
<td>treated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Wright et al., 2000</td>
<td>95</td>
<td>III</td>
<td>CR</td>
<td>treated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Elsaleh et al., 2000a</td>
<td>255</td>
<td>III</td>
<td>CR</td>
<td>untreated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Elsaleh et al., 2000b</td>
<td>656</td>
<td>III</td>
<td>CR</td>
<td>both</td>
<td>longer survival</td>
</tr>
<tr>
<td>Watanabe et al., 2001</td>
<td>384</td>
<td>III</td>
<td>CR</td>
<td>untreated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Elsaleh et al., 2001</td>
<td>272</td>
<td>III</td>
<td>CR</td>
<td>treated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Elsaleh et al., 2001a</td>
<td>229</td>
<td>III</td>
<td>colon</td>
<td>treated</td>
<td>longer DFS (not OS)</td>
</tr>
<tr>
<td>Samowitz et al., 2001a</td>
<td>185</td>
<td>III</td>
<td>prox.colon</td>
<td>both</td>
<td>longer survival</td>
</tr>
<tr>
<td>Ribic et al., 2003</td>
<td>462</td>
<td>III</td>
<td>prox.colon</td>
<td>untreated</td>
<td>longer survival</td>
</tr>
<tr>
<td>Carethers et al., 2004</td>
<td>268</td>
<td>III</td>
<td>CR</td>
<td>treated</td>
<td>longer survival</td>
</tr>
<tr>
<td></td>
<td>338</td>
<td>III</td>
<td>colon</td>
<td>?</td>
<td>longer survival</td>
</tr>
<tr>
<td></td>
<td>570</td>
<td>II/III</td>
<td>colon</td>
<td>both</td>
<td>longer survival</td>
</tr>
<tr>
<td></td>
<td>287</td>
<td>II/III</td>
<td>colon</td>
<td>untreated</td>
<td>longer survival</td>
</tr>
<tr>
<td></td>
<td>283</td>
<td>II/III</td>
<td>colon</td>
<td>treated</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>II/III</td>
<td>CR</td>
<td>untreated</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>II/III</td>
<td>CR</td>
<td>treated</td>
<td>ns</td>
</tr>
</tbody>
</table>

* adjuvant 5-FU based chemotherapy, untreated or including both groups, ns: not significant, CR colon and rectum

Others showed that MSI-H tumours are significantly more common in tumours with lower nodal burden or stage II tumours without nodal involvement and in tumours originating from the proximal colon (Goel et al., 2003; Elsaleh et al., 2000a). In a group of 209 stage II/III CRCs, Goel et al (2003) found MSI-H in 25% (12/49) and 11% (18/160) in stage II and III tumours (P=0.02), respectively. Controlling for adjuvant chemotherapy MSI-H showed a significant positive predictive stage independent effect on overall survival for patients, who did not receive chemotherapy compared to those, who received chemotherapy. Several studies support the hypothesis that patients with MSI-H stage III colon cancer treated with 5-FU based chemotherapy have a lower risk of cancer recurrence and a better survival than those with MSS tumours. Apparently, the favourable response of colon cancer with MSI-H to adjuvant chemotherapy differs from the response of microsatellite stable (MSS) tumours and adjuvant treatment can be spared in tumours with a very low risk of recurrence (Hemminki et al., 2000; Watanabe et al., 2001; Elsaleh et al., 2000a). In a pooled re-analysis of five trials, Ribic et al (2003), however, found that adjuvant chemotherapy does not significantly improve survival in patients with MSI-H instability.

**Prediction of clinical outcome in CIN colon cancers**

Most tumours have a CIN phenotype as found in tumours arising due to the FAP syndrome. The genetic alterations found in the sporadic colon cancers are therefore thought to be similar to those found mutated in FAP tumours. The most commonly studied genetic alterations in relation to survival are briefly reviewed here.
**KRAS**

KRAS mutations occur early in the adenoma-carcinoma sequence and are present in 30% to 40% of the colon tumours (Fearon and Vogelstein, 1990; Vogelstein et al., 1988; Forrester et al., 1987; Hardingham et al., 1998; Andreyev et al., 1998). The prognostic value of KRAS mutations on survival and in predicting response to adjuvant treatment in colon cancer patients is controversial. Several authors did not find a correlation between presence of KRAS mutation and survival (benefit from treatment) in stage III CRC patients (Tortola et al., 1999; Ahnen et al., 1998; Esteller et al., 2001). In a large multi-centre study of more than 4000 patients an association was found between presence of KRAS mutation with an increased risk of relapse or death of disease (26%; P=0.004) (Andreyev et al., 2001). Ahnen et al (1998) showed a positive effect of adjuvant chemotherapy in patients with stage III colon cancer who exhibited wildtype KRAS, an effect that was not found in patients with stage II CRC. Font et al (2001) however, demonstrated a worse prognosis in those patients with stage II tumours with aspartic and serine mutations.

**TP53**

TP53 mutations, which occur in 40-60% of the CRC, are more frequently found in tumours distal to the splenic flexure (Breivik et al., 1997; Soong et al., 1997; Soong et al., 2000; Kressner et al., 1999). As the ‘guardian of the genome’ p53 blocks cell proliferation in the presence of cell-damage stimulating DNA repair or promoting apoptotic cell death in case of defective repair function. Generally, TP53 mutations are late events. Mutations in the conserved regions are more aggressive than mutations outside this domain (Goh et al., 1999; Pricolo et al., 1997; Arends et al., 2000; Kressner et al., 1999; Slebos et al., 1996). Deletions at the chromosomal region 17p13 where TP53 is located, are common in colon cancer and have been associated with poor patient survival (Pricolo et al., 1997; Smith et al., 1999; McLeod et al., 1999; Petersen et al., 2001). In a meta-analysis of 28 studies on p53 alterations, p53 appeared to be an independent significant factor in the overall survival in patients treated with surgery alone (Petersen et al., 2001). TP53 mutation has been associated with poor sensitivity to 5-FU-based chemotherapy (Elsaleh et al., 2000b). In a retrospective study of 891 stage III CRC patients of which 270 received 5-FU-based chemotherapy, a positive staining for p53 is associated with a higher risk of cancer-related death (Elsaleh et al., 2001).

**Allelic losses or loss of heterozygosity**

Allelic losses or loss of heterozygosity (LOH) at 18q (DCC/SMAD2/SMAD4; Table 3a) (Carethers et al., 1998; Watanabe et al., 2001; Halling et al., 1999; Jen et al., 1994; Lanza et al., 1998; Ogunbiyi et al., 1998; Jernvall et al., 1999; Barratt et al., 2002; Choi et al., 2002; Diep et al., 2003), 8p (Table 3b) (Halling et al., 1999; Choi et al., 2002) and 17p (TP53: Table 4a/b) (Halling et al., 1999; Elsaleh et al., 2000b; Barratt et al., 2002; Choi et al., 2002; Diep et al., 2003; Dix et al., 1994; Soong et al., 1997; Soong et al., 2000; Goh et al., 1999; Pricolo et al., 1997) also seem to play an important role in the clinical results. In patients with stage III colon cancer, Watanabe et al (2001) found a significantly higher relative risk of death in MSS tumours with LOH of 18q versus those with retained 18q alleles (RR 2.75; 95% CI 1.34-5.65; P=0.006). Jen et al (1994) showed a similar 5-year
survival in patients with stage II without LOH at 18q compared to that of patients with stage III. Originally DCC ‘deleted in colorectal cancer’ was considered as the main responsible tumour suppressor gene in this region, but mutant alleles of DCC are rarely observed, whereas mutations in SMAD2 and SMAD4 occur at frequencies of 11% and 30%, respectively (Arends et al., 2000). Diep et al (2003) also found a significantly worse outcome in patients with a stage II colon tumour containing deletion of 17p or 18q, another region often deleted in colon cancer. Laurent-Puig et al (1992) and Font et al (2001) found that in patients with stage II CRC, allelic loss of chromosome 18q had a worse prognosis, which could not be demonstrated in stage III disease. The impact of both mutated 17p and mutated 18q on the outcome in CRC remains unclear as a shorter survival has been reported in several studies (Watanabe et al., 2001; Jen et al., 1994; Lanza et al., 1998; Oggunbiyi et al., 1998; Jernvall et al., 1999; Massa et al., 1999; Iino et al., 1994; Martinez-Lopez et al., 1998), while others found an association with favourable outcome (Carethers et al., 1998; Halling et al., 1999; Dix et al., 1994; Khine et al., 1994). Also amplifications are found to be associated with survival, as increase in 20q copynumber from results published by Diep et al (2003) in which gain of 20q is associated with longer survival in stage III CRC (N=48).

Table 3a: LOH of 18q (DCC) as a factor for survival of patients with stage II/III CRC

<table>
<thead>
<tr>
<th>reference</th>
<th>number of patients</th>
<th>stage</th>
<th>prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jen et al., 1994</td>
<td>145</td>
<td>II/III</td>
<td>shorter survival (II)</td>
</tr>
<tr>
<td>Carethers et al., 1998</td>
<td>70</td>
<td>II</td>
<td>ns</td>
</tr>
<tr>
<td>Halling et al., 1999</td>
<td>386</td>
<td>II/III</td>
<td>ns</td>
</tr>
<tr>
<td>Lanza et al., 1998</td>
<td>118</td>
<td>II 61/III 57</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Oggunbiyi et al., 1998</td>
<td>151</td>
<td>I-IV</td>
<td>shorter survival (II/III)</td>
</tr>
<tr>
<td>Jernvall et al., 1999</td>
<td>123</td>
<td>78/III 45</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Watanabe et al., 2001</td>
<td>460</td>
<td>II/III</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Barratt et al., 2002</td>
<td>230</td>
<td>II/III</td>
<td>longer survival</td>
</tr>
<tr>
<td>Choi et al., 2002</td>
<td>145</td>
<td>II 73/III 72</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Diep et al., 2003</td>
<td>220</td>
<td>I-IV (II 79)</td>
<td>shorter survival</td>
</tr>
</tbody>
</table>

ns: not significant

Table 3b: LOH at 8p as a factor for survival of patients with stage II/III CRC

<table>
<thead>
<tr>
<th>reference</th>
<th>number of patients</th>
<th>stage</th>
<th>prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halling et al., 1999</td>
<td>226</td>
<td>II 68/ III 158</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Choi et al., 2002</td>
<td>73</td>
<td>II</td>
<td>ns</td>
</tr>
<tr>
<td>Choi et al., 2002</td>
<td>72</td>
<td>III</td>
<td>shorter survival</td>
</tr>
</tbody>
</table>

ns: not significant

Table 4a: LOH of 17p (TP53) as a factor for survival of patients with stage II/III CRC

<table>
<thead>
<tr>
<th>reference</th>
<th>number of patients</th>
<th>stage</th>
<th>prognosis</th>
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</thead>
<tbody>
<tr>
<td>Halling et al., 1999</td>
<td>321</td>
<td>II/III</td>
<td>ns</td>
</tr>
</tbody>
</table>


LIST OF PUBLICATIONS

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Patients</th>
<th>Stage</th>
<th>Site</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dix et al., 1994</td>
<td>100</td>
<td>II/III</td>
<td>CR</td>
<td>ns</td>
</tr>
<tr>
<td>Soong et al., 1997</td>
<td>541</td>
<td>II/III</td>
<td>CR</td>
<td>trend to longer survival</td>
</tr>
<tr>
<td>Soong et al., 2000</td>
<td>665</td>
<td>III</td>
<td>CR</td>
<td>ns*</td>
</tr>
<tr>
<td>Goh et al., 1999</td>
<td>328</td>
<td>I-IV</td>
<td>CR</td>
<td>shorter survival; stage III</td>
</tr>
<tr>
<td>Pricolo et al., 1997</td>
<td>70</td>
<td>III</td>
<td>colon</td>
<td>shorter survival</td>
</tr>
<tr>
<td>Elsaleh et al., 2000b</td>
<td>388</td>
<td>III</td>
<td>colon</td>
<td>ns</td>
</tr>
<tr>
<td>Tang et al., 2004</td>
<td>138</td>
<td>II/III</td>
<td>CR</td>
<td>ns?</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td>only surgery</td>
<td>shorter survival</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td></td>
<td>adj. treated</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = positive association with distal tumours.
ns: not significant
CR colon and rectum

Table 4b: TP53 mutation as a factor for survival of patients with stage II/III CRC

MGMT

Methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation is most likely associated with MSI-L phenotype (not specifically with MSI-H) and presence of G to A KRAS mutations. MGMT hypermethylation probably leads to more frequently transition of G:C to A:T transitions in the TP53 gene (Whitehall et al., 2001; Esteller et al., 2001). Nagasaka et al (2003) found that MGMT promoter hypermethylation might be predictive to a low risk of recurrence in CRC patients, who received adjuvant therapy. They found that CRC patients with unmethylated MGMT promoters were more likely to experience a recurrence sooner than patients with methylated MGMT promoters.

Prediction of clinical outcome in colon cancer related to 5-FU chemosensitivity markers thymidylate synthase and dihydropyrimidine dehydrogenase

5-FU has proven to be effective in the adjuvant treatment of patients with stage III colon cancer increasing the survival rates with 15% (Moertel et al., 1995; IMPACT, 1995). The activity of 5-FU mainly depends on the intracellular conversion to its active metabolite, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which inhibits DNA synthesis by forming a stable complex with thymidylate synthase (TS) in the presence of folates (Pinedo and Peters, 1988). Inhibition of FdUMP by TS leads to depletion of 2'-deoxythymidine-5'-monophosphate (dTMP) 2'-deoxythymidine-5'-triphosphate (dTTP), inhibiting DNA synthesis and initiating cell cycle arrest or cell death a process in which p53 also plays an important role (Leichman et al., 1997; Etienne et al., 2002; Aschele et al., 2002). Dihydropyrimidine dehydrogenase (DPD), the first and rate-limiting enzyme in the three-step pathway of uracil and thymine catabolism, is also important in the degradation and inactivation of 5-FU (Diasio and Harris, 1989). DPD converts over 85% of the clinically
administered 5-FU into the inactive metabolite dihydrofluorouracil, a process which takes place mainly in the liver (Lu et al., 1992). As TS is the target enzyme for 5-FU and DPD is the rate-limiting enzyme in the catabolism of 5-FU, the differences in chemosensitivity of colon tumours on 5-FU likely depend on the protein expression levels of TS and DPD. Therefore, TS and DPD may predict disease-free survival in patients treated with 5-FU regimens and antifolates.

Several authors have shown that TS is an independent prognostic survival factor (Table 5a). High levels of intratumoural TS expression in stage III CRC patients, receiving adjuvant 5-FU-based chemotherapy, were associated with a longer overall survival than patients with low intratumoural TS expression (Johnston et al., 1994; Edler et al., 2002; Kornmann et al., 2002). A few groups (Allegra et al., 2003; Sakamoto et al., 2003), however, reported a shorter overall survival of patients with high TS levels than of patients with low TS levels, while others reported no survival difference according to TS expression levels (Tomiak et al., 2001; Nanni et al., 2002; Allegra et al., 2002). From the results of all these studies it can be concluded that the role of TS as a prognostic marker is inconclusive.

Low levels of intratumoural DPD expression have been suggested to be associated with a longer survival (Tokunaga et al., 2003; Tsuji et al., 2004), while others (Kornmann et al., 2002; Ikeguchi et al., 2002) found no prognostic advantage (Table 5b). In addition, Kornmann et al. (2003) found that the combination of TS and DPD expression levels predicted differences in prognosis, where adjuvantly treated CRC patients with high TS levels and low DPD levels had the longest survival.
### Table 5a: TS expression as a factor for survival of patients with stage II/III CRC (predicting efficacy of 5-FU treatment)*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Patients</th>
<th>Stage</th>
<th>Chemotherapy</th>
<th>Method</th>
<th>Prognostic Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnston et al., 1994</td>
<td>294</td>
<td>II-IIIrectal</td>
<td>untr+adj</td>
<td>IHC</td>
<td>Yes, high TS improved survival</td>
</tr>
<tr>
<td>Lenz et al., 1998</td>
<td>45</td>
<td>II</td>
<td>untreated</td>
<td>IHC</td>
<td>Yes, low TS longer survival</td>
</tr>
<tr>
<td>Yamachika et al., 1998</td>
<td>86</td>
<td>I-III</td>
<td>untr+adj</td>
<td>IHC+ activity</td>
<td>Yes, high TS improved survival</td>
</tr>
<tr>
<td>Cascinu et al., 2001</td>
<td>100</td>
<td>III</td>
<td>untr+adj</td>
<td>IHC</td>
<td>Yes, low TS low recurrence rate</td>
</tr>
<tr>
<td>Allegra et al., 2002</td>
<td>465</td>
<td>220 II/245 III</td>
<td>untr151+ adj</td>
<td>IHC</td>
<td>No NS</td>
</tr>
<tr>
<td>Nanni et al., 2002</td>
<td>330</td>
<td>II-III</td>
<td>adj</td>
<td>IHC</td>
<td>No NS</td>
</tr>
<tr>
<td>Edler et al., 2002</td>
<td>862</td>
<td>II-III</td>
<td>untreated</td>
<td>IHC</td>
<td>Yes, no high TS improved survival</td>
</tr>
<tr>
<td>Allegra et al., 2003</td>
<td>706</td>
<td>291 II/414 III</td>
<td>adj</td>
<td>IHC</td>
<td>Yes, MoAbTS106</td>
</tr>
<tr>
<td>Kornmann et al., 2003</td>
<td>295</td>
<td>44 II/251 III</td>
<td>adj</td>
<td>RT-PCR</td>
<td>No high TS in combination with DPD</td>
</tr>
<tr>
<td>Sakamoto et al., 2003</td>
<td>229</td>
<td>I-III</td>
<td>adj</td>
<td>IHC</td>
<td>Yes, OS and DFS MoAb RTSMA2</td>
</tr>
</tbody>
</table>

* determined in primary tumour tissues; untr= only surgery; adj= adjuvant chemotherapy; NS= not significant; IHC= immunohistochemical analysis

### Table 5b: DPD expression as a factor for survival of patients with stage II/III CRC (predicting efficacy of 5-FU treatment)*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Patients</th>
<th>Stage</th>
<th>Chemotherapy</th>
<th>Method</th>
<th>Prognostic Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kornmann et al., 2002</td>
<td>348</td>
<td>51 II/297 III</td>
<td>adj</td>
<td>RT-PCR</td>
<td>No NS</td>
</tr>
<tr>
<td>Ikeguchi et al., 2002</td>
<td>189</td>
<td>I-IV</td>
<td>adj 120</td>
<td>ELISA</td>
<td>No NS (and not in subgroup 51 II )</td>
</tr>
<tr>
<td>Tokunaga et al., 2003</td>
<td>100</td>
<td>II-IV</td>
<td>adj</td>
<td>IHC</td>
<td>Yes, only univariate</td>
</tr>
<tr>
<td>Kornmann et al., 2003</td>
<td>295</td>
<td>44 II/251 III</td>
<td>adj</td>
<td>RT-PCR</td>
<td>Yes in combination with high TS expression</td>
</tr>
<tr>
<td>Tsuji et al., 2004</td>
<td>182</td>
<td>107 II/75 III</td>
<td>adj 93</td>
<td>ELISA</td>
<td>Yes, no low DPD shorter survival</td>
</tr>
<tr>
<td>Oi et al., 2004</td>
<td>64</td>
<td>64 III</td>
<td>adj</td>
<td>ELISA</td>
<td>NS</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>IHC</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* determined in primary tumour tissues, NS= not significant; adj= adjuvant chemotherapy, untr= only surgery

### Prediction of clinical outcome in colon cancer related to other (potential prognostic) molecular expression markers

Also the expression levels of many other proteins different from the previously described chemosensitive TS and DPD markers are described as having potential prognostic value. Examples are the preoperative serologic marker carcinoembryonic antigen (CEA), the oncogene c-myc, apoptosis markers Bcl-2 and Bax, proliferation marker Ki-67, angiogenesis marker VEGF, growth factors TGF-alfa, -betaRII and EGF-R and so on. Most of these markers, however, seem less promising, because of inconsistencies found between the different studies published or due to the limited size of studies.
FINDING DEFINITE PROGNOSTIC FACTORS IN COLON CANCER

As is clear from the previous paragraphs finding prognostic factors is difficult. Many studies have been conducted, however none of them have resulted in clinical use, mainly due to inconclusiveness or inconsistencies. One of the problems in the interpretation of all these data is the fact that many studies are heterogeneous in terms of tumour stage or even include rectal tumours with a different treatment-strategy and prognosis. Moreover, the number of patients or tumours screened is often small. Another problem is that different methods of analysis are used, which may hamper a direct comparison between studies.

Ideal would be to design and investigate a clearly defined large prospective trial including large numbers of patients with colon cancer homogeneously staged with respect to clinicopathological characteristics, including preferably one treatment strategy/schedule such as adjuvant treatment vs. no adjuvant treatment and for which all molecular analyses and its interpretation should be standardised.
REFERENCES


Esteller M, Risques RA, Toyota M, et al. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T


LIST OF PUBLICATIONS


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APPENDIX

Glossary of microsatellite instability target genes

**APAF1** apoptotic protease activating factor 1 at 12q23, alias CED4 downstream effector of p53 mediated-apoptosis

**BAX** BCL2-associated X protein, proapoptosis gene at 19q13.3-q13.4

**BCL10** B-cell CLL/lymphoma 10, at 1p22, aliases CARMEN, CIPER, mE10, c-E10, CLAP; regulatory gene for apoptosis

**CASP5** caspase 5, apoptosis-related cysteine protease at 11q22.2-q22.3 alias ICE (rel) III caspase-5 member of the caspase family of proteases with (A) 10 tract precise function unknown but proteases related to apoptosis and immune and inflammatory responses

**CHK1** CHK1 checkpoint homolog (S. pombe) at 11q24 alias CHEK1 Cell cycle regulator, important role in G2 checkpoint

**E2F4** E2F transcription factor 4, p107/p130-binding at 16q21-q22 inhibiting transcription factor, forms complex with p130 which is pRB related

**GRB14** growth factor receptor-bound protein 14 at 2q22-q24 adapter protein member of GRB 7 family negative regulator of insulin (receptor) signal(ing) transduction

**IGFIIIR** insulin-like growth factor II receptor at 6q25-q27

**MBD4** methyl-CpG-binding domain protein 4, also known as MED1, at 3q

**MSH3** MutS homolog 3 (E. coli), mismatch repair gene, at 5q

**MSH6** MutS homolog 6 (E. coli), mismatch repair gene, 2p16

**PTEN** phosphatase and tensin homolog (mutated in multiple advanced cancers 1) at 10q23 aliases MMAC1,TEP tumour suppressor gene inhibits cell proliferation

**RAD50** RAD50 homolog (S. cerevisiae) 5q23-31 alias hRAD50

**RHAMM** HMR hyaluronan-mediated motility receptor, located on 5q33.2-qter

**RIZ** retinoblastoma protein interacting zinc finger gene at 1p36 aliases RIZ1/RIZ2/PRDM2 (PR domain containing 2, with ZNF domain) a candidate tumour suppressor gene

**TCF4** transcription factor 4, at 18q21.1 aliases SEF2-1B, ITF2; a crucial member of the Apc/beta catenin/ T-cell factor (TCF) pathway

**TGFRII** transcription growth factor beta receptor type II, at 3p22

**TNFRSF6** tumour necrosis factor receptor superfamily member 6, at 10q24.1 aliases FAS/CD95/APO-1, death receptor involved in apoptosis