CHAPTER 6

SUMMARY AND GENERAL DISCUSSION
LIST OF PUBLICATIONS

PROGNOSTIC FACTORS FOR ADJUVANTLY TREATED PATIENTS WITH STAGE III COLON CANCER

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, such as 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 decreases with more advanced stage 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, particularly in patients with stage III colon cancer (Moertel et al., 1995; IMPACT, 1995; IMPACT B2, 1999). Although disease-free survival (DFS) has increased during the last decades, about half of the patients will still develop recurrent disease, usually presented as liver metastases (Gatta et al., 1998). Currently, it is still not possible to predict in which patient adjuvant chemotherapy will be beneficial. Within the group of stage III colon cancer patients the clinical outcome is variable and predicting biological behaviour of these colon cancers is difficult and remains a great clinical problem.

In this thesis we focussed on the search for molecular prognostic markers that might be helpful in discriminating between long-term and short-term survivors. As mentioned in Chapter 1 a large number of studies have and are being undertaken to find prognostic and predictive markers in particular in stage II and III CRC patients, but mostly with contradicting results. These contradicting findings might be explained by various factors, such as heterogeneity in terms of tumour stage between the various studies and the often small numbers of patients or tumours that are screened. So far, no single molecular marker has been identified with sufficient relevance for CRC prognosis, nor is one currently used in clinical practice.

To overcome some of these problems, which are mentioned in greater detail in Chapter 1, we have collected a large well-defined group of patients with stage III colon cancer (N=391), randomised for adjuvant treatment with two 5-fluorouracil-based chemotherapy regimens to possibly identify somatic aberrations or molecular expression markers, which are associated with clinicopathological parameters of these patients and in particular with disease-free survival (DFS).

We first classified the tumours according to microsatellite instability (MIN) or presence of TP53 and KRAS mutations, markers commonly associated with chromosomal instability (CIN).

MSI-H, as shown in Chapter 2, was identified in 16% (44/273) of the colon tumours. An MSI-H tumour phenotype was found associated with a proximal location of the tumour, poor cellular differentiation and mucinous tumour histology. In a univariate analysis, MSI-H was found to be associated with a longer DFS. In a multivariate model, adjusting for extent of nodal involvement, histology, invasion and tumour grade, the association of MSI and DFS did no longer reach statistical significance. This is not surprising given the low frequency of the MSI-H phenotype (about 15%) and the many different subgroups. We also found that mutations in TP53, a gene suggested as often mutated in tumours belonging to the CIN phenotype (Livingstone et al., 1992; Yin et al., 1992; Agapova et al., 1996; Leslie et al., 2003), are associated with a reduced DFS. TP53 mutations were identified in 53%
(116/220) of the analysed tumours. Presence of a TP53 mutation was furthermore associated with a higher frequency of non-mucinous tumours. Both in univariate and multivariate analyses, mutant TP53 appeared to be a moderate independent negative predictor for prognosis in adjuvantly treated stage III colon cancer patients. The combined analysis of MSI and TP53 had a relatively low statistical power because of the reduced number of tumours (N=194) and the increased number of subgroups. The predictive value of presence of a TP53 mutation for DFS was higher than that of MSI, with TP53 still reaching borderline significance. Although the risk estimate for MSI did not change much compared to the estimate derived from a univariate analysis, the 95% confidence interval around this estimate, however, was very wide. This is largely explained by the low frequency of MSI-H (about 15%). To obtain a comparable power to detect a similar survival difference for MSI as for TP53, one would require a far larger study size, as the frequency of TP53 mutation was about 50% (see also next section). KRAS mutations were identified in 28% (58/205) of analysed tumours. Presence of a KRAS mutation, however, did not correlate with any investigated clinicopathological parameter, nor with DFS.

Secondly we looked at the prognostic value of specific chemosensitive markers, such as TS and DPD (Chapter 5). Low TS protein levels, determined by immunohistochemical analysis using tissue microarrays, were associated with a mucinous histology of the tumour and low DPD protein levels were associated with a younger age at the time of randomisation. We found, however, no prognostic value for expression of TS and of DPD proteins in this 5-FU adjuvantly treated group of stage III colon cancer patients, neither when expression was determined in primary tumours nor when that was done in lymph node metastases.

In conclusion, we have shown that TP53 mutation is a moderate negative prognostic factor in colon cancer patients adjuvantly treated with 5-FU-based chemotherapy. MSI-H status appears to be a positive prognostic indicator, although we could not confirm this in multivariate analysis due to the small number of MSI-H tumours (lack of power). In contrast to MSI and TP53 (mutation) status, KRAS mutation and expression of TS and of DPD proteins have no prognostic value in the 5-FU adjuvantly treated patients with stage III colon cancer.

Lack of power?
As mentioned, we found MSI to be associated with patient prognosis in a univariate model. In a multivariate model however MSI was no longer significant. Clearly, the power of our study to confirm the prognostic effect of MSI status was limited. Actually, our study had a power of about 80% to detect a 20% absolute difference in DFS (80% versus 60%) associated with MSI-H status. To increase the power of our study we could enlarge the number of analyzed samples. By enlarging the sample size, i.e. enlarging power, the detectable effect-size would become smaller. In our study, the confidence limits for the effect-size of MSI-H would become narrower, but the size of the effect would probably not change much. Generally, a low frequency phenotype such as MSI-H requires a larger number of patients to study survival difference than a fairly frequent molecular marker such as TP53 mutation.
This is probably the main reason why TP53 mutation was found to be a predictor for DFS, but MSI not.
Enlarging the group size will be required when studying the combined effect of the clinical parameters and both MSI and TP53 (mutation) status, since we showed that with exception of the extent of nodal involvement all statistical significant associations were lost in this multivariate analysis (see Chapter 2).

### Table 1: Effect statistics

<table>
<thead>
<tr>
<th></th>
<th>Trivial</th>
<th>small</th>
<th>Moderate</th>
<th>large</th>
<th>very large</th>
<th>Nearly perfect</th>
<th>Perfect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation</td>
<td>0.0</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>Effect size</td>
<td>0.0</td>
<td>0.2</td>
<td>0.6</td>
<td>1.2</td>
<td>2.0</td>
<td>4.0</td>
<td>Infinite</td>
</tr>
<tr>
<td>Frequency difference</td>
<td>0</td>
<td>10</td>
<td>30</td>
<td>50</td>
<td>70</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Relative risk</td>
<td>1.0</td>
<td>1.2</td>
<td>1.9</td>
<td>3.0</td>
<td>5.7</td>
<td>19</td>
<td>Infinite</td>
</tr>
<tr>
<td>Odds ratio</td>
<td>1.0</td>
<td>1.5</td>
<td>3.5</td>
<td>9.0</td>
<td>32</td>
<td>360</td>
<td>Infinite</td>
</tr>
</tbody>
</table>


The observed RRs associated with MSI and TP53 (mutation) status are 1.8 (95% CI 0.9-3.3) and 1.7 (95% CI 1.1-2.6), respectively. Their effect-sizes might be considered small to moderate (Table 1). How large are these effects of MSI and TP53 mutation compared to known prognostic factors? The best known prognostic factor with respect to stage III colon cancer is the number of tumour-positive lymph nodes. The relative risk found associated with the number of tumour-positive lymph nodes was 2.3 (95% CI 1.6-3.4) or 3.1 (95% CI 2.0-4.8), in the groups analysed for MSI and TP53, respectively. Thus, the number of positive lymph nodes, would in terms of effect-size have a moderate to large effect (Table 1) one being stronger than the one found for MSI and TP53.

Although the effects of MSI and TP53 might be considered small, they still were stronger than the effects of tumour invasion, tumour grade/differentiation or histology, three histopathological factors which have been frequently reported as being associated with DFS (Newland et al., 1994; Hermanek et al., 1995; Compton, 2003).

In order to expand the sample size, one might also consider to perform a meta-analysis. Next to homogeneously well-staged tumour samples according to the pTNM staging system, strictly standardised protocols for molecular analyses and their interpretation are required for that. Whether this is a realistic and financially feasible option, is doubtful.

**Additional candidate markers**

A future search for strong markers in the material available remains a worthwhile approach. Possible further prognostic candidates are the proliferation marker Ki-67, apoptosis markers such as bc1-2, bax, bag-l (Manne et al., 1997; Ogura et al., 1999; Kichuchi et al., 2002) all involved in the p53 pathway, M30 cytodeath, determination of the amount of apoptotic tumours cells; c-myc (Bhatavdekar et al., 1997), angiogenesis markers such as VEGF (Takahashi et al., 1995); cell cycle regulators p16, p21 and p27 (Zhao et al., 2003; Zirbes et al., 2000; Loda et al., 1997); and metastasis markers such as nm23, S100A4 and mucins such as MUC1 (Campo et al., 1994; Gongoll et al., 2002; Bald et al., 2002; Byrd et al., 2004). All these proteins are part of important different functional processes that are involved in colon tumour behaviour and have been suggested to be associated with
prognosis. Expression of these proteins could be determined by immunohistochemical analysis using the tissue microarrays (TMAs) constructed and used in this thesis for determination of protein levels of TS and DPD (Chapter 5). These TMAs contain all 391 collected primary tumours and part of the matching lymph node metastases. Where other more complex stainings need more strict definitions and criteria of interpretation with respect to intensity, percentage scales or different localisation, this would be a quick and straightforward approach, especially in cases where presence or absence of proteins can be easily assessed. However, focal staining could be missed more easily by use of TMAs and their use also gives no insight into the histology of the tissue structures surrounding the tumour. Screening seems justified, as again most studies screened limited sets of patients, especially with respect to tumour stage.

Further suggestions are to determine DNA ploidy (Witzig et al., 1991; Lanza et al., 1998) and LOH, the latter at least at chromosome arms 18q, 17p, 5q, 8p, 1p (and possibly at 2p, 22q, 14q, or 9p as well) (Ogunbiyi et al., 1997; Weber et al., 1999, Iino et al., 1994; Massa et al., 1999; Choi et al., 2002), which seem to be involved in colon cancer and are possibly associated with survival as well. One could also screen for other chromosomal aberrations, such as the gains at 20q and at 13q that seem associated with both an MSS phenotype and presence of TP53 mutation, as shown in Chapter 3. The use of a whole genome array, may give more information since interstitial chromosomal aberrations would also be included. Nevertheless, since most chromosomal aberrations will affect the ends of the chromosomes, a subtelomeric array used by us can be sufficient to discriminate between near-absence and prominent presence of chromosomal instability.

Screening for candidate markers may be useful, but it might be more interesting to use a more open approach as one might do by using expression arrays that can assess expression levels of all human genes in a tumour sample. One might consider to perform genome-wide gene expression profiling of stage III colon cancer specimens, including both primary tumour and lymph node metastases, by use of expression array and compare the expression profile with that of matched normal tissue. Such an approach might lead to identification of yet unknown differentially expressed genes with a strong prognostic value. It might also give clues to unknown important functional routes or genes that influence survival. For our selected group of patients, however, we only have formalin-fixed paraffin-embedded specimens available from which isolation of RNA for expression profiling is not possible. We would have to select a new patient population for which at least fresh-frozen tumour tissue would be available. Since this is currently no standard procedure, it would ask for designing a new large well-defined prospective trial with a large sample size ensuring sufficient power to perform statistically significant multivariate analyses. Such a study should include clinicopathologically well-defined tumours staged according to the pTNM staging system and include all extra known clinicopathological factors. Blood and fresh-frozen primary tumour, lymph node metastases and matched normal tissue samples from all patients should be available. Tumour material should be obtained by microdissection in stead of macrossection, to ensure the highest possible tumour cell content. Interesting would also be the inclusion of a group of patients not adjuvantly treated with 5-FU. Since, however, currently all stage III colon cancer patients should receive adjuvant chemotherapy according to current treatment guidelines, such a control group will be hard to find. Still, the effect of adjuvant chemotherapy may differ considerably in various subgroups as determined by extent of nodal involvement and TP53 and MSI-H status. A more feasible approach would be to determine if these prognostic markers could identify subgroups of localized colon cancer patients with a worse prognosis who may benefit from
adjuvant therapy. It will, however, remain difficult to identify genuine predictive markers for response to adjuvant chemotherapy, genes that discriminate between responders and non-responders.

Do **TP53** and **KRAS** mutations point to CIN?

As 9 of the 25 MSI-H tumours (36%) had a **TP53** mutation (Chapter 2), we found a substantial degree of overlap between an MSI-H phenotype and presence of a **TP53** mutation. Thus, characteristics that have been suggested as opposing each other (**TP53** mutation as CIN characteristic and MSI-H as MIN characteristic) are not mutually exclusive. This brought us to determine the extent of chromosomal instability (CIN) in MSI-H and **TP53** mutation-containing tumors as described in Chapter 3. We found that presence of a **TP53** mutation in MSI-H tumours cannot be considered as an indicator of chromosomal instability, as determined by use of subtelomere-specific CGH-based arrays. We have shown that the number of chromosomal abnormalities in MSI-H colon tumours is low and clearly different from MSS tumours and that tumours characterised by both MSI-H and presence of a **TP53** mutation hardly showed any chromosomal instability. A certain degree of overlap was also found between **KRAS** mutations and MSI-H status. A **KRAS** mutation was exhibited by 4/25 MSI-H tumours (16%) compared to 42/126 of MSS tumours (33%) (Chapter 2). This finding also prompted us to investigate the **KRAS** mutation frequency and spectrum in hereditary (HNPCC) and sporadic (MSI-H and MSS) CRCs as described in Chapter 4. As also shown in Chapter 4, the **KRAS** mutation frequency in MSI-H tumours was lower than that in the MSS and HNPCC tumours, but a negative correlation was missing. There was a dependence on h**MLH1** methylation status since MSI-H tumours with hypermethylation had the lowest mutation frequency (18%), whereas MSI-H tumours without hypermethylation showed a mutation frequency of 36%, which equals the mutation frequencies observed in MSS and HNPCC tumours. Thus, a **KRAS** mutation, considered as CIN characteristic and MSI-H, as a MIN characteristic, are also not mutually exclusive.

Presence of a **KRAS** mutation in MSI-H tumours can, just as presence of a **TP53** mutation, not be considered an indicator of chromosomal instability, since we found no chromosomal instability (i.e. less than 3 chromosomal aberrations) in 100% (3/3) of the **KRAS** mutation-containing MSI-H tumours with our arrays (data not shown). Interestingly, two of these three **KRAS** mutation-containing MSI-H tumours also had a **TP53** mutation. Our data therefore show that **TP53** and **KRAS** mutations are not restricted to the CIN phenotype as often has been suggested. We found no evidence, however, for an overlap of CIN and MIN. We did notice that tumours exist that are both microsatellite and chromosomally stable. This points to the existence of a third group of tumours with respect to genome stability/instability (microsatellite and chromosomally stable = MACS).
REFERENCES


Baldus SE, Monig SP, Hanisch FG, et al. Comparative evaluation of the prognostic value of MUC1, MUC2, sialyl-Lewis(a) and sialyl-Lewis(x) antigens in colorectal adenocarcinoma. Histopathology 2002;40:440-449.


LIST OF PUBLICATIONS


