PATHOLOGICAL AND BIOLOGICAL ASPECTS OF COLORECTAL CANCER TREATMENT
The studies presented in this thesis were performed at the department of pathology of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, the department of pathology, PAMM laboratories Eindhoven, the Netherlands and the department of surgery, Catharina hospital, Eindhoven, the Netherlands.

Cover illustrations: Front cover, from top to bottom: immunofluorescence (IF) double staining of normal colorectal mucosa with the monoclonal Ber-EP4 and polyclonal anti Ep-CAM antibodies, IF staining of a colorectal carcinoma with the Ber-EP4 antibody, hematoxylin and eosin staining of a whole mount slide of a rectal cancer specimen, IF staining of a colorectal carcinoma with the HECD-1 anti E-cadherin monoclonal antibody. Back cover: IF staining of normal colorectal mucosa with the Ber-EP4 antibody.

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Colofon
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PATHOLOGICAL AND BIOLOGICAL ASPECTS OF COLORECTAL CANCER TREATMENT

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van Rector Magnificus prof. mr. S.C.J.J. Kortmann, volgens besluit van het College van Decanen in het openbaar te verdedigen op woensdag 10 september 2008 om 15:30 uur precies

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‘In commendation of ye microscope’

‘Of all the Inventions none there is Surpasses
the Noble Florentine’s Dioptrick Glasses
For what a better, fitter guift Could bee
in this World’s Aged Luciosity.
To help our Blindnesse so as to devize
a paire of new & Artificial eyes
By whose augmenting power wee now see more
than all the world Has ever dounn before’

Henry Powers 1664 (in the original old English)
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GENERAL INTRODUCTION
§1 COLORECTAL CANCER: EPIDEMIOLOGY AND PROGNOSTIC FACTORS

Colorectal cancer is the second most common malignancy in women and the third most common in men. In the Netherlands, approximately 10,000 people are diagnosed for colorectal cancer each year and about 30% of these tumors originated from the rectum (www.IKCnet.nl). In the Western world, the incidence of colorectal cancer is increasing due to changes in lifestyle. The incidence of both colon and rectal cancer is higher in men than in women. Food intake rich in meat and fat and low in fiber is an important risk factor for the development of colorectal cancer. In addition, decreased physical activity, smoking and high alcohol consumption play an important role in the epidemiology of this disease. The connection between lifestyle and colorectal cancer epidemiology is clearly illustrated by the increased incidence of this malignancy in Third World countries adopting the Western lifestyle.

About 4,000 patients die of colorectal cancer per year in the Netherlands (www.IKCnet.nl). The 5-years overall survival is approximately 50-60%. Factors prognostic for outcome after treatment of colorectal cancer can be roughly divided in tumor-and treatment-related factors. Some tumors are more aggressive than others; this is, at least partly reflected in tumor stage, type and differentiation grade as well as in molecular features (paragraph 2 and 3). However, treatment is one of the most important factors for prognosis. With the right treatment, even aggressive tumors can sometimes have an excellent prognosis. On the other hand, if small, potentially harmless tumors are not adequately treated their prognosis can become terrible poor (paragraph 4).

§2 PATHOLOGY OF COLORECTAL CANCER

Pathologically, tumors can be characterized by stage, type and grade. Staging of colorectal tumors has a long history and is still based on the system devised by Cuthbert Dukes in the 1930’s. He described the 2 main components; invasion depth and nodal status. In later years, the distant metastases were added as a third component and this formed the basis of the current Tumor, Node, Metastasis (TNM) systems of the international union against cancer (uicc). Combinations of these three components are grouped in 4 stage categories, having each a different prognosis. The TNM system is one of the most classic tumor-related prognostic factors (Figure 1).
In addition to the TNM stage, patients’ prognosis might also depend on the tumor type. There are several histological tumor types with different prognoses. The majority of colorectal carcinomas (approximately 90%), are classified as adenocarcinomas. The remaining 10% are mucinous carcinomas. Furthermore, there are some very rare epithelial tumor types (less than 1%) such as signet ring cell carcinomas, adenosquamous carcinomas and undifferentiated carcinomas. According to the World Health Organization (WHO) guidelines a tumor is classified as mucinous when the proportion of the mucinous component is \( \geq 50\% \). This mucinous component usually consists of mucinous lakes that either contain tumor cells or are sterile. Sometimes, mucinous carcinomas with signet ring cell dedifferentiation can be observed. These signet ring cells contain intracellular mucin that pushes the nucleus to the cell membrane, thus resembling a signet ring. A carcinoma containing signet ring cells not arising from dedifferentiation of a mucinous carcinoma is called a \textit{de novo} signet ring cell carcinoma and can be recognized by the absence of mucinous lakes \(^6\). The prognosis of an adenocarcinoma, mucinous and signet ring cell carcinoma, with
similar tumor stages, ranges from good to poor respectively \(^{7,9}\). A third important tumor-related prognostic factor is tumor grade. Tumor grade is traditionally classified as undifferentiated, poorly, moderately or well differentiated according to the WHO guidelines. The classification of the tumor grade is based on the percentage of gland formation. Poorly or undifferentiated tumors have a poorer prognosis than moderately or well differentiated tumors. Dedifferentiation of tumor cells at the invasive front, also referred to as tumor sprouting or budding \(^{10,11}\) is associated with increased tumor spread and poor prognosis \(^{12,13}\). On a molecular level, tumor budding is accompanied by loss of adhesion molecules and an increased expression of metalloproteinases \(^{14,15}\).

§3 GENETIC BACKGROUND OF COLORECTAL CANCER

Carcinogenesis of colorectal cancer is a very complex mechanism that is largely influenced by the genetic background. In general, the development of sporadic colorectal cancer is traditionally described by two different pathways; the gatekeeper and the caretaker pathway (Figure 2).

*Figure 2: The two most common pathways involved in tumor development with their sporadic and most common hereditary variants. The flagged arrows indicate the accelerated process of carcinogenesis in the hereditary variants. HNPCC: Hereditary Non-Polyposis Colorectal Cancer, FAP: Familial Adenomatous Polyposis.*
Ninety to ninety-five percent of cancers arising in the colon or rectum have no defined hereditary basis and are called sporadic tumors. Approximately 85% of these tumors have been developed through the gatekeeper pathway (white filled arrows, Figure 2). This pathway is initiated by mutations in the gatekeeper gene *adenoma polyposis coli* (*APC*), located on the long arm of chromosome 5. *APC* is a tumor suppressor gene and the most important function of the APC protein is to control the Wnt signal transduction pathway. In addition, this protein has also been described to interfere with the microtubule formation causing increased mitotic abnormalities explaining that carcinogenesis via the gatekeeper pathway often results in aneuploid tumors. Chromosomal deletions (loss of heterozygosity), amplifications and translocations are frequently observed. This chromosomal instability regularly affects the *Kirsten-RAS (K-RAS)* oncogene and the tumor suppressor genes *mothers against decapentaplegic homolog 4 (Drosophila) (SMAD-4)* and *p-53*.

The second pathway of cancer development is primarily initiated by mutations in the mismatch repair genes (MMR genes) and is also referred to as the caretaker pathway. About 15% of sporadic tumors arise due to disfunctioning of caretaker genes. Examples of these genes are *human MutL homologue 1* (*hMLH1*), *human MutS homologue 2* (*hMSH2*), *human MutS homologue 6* (*hMSH6*) and *human post-meiotic segregation* (*hPMS2*). Normally, the MMR proteins repair errors of 1 to 3 base mismatches that occurred during DNA replication. However, mutated or epigenetically changed caretaker genes will result in genetic instability leading to an increased rate of carcinogenesis (black filled arrows, Figure 2). Short tandemly repeated DNA sequences are very susceptible to errors in replication. Increased hypermutatability (due to stand slippage) of markers with these short tandem repeats, also referred to as micro satellite instability (MSI), reveals the presence of genetic instability caused by disfunctioning caretaker genes. Examples of hypermutated functional genes, involved in cell growth and survival that also contain these tandemly repeat sequences are *type II transforming growth factor-beta receptor (TGFβRII)*, *Bax* and *Caspase-5*.

The inactivating mutations in these genes have an important impact on carcinogenesis. In contrast to the gatekeeper pathway, tumors with defective caretaker genes are generally diploid with no significant gross chromosomal changes but subtle sequence alternations. In addition, aberrant gene silencing by means of methylation (e.g. methylation of the *MLH1* gene) is commonly observed in tumors that have developed following the caretaker pathway. A poor differentiation grade, extensive infiltration of lymphocytes and the presence of extracellular mucin are morphological characteristics that can be appreciated in tumors exhibiting MSI.

Only 5-10% of colorectal cancers arise due to underlying germline mutations. In the case of an inherited mutation, the process of carcinogenesis will occur at an increased
rate (Figure 2) because only one mutation, in the normal allele, is required for loss of function, as described by the two-hit theory \(^3\). Hereditary variants of both the gatekeeper and caretaker pathways can be found. Patients with familial adenomatous polyposis (FAP) have a germline mutation in the gatekeeper gene \textit{APC}. Hereditary non-polyposis colorectal cancer (HNPCC, or Lynch syndrome) is caused by germline mutations in one of the caretaker genes \textit{mlh-1, msh-2, msh-6} or \textit{pms-2}.

Recent studies have shown that these different pathways of carcinogenesis not only result in different tumors with genetic profiles but also affect prognosis. For example, patients with MSI tumors have a more favorable prognosis but are less likely to benefit from chemotherapy \(^{33-35}\). However, investigations on the gatekeeper pathway have shown that this type of carcinogenesis seems to have less prognostic potential.

\section*{§4 TREATMENT OF COLORECTAL CANCER}

\subsection*{§4.1 Surgery}
Surgery is the mainstay of treatment for both colon and rectal cancer. The surgical approach of colon cancer is aimed not only on achieving wide margins of resection (a proximal and distal margin of at least 5 cm) but also on harvesting regional lymph nodes located in the mesocolon. If a tumor is located in the cecum, ascending colon, hepatic flexure or the transverse colon, a right hemicolecotomy, including a dissection of the mesenteric lymph nodes, will be performed. Tumors located in the splenic flexure or the descending colon are treated with a left hemicolecotomy. Tumors in the sigmoid colon are operated on with a segmental (low anterior) resection. Colon cancer can be treated with open surgery but can also be safely treated laparoscopically \(^{36,37}\). Laparoscopic surgery of rectal carcinoma is under investigation and is currently not routinely applied in clinical practice but is still in the experimental phase.

The rectum requires a different surgical approach than the colon because of the anatomy and location in the pelvic area, beneath the peritoneal reflection. Traditionally, this kind of surgery was difficult to perform and, as such, resulted in a large number of local recurrences. A major step forward with respect to surgery of rectal cancer is the introduction of the total mesorectal excision (TME) surgical technique. This technique was described by Heald in 1986 and aims to remove as much mesorectal fat as possible by sharp dissection following the mesorectal fascia (the “holy” plane, Figure 3) \(^{38}\). The introduction of TME surgery resulted in a decrease in local recurrence rates of 40/50% to <10% because more tumors could be completely excised by this technique \(^{39-41}\). Tumor location determines whether a low anterior resection (LAR) or an abdominoperineal resection (APR) should be performed (Table 1). A LAR is performed in case of middle
and upper rectal cancer in which the tumor is located 5-15 cm from the anal verge. If the tumor is located more distally (0-5 cm from the anal verge), an APR will be required, amputating the anal sphincter and forming a permanent colostomy. Traditionally, many rectal cancers were treated with an APR resection, however, improved imaging techniques and surgical expertise enabled successful local, sphincter saving excisions using the LAR surgical approach. In addition to retention of the anal sphincter, local recurrence rates are lower and survival rates are higher after a LAR procedure compared to patients who underwent APR. In case of small (T1, N0) superficially growing, well or moderately differentiated rectal tumors, TME surgery is not required. Instead, the tumor can be dissected by transanal endoscopic microsurgery (TEM) (Table 1).

A very important treatment-related factor in rectal cancer related to surgery is the circumferential margin (CRM). The CRM is the shortest distance (in mm) between the circumferential resection plane and the tumor (Figure 3). The circumferential margin is assessed microscopically. A specimen with tumor $\leq 1$ mm from the inked margin is considered as having a positive CRM. The value of CRM assessment in rectal cancer was first demonstrated by Quirke et al in 1986 and has been confirmed by numerous other studies over time. The colon is for the most part surrounded by the serosa, which is less easily penetrated by tumor cells than the mesorectal fat. The anatomy of the colon is such that a circumferential margin is generally not present but surgical resection planes of the cecum, descending colon and ascending colon can be quite large compared to other parts of the colon. The prognostic implications of retroperitoneal surgical resection margin (RSRM) involvement of colon segments that are partially fused to the peritoneum are limitedly studied. However, Scott et al demonstrated that RSRM involvement was present in 19 of 228 right hemicolectomies and was correlated with advanced tumor stage and a high incidence of synchronous and metachronous distant metastasis. In conclusion, circumferential margin involvement is a well established treatment-related factor with profound prognostic value in rectal cancer but the prognostic implications of RSRM involvement in colon cancer is currently under investigation and is not well established (yet).
§4.2 (Neo)adjuvant therapy

Traditionally, adjuvant therapy was applied to selected cases of colorectal cancer after surgery. Chemotherapy has some effect on colon cancer, but the effects on rectal cancer were long in doubt. Currently, colon cancer patients are eligible for postoperative 5-fluorouracil (5-FU) based chemotherapy if positive lymph nodes (stage III) are found in the resection specimen (Table 1). But in the case of high-risk stage II colon (T4, poor differentiation grade, perforation, obstruction, angioinvasion and/or less than 10 lymph nodes investigated) adjuvant chemotherapy should be under serious consideration (www.oncoline.nl).

Radiotherapy is only possible in rectal cancer. Trials in Sweden in the 1990s have
demonstrated that the use of short-term neoadjuvant radiotherapy causes improved local control and survival and is more effective than postoperative radiotherapy. The beneficial effects on patients’ prognosis by preventing local recurrence is a major advantage of preoperative radiotherapy. In addition, preoperative radiotherapy has been shown to be more effective than postoperative radiotherapy with a similar dose, which could be explained by the fact that well-oxygenated cells are more sensitive to radiotherapy than hypoxic cells.

Another major advantage of neoadjuvant treatment is the achievement of tumor down-staging, facilitating the resection of advanced rectal tumors. Down-staging is dependent on fraction sized and the total irradiation dose applied. These two factors largely determine the overall treatment time (time between start of irradiation and surgery). The overall treatment time of short-term radiotherapy (approximately 10 days) is too short to achieve down-staging since the overall treatment time to achieve this should be at least 4 weeks. Therefore, optimal neoadjuvant treatment of advanced rectal cancer consists of long-term radiotherapy with or without chemotherapy.

Patient prognosis can be improved and over-treatment can be reduced if patients are selected for the appropriate neoadjuvant or adjuvant treatment. Rectal cancer can roughly be divided in three categories with each a different treatment strategy; small well differentiated tumors, mobile tumors (T1-T3) and locally advanced rectal cancer (LARC).

However, the term LARC is not clearly defined. Definitions range from patients who received long-term neoadjuvant radiotherapy (RT) or radiochemotherapy (RCT), to patients with positive lymph nodes, advanced cT3 or cT4 or patients with a threatened or invaded CRM. The population of patients with LARC described in the present thesis is defined as patients with a rectal carcinoma with an invaded or threatened CRM (a predicted CRM of less than 2 mm on MRI). A clear definition of LARC and application of the proper therapy regimen is crucial since it improves the outcome of this specific patient population.

Small (T1, No) superficially growing, well or moderately differentiated rectal tumors only require limited surgery. However, if after inspection of the TEM specimen the margins appear to be involved or lymphangio or vascular invasion is detected, patients will nevertheless receive short-term radiotherapy followed by TME surgery (www.oncoline.nl). Mobile (T1-T3, No/N+) rectal tumors receive short-term preoperative radiotherapy (5x5 Gray) followed by surgery within 5 days after completion of radiotherapy. This therapy became the standard regimen in the Netherlands after the Dutch TME trial which demonstrated that the addition of preoperative radiotherapy to standardized TME surgery could decrease local recurrence rates from 8.2% to 2.4%.
case of tumor fixation (non-mobile tumors), short-term radiotherapy followed by TME surgery is not sufficient. This subpopulation of rectal tumors, also referred to as locally advanced rectal cancer (LARC), require extensive preoperative radiochemotherapy (RCT) consisting of long-term radiotherapy (45-50 Gy in fractions of 1.8-2 Gy) combined with 5-FU based chemotherapy (Table 1). The aim of this more aggressive regimen is to increase local control.

<p>| Table 1: Overview of the Current Treatment Guidelines for Colorectal Cancer in the Netherlands |</p>
<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Tumor Stage</th>
<th>Tumor Location</th>
<th>Surgical Approach</th>
<th>(Neo) Adjuvant Therapy</th>
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</thead>
<tbody>
<tr>
<td>Colon</td>
<td>Stage I/II</td>
<td>na</td>
<td>Open or laproscopic</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Stage III or high-risk stage II*</td>
<td>na</td>
<td>adjuvant 5-FU based chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>T1/No well to moderately differentiated, absence of lymphangio or vascular invasion</td>
<td>TEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile: T1/T3, No/N+</td>
<td>0-5 cm from anal verge</td>
<td>APR</td>
<td>Neoadjuvant short-term RT (5x5 Gy)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-15 cm from anal verge</td>
<td>LAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced, fixed rectum tumors (T4, No/N+)</td>
<td>LAR, APR, ASR of PE</td>
<td>Neoadjuvant long-term RCT (45-50 Gy in fractions of 1.8-2 Gy combined with 5-FU) based chemotherapy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*High-risk stage II is defined as tumors having at least one of these characteristics: T4, poor differentiation grade, perforation, obstruction, angioinvasion and/or less than 10 lymph nodes investigated. Abbreviations: TEM: transanal endoscopic microsurgery, APR: abdominoperineal resection, LAR: low anterior resection, ASR: abdominal-transsacral resection, PE: pelvic exenteration, RT: radiotherapy, RCT: radiochemotherapy, 5-FU: 5-fluorouracil, na: not applicable. Source: www.oncoline.nl

§5 Predictive Factors for Response to (Neo)Adjuvant Therapy

Tumor response to (neo)adjuvant therapy affects treatment outcome. Predictive factors for therapy response are often tumor-related factors that can predict the extent of tumor response. Foreseeing the modifying effects of therapy on the clinical outcome has several advantages such as prevention of under and over-treatment. However, in order to use these predictive factors in clinical practice, these markers will have to be
very sensitive and specific. And despite much effort, only very few predictive factors, also referred to as markers, are currently used as diagnostic tools for routine testing. HER-2/neu (HER2, ERBB2) is for example currently used as a marker to predict the clinical benefit from trastuzumab (Hercpentin) in patients with metastasis breast cancer. 

At a biological level, there are numerous candidate markers involved in different cellular processes related to therapy resistance and are therefore interesting candidate markers for studying tumor response. Important categories are (Figure 4):

1. Drug influx and efflux pumps.
2. Detoxification processes.
3. Modifications of the drugs target.

Figure 4: Schematic drawing of different cellular processes related to therapy resistance. These processes are numbered 1 to 3. 1: drugs influx and efflux pumps, 2: detoxification processes, 3: modifications of the drugs target. All these processes influence apoptosis. The different apoptosis regulating proteins studied in this thesis are also depicted. White arrow: pro-apoptotic effect, black arrow: anti-apoptotic effect.
P-glycoprotein is a well known example of a transmembrane transporter. Other examples of pumps with functions similar to that of P-glycoprotein are multi resistance protein (MPR-1) and lung resistance related protein (LRP). These pumps influence the intracellular levels of the active drug. Resistance can occur when these transmembrane transporters pump the active drug out of the cell. However, other pumps are often needed for transportation of larger drug particles into the cell\textsuperscript{54,55}. When the chemotherapeutic drug has arrived in the tumor cells, detoxification processes in the cell by for example a member of the enzyme detoxification family of the glutathione s-transferases (GSTs). These enzymes have been described to break down several types of drugs and decrease the effective drug levels\textsuperscript{56,57}. Modifications of the drug target also influences drug efficiency. 5-FU, the major component of chemotherapy regimens for colorectal cancer, inhibits the enzyme thymidylate synthase (TS). TS is a central enzyme in DNA synthesis and its protein expression is affected by three different functional polymorphisms in the untranslated regions (UTRs) of the gene. Sensitivity to 5-FU based therapy might be largely influenced by the intra-cellular levels of the TS protein. Tumors with higher protein levels have been described to be associated with a poorer response to 5-FU than tumors with lower TS protein levels\textsuperscript{58,59}. Eventually, the downstream effect of almost all chemotherapeutic agents is the triggering of apoptosis (programmed cell death). This event is regulated by the interplay of different proteins (Figure 4), such as p53, (the name p53 is in reference to its apparent molecular mass of 53 kDa) cyclooxygenase-2 (Cox-2), B-cell lymphoma 2 (Bcl-2), Bcl-2--associated X protein (Bax) and mammaryserine protease inhibitor (maspin). All these factors have pro- or anti apoptotic properties and can therefore affect the apoptotic balance and finally apoptosis\textsuperscript{60-64}. The fact that some of these factors also influence each other, e.g. Cox-2 and Bcl-2, makes this delicate interplay only more complex. Moreover, the proteins that are depicted in Figure 4 are obviously only a small representation of all proteins involved in the regulation of apoptosis.

§6 FUTURE STAGING OF COLORECTAL CANCER

As described in the former paragraphs, patients’ outcome after diagnosis of colorectal cancer depends on several different factors. The total population of patients with this malignancy will have an intermediate outcome (Figure 5A). Application of traditional staging will split this group into good and relatively poor prognosis (5B). Evaluation of treatment-related prognostic factors will lead to further refinement of prognosis (5C), as has been described in this thesis. In the future, factors predictive for therapy response will finally lead to a very detailed refinement in foreseen outcome (Figure 5D); when every patient can receive optimal individualised treatment (tailor made therapy). Accurate estimation of tumor invasion depth, N status and CRM involvement by imaging techniques is becoming increasingly important in order to provide this
In this thesis pathological and biological aspects of colorectal cancer are discussed, focusing on improving staging and treatment. The present thesis consists of two major parts. The first part (chapter 1-3) deals with the pathological aspects of colorectal cancer treatment and the second (chapter 4-8) with the biological aspects. For this purpose, 3 different patient populations were used.

The first population consists of patients with mobile rectal (T1-T3) cancer that were included in the Dutch TME trial. These patients were randomized for either TME surgery alone or short-term radiotherapy (5X5 Gy) followed by standardized TME surgery. The conclusions that could be drawn based on studying this population are described in chapter 2, 4, 5 and 6.

The second population consists of patients with locally advanced rectal cancer (advanced T3 and T4) that had a threatened circumferential margin. All patients received long-term radio(chemo)therapy comprising of 50.4 Gy in 1.8 Gy fractions and 5-FU based chemotherapy. Studies based on this population are described in chapter 3 and 8.

Finally, patients with stage III colon cancer were investigated (chapter 7). These Patients received adjuvant 5-Fluorouracil (5-FU) based adjuvant chemotherapy.

In chapter 1 the future of tumor staging is discussed, with special emphasis on the use of treatment-related factors in staging of rectal cancer. This critical comment is based on the data described in chapter 2. In this chapter, we compared the traditional staging system with a new developed system including circumferential margin status, in two independent populations. In chapter 3 we investigated the best way to evaluate the therapy-effect in locally advanced rectal cancer.
The first chapters dealing with the biological aspects, chapter 4 and 5 describe the role of adhesion molecules (epithelial cell adhesion molecule, E-cadherin and β-catenin) in tumor progression and shed some light on genetic mechanisms of tumor differentiation. Chapter 6 describes the investigations on the prognostic and predictive value of Cox-2 in patients with rectal cancer distinguishing between patients who did and did not receive radiotherapy. Technical aspects of determining the predictive value of TS in patients with colon cancer are mentioned in chapter 7. Finally the potential value of apoptosis for the prediction of response to long-term radio(chemo)therapy is evaluated in chapter 8.
REFERENCE LIST

CHAPTER 1

RECTAL CANCER: THE COMBINATION OF TREATMENT AND TUMOR-RELATED FACTORS GIVES MORE INFORMATION THAN THE TRADITIONAL TNM STAGING BASED ON TUMOR CHARACTERISTICS ALONE. TIME FOR A REVISION OF CURRENT STAGING SYSTEMS?

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Comments and controversies
Treatment of rectal cancer has radically changed over the last decade. The introduction of the surgical technique Total Mesorectal Excision (TME) worldwide has resulted in a decline of local recurrence rate, due to the fact that more tumors were completely excised, along with regional metastatic disease in the mesorectal fat. In addition, the role of neoadjuvant treatment either by radiotherapy or by radiochemotherapy has been established over the last 10 years. In Europe, overwhelming evidence has been gathered from large randomized trials (TME, SRCT, CR07) with a total number of 4,427 patients showing that for primarily resectable rectal cancer short-term pre-operative radiotherapy (5x5 Gy) resulted in local recurrence rates lower than 5%, especially in combination with TME surgery. For locally advanced tumors, long-term radiotherapy (approximately 50 Gy) in combination with neoadjuvant chemotherapy is the treatment of choice.

The combination of the above mentioned changes in therapy results in improved prognosis of patients with rectal cancer, especially with respect to local recurrence, but these advances have not yet been included in staging of rectal carcinoma. In fact, these innovations in therapy call for a change in approach of staging. Due to the application of neoadjuvant therapy both the function of staging systems as well as the factors used for staging have changed, which complicates the current practice.

Initially postoperative pathological staging was used for the prediction of prognosis as well as for the indication of adjuvant therapy. Nowadays, clinical staging determines whether and which preoperative therapy should be applied and postoperative staging is used to evaluate the effects of therapy in addition to the above mentioned goals. The consequence of these changes is a divergence between cTNM and pTNM. Moreover, the current pTNM is essentially different from the pTNM of last century. Still, the staging system for rectal cancer uses the same rules as Cuthbert Dukes proposed in 1932.
IMPACT OF NEOADJUVANT THERAPY

Long-term radiotherapy and chemoradiation schemes are aimed at tumor downstaging, to facilitate complete surgical removal. Pronounced changes in tumor histology are observed in the operation specimen, indicative of tumor response or regression. In many of these cases the pT stage is lowered compared to the initial cT stage, but it is not clear which of these two is the best predictor for prognosis. The current guidelines of the AJCC/TNM staging systems 7,8 acknowledge preoperative treatment by adding the prefix y, but the clinical consequences are not clear.

The ypT stage can be used as a measurement for tumor downstaging, however, after removing locally advanced tumors, tumor remnants might be left behind in the surrounding tissue, resulting in inadequate determination of T stage. Moreover, there is a large variability between the pT3 tumors with regard to tumor load. Alternatively, response can be indicated by determining the grade of tumor regression. Various systems have been suggested to grade tumor regression 9-11, but the majority is not able to demonstrate a relation with prognosis. In addition, reproducibility of regression grading is poor 12,13.

Since the goal of long-term neoadjuvant therapy is the facilitation of surgical removal, we suggest inclusion of surgery related factors in the staging after this kind of treatment.

SURGICAL FACTORS IN THE 21ST CENTURY

The recognition of TME as a superior surgical technique is preceded by the recognition of circumferential margin (CRM) involvement as the best prognostic factor, not only for local recurrence, but even for development of metastases, as well as for survival. A recent review with data of over 17,500 patients 14 demonstrated that the prognostic value of an involved CRM for local recurrence is even stronger after neoadjuvant therapy (hazard ratio 6.3 (95%CI 3.6 -16.7) versus hazard ratio 2.2 (95% CI 1.5 -3.2) without neoadjuvant therapy).

A positive CRM after surgery can be caused by various factors; the most important of which are suboptimal quality of surgery, aggressive tumor growth, therapy resistance and inadequate preoperative imaging. The quality of surgery is analyzed by the assessment of plane of resection. This is correlated with both local recurrence and overall survival and its value has recently been confirmed in another large multicenter trial 15. The finding that CRM involvement can predict the development of distant
metastases as well as survival may suggest that aggressive tumor growth is most important. However, the fact a positive CRM due to poor quality surgery is correlated with survival as well, indicates that for prognosis the cause of margin involvement does not seem to matter.

**STAGING SYSTEMS: WHERE SHOULD WE GO?**

In the era of neoadjuvant therapy, the existing staging systems are suboptimal. There is a need for the implementation of treatment-related factors, which will improve both staging and prognostification. The result of treatment is one of the most relevant features for predicting final outcome, therefore modern staging systems should take both tumor and treatment factors into account. The incorporation of these factors should of course be evidence based. Before we can propose a new staging system, we have to address the following questions: Which factors can reliably predict prognosis? Are these factors generally applicable? Can these factors be assessed in a reliable and simple way? Is there a combination of factors that adequately divides patients in large, homogeneous groups with highly divergent survival curves?

**Which factors can reliably predict prognosis?**

First we have to question the value and reliability of established tumor factors as invasion depth and lymph node status in the current situation. As mentioned above, the reliability and relevance of ypT is questionable. The presence of lymph node metastases after neoadjuvant therapy is still a major prognostic factor. However, an unknown number of node negative patients will have had positive nodes that are sterilized by neoadjuvant therapy. Therefore, ypNo consists of a heterogeneous group of patients who were initially node negative and patients whose metastatic tumors responded well to treatment. Although the meaning of ypNo might be different from pNo, the prognostic impact is still there. Although a multivariate analysis of 182 patients suggests that after neoadjuvant therapy CRM is more important for prognosis than lymph node involvement. Since neoadjuvant therapy is mainly aimed on local control, we can, at current, leave the presence of metastatic disease (TNM IV) out of this discussion.

Treatment-related factors are CRM, tumor regression and quality of surgery. The results of tumor regression grading are variable and no consistent relation with prognosis has been demonstrated. Moreover, 4 different studies including a total number of 490 patients demonstrate superiority of CRM-assessment above regression grading. Quality of surgery evaluation in two independent randomized trials demonstrates prognostic value for both local recurrence and survival. However, CRM involvement is more important than plane of surgery.
Finally, there are many biomarkers described, but none of them have reached the standard assessment of rectal cancer specimens and therefore remain beyond the scope of this comment.

**Are these factors generally applicable?**

Although in most cases of rectal cancer preoperative neoadjuvant therapy will be applied, some patients will be operated right away. The new staging should be applicable in all situations. Tumor invasion, lymph node metastases, CRM involvement and quality of surgery can be evaluated with and without neoadjuvant therapy and in any laboratory of pathology. One could argue that this is the case for tumor regression as well, and that without therapy there will be no regression. As a result, although the absence of regression after therapy may be a bad sign, the absence of regression without therapy has no meaning at all.

**Can these factors be determined in a reliable and simple way?**

The stage of the tumor is relatively simple determined provided that an adequate sampling of the tumor area is performed. Especially for the determination of ypT0 (complete regression) a standardized protocol is required. Careful examination of the resection specimen will reveal possible involvement of the circumferential margin and presence of lymph node metastasis. Detailed protocols are available\(^\text{25,26}\).

Determination of tumor regression is much more difficult and reproducibility studies show kappa values as low as 0.30\(^\text{13}\). One of the reasons is that there is no consensus about the definitions that should be used, apart from the definition of complete response. Disappointingly, up to now none of the reported studies used this definition.

**Is there a combination of factors that adequately divides patients in large, homogeneous groups with highly divergent survival curves?**

In a recent study\(^\text{27}\) based on the data from a randomized clinical trial\(^\text{1}\) we demonstrated in a multivariate model that CRM rather than pT stage is important for the prognosis of rectal cancer. This is true for patients without preoperative treatment as well as for patients treated with short-term preoperative radiotherapy. Based on these findings we designed a staging system, including both CRM (treatment-related factor) as well as nodal status (tumor-related factor) (Figure 1). Using this system we created highly divergent survival curves, with a small group of patients with a poor prognosis (n = 93 (7%), 36% 5-year survival) and a large group of patients with a good prognosis (n = 743 (57%), 92% 5-year survival). We confirmed our findings in an independent dataset, derived from another randomized trial\(^\text{28}\) (Figure 1b). In this group of patients (with short-term and long-term neoadjuvant therapy) the new staging system performed significantly better than TNM.
Figure 1: Hazard ratio and 95% confidence interval of TNM staging versus a new staging method which is based on a combination of nodal staging (N) and circumferential margin status (NCRM). (A) TME trial (n = 1530; follow-up 67 months). (B) Polish rectal cancer trial (n = 316; follow-up 48 months). y: pre-operative treatment, p: pathologic.
CONCLUSION

In modern staging of cancer there should be an important place for treatment-related factors, since the result of treatment is one of the most important prognostic factors. For rectal cancer we demonstrated that in the era of neoadjuvant therapy free circumferential resection margins are more important than the classic factor of invasion depth, and that incorporation of this factor in staging systems leads to better prognostification and selection of patients.
Chapter 1

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CHAPTER 2

IMPROVEMENT OF STAGING BY COMBINING TUMOR AND TREATMENT PARAMETERS: THE VALUE FOR PROGNOSTICATION IN RECTAL CANCER

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For the Cooperative Clinical Investigators and the Pathology Review Committee

Background
Staging of cancer is based on the TNM system. This valuable system only takes tumor-related parameters into account, but in the era of refined surgery and preoperative therapy treatment-related factors are of equal importance. By rectal cancer as a model we explored the hypothesis that a combination of tumor- and treatment-related parameters will result in improved prognostication.

Patients and methods
Standardized clinicopathological and histological factors considered predictive for survival were studied in eligible patients treated in a trial for rectal cancer (n = 1324). These factors were analyzed in relation to survival using log-rank tests, Kaplan-Meier curves and Cox regression both individually and in combination, the latter including TNM staging. A second dataset from an independent trial (n = 316) was used for data validation.

Results
Multivariate analysis identified nodal status (p=0.001) and circumferential margin (p=0.001) involvement as most important prognostic factors for survival. Combination of these factors formed an improved staging system (node status and circumferential margin NCRM) compared to the present TNM-staging with respect to 5-year cancer-specific survival. The results were confirmed in our independent patient population.

Conclusions
NCRM staging of rectal cancer results in a broad range of survival rates and favorable patient grouping. Our data give strong evidence that a staging system combing tumor- and treatment-related factors provides better prognostic information than the classic TNM system, which is based solely on tumor-related factors. Similar results might be obtained in other types of cancer where quality of treatment is important for outcome.
Staging of cancer patients is an important tool for the selection of patients for adjuvant treatment. Ideally, stage grouping results in homogeneously divided groups incorporating patients with similar prognoses. The most important staging system is the TNM system that is used worldwide for treatment selection in many tumor types. The strength of this system is that it is easily applicable and well defined. Another important feature is the regular adaptation due to new data, leading to the 6th edition in 2002. In general, the factors included in the TNM system are purely tumor-related. Currently, in many patients neoadjuvant treatment is given, which may alter stage-related outcomes. We hypothesize that treatment-related factors might be relevant for identification of high-risk patients.

Recent developments in the treatment of rectal cancer include total mesorectal excision and the switch from postoperative to preoperative radiotherapy. The combination of these modalities reduced local recurrence risk significantly. One of the major prognostic factors in rectal cancer is involvement of the circumferential margin (CRM), which is an important surgery-related factor. This factor is not only prognostic for local recurrence, but also for the development of distant metastases. Therefore, this might be an important factor in the selection of patients for adjuvant therapy. The current TNM staging system does not take this parameter into account.

We decided to explore the hypothesis that a staging system that includes both tumor and treatment-related prognostic factors results in improved prognostication. These treatment-related factors should be easily identifiable and uniform in order to eliminate interpretation variation in pathological practice. The development of a staging system should be evidence-based, developed from multicenter randomized trials, and be tested by multivariate analysis.

In the current study we used the patient population of a large multicenter randomized trial to develop a system with increased prognostic value, based on both tumor-and treatment-related factors. A second dataset from an independent trial was used for verification. Our approach can be regarded as a model for other types of cancer where preoperative treatment is given and where quality of surgery determines outcome significantly.
METHODS

Study population
Data from patients from the radiotherapy (RT) + Total Mesorectal Excision (TME) trial, which was a large multicenter trial, are the basis of this study. This trial compared TME surgery and preoperative short-term radiotherapy with TME surgery and has been described extensively. Informed consent was obtained from all included patients and the medical ethics committees of all participating hospitals have approved the trial.

Patient selection
For the current study, the data of the eligible Dutch patients in the trial (n = 1474 from 83 Dutch hospitals) as described earlier were analyzed. Patients were excluded from the analysis for the following reasons: no resection (n = 37), macroscopic resection locally not complete (n = 5), distant metastases at operation, TNM stage IV (n = 91) and no tumor at operation (n = 15). For the final analysis 1324 patients could be evaluated. In case of cancer-specific survival analysis, cause of death was unknown in 7 patients. These patients were excluded for this analysis. The median follow-up period at the time of the analysis was 67.2 months.

Independent test population
Data from patients from the Polish rectal cancer trial were used as an independent test population. This trial compared short-term preoperative radiotherapy (5 x 5 Gy) followed by TME with chemoradiation (50.4 Gy, 5-fluorouracil and leucovorin) followed by TME. 316 patients were included with a median follow-up period of 48 months.

Treatment and diagnostic procedures
The short course of preoperative radiotherapy and the surgical technique are described extensively elsewhere. In brief, the main principles of this technique involve sharp dissection within the true pelvis around the integral mesentery under direct vision, envelopment of the entire midrectum and preservation of the hypogastric plexus. The majority of patients were treated with low anterior resection (69%). Pathological procedures were performed as have been described by Quirke et al. Special attention has been given to the examination of the circumferential resection margin. A margin of 1 mm or less was considered positive. In addition, all cases were reviewed by the Pathology Review Committee.

Data collection and statistics
All case record forms were sent to the central data office at the Department of Surgery of the Leiden University Medical Center in the Netherlands. The data were checked and
entered in a database and analyzed with the SPSS package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA). Relations between various parameters were analyzed using \( \chi^2 \)-methods and Mann-Whitney nonparametric testing procedures. Univariate survival analyses of time to cancer-related death were performed using the Kaplan-Meier method, with the time of surgery as the entry date. Differences in observed survival between groups were tested for statistical significance using log-ranks tests. Factors were included in the multivariate analysis if the p-value of the univariate analysis was less than 0.10. Multivariate analysis was performed using the Cox proportional hazards regression model. A p-value of 0.05 or less was considered statistically significant.

The performance of the prognostic systems was evaluated by the integrated Brier score. The integrated Brier score is a measure comparing the actual survival outcomes of individuals with the predictions based on a prognostic system, accounting for possible censoring. The integrated brier score of the whole group (using the Kaplan-Meier estimates as a prediction) was used as an estimate of the total variance; the integrated Brier score taking into account the prognostic system was used an estimate of the residual variance. This leads to the percentage of variance explained. A prognostic system is better when within a prognostic group individuals are more homogeneous (residual variance is smaller) and as a consequence the percentage of variance explained is higher.

RESULTS

Univariate analysis
Patient-, treatment- and tumor-related factors as well as histologic factors were analyzed in relation to cancer-specific 5-year survival (Table 1).

Patient-related factors (age and gender) did not influence survival. Both surgery-related factors, distal and circumferential margins, were strongly associated with survival. Although only a small group of patients presented with a positive distal margin \( (n = 13) \), survival in this group was very low at 38%, compared to 78% in the negative margin group \( (p < 0.0001) \). Quality of surgery, as measured by the completeness of mesorectal excision or plane of resection, was not taken into account due to the large number of missing data in the whole series.

The size of the tumor was not a relevant factor for cancer-specific survival. As expected, the classic factors of invasion depth and lymph node status were relevant. Tumor location (i.e. distance from the anal verge) showed a decreased survival for the low rectal carcinomas (74% vs 78% and 82% for the mid- and upper rectum, \( p = 0.009 \)).
Histological factors such as differentiation grade (2-tiered), growth pattern, lymphoid reaction and eosinophilic infiltrate also were significant predictors of cancer-specific survival in the univariate analysis, which was also reported by Nagtegaal et al.\textsuperscript{15} This shows the validity of our material.

**Multivariate analysis**
Factors included in the multivariate analysis are depicted in Table 2. The most relevant significant factors were circumferential margin involvement and lymph node status (in both cases \( p < 0.0001 \)), with a relative risk of 1.7 for cancer-related death in case of positive CRM and a relative risk of 5.3 in case of more than 3 positive lymph nodes (N2). Furthermore, the location of the tumor was important, as well as distal margin involvement. Invasion depth showed a difference in cancer-specific survival between the tumors limited to the bowel wall (T1, T2) and those that invaded the surrounding fat (T3). In the T4 group only 37 patients were present, accounting for a non-significant decrease in cancer-specific survival. Of the histologic factors, only the presence of eosinophilic infiltrate and lymphoid reaction seems to protect against cancer-related death.

**Node status and circumferential margin staging**
With the results of the multivariate analysis, an improved staging system based on the combination of lymph node status (N) and CRM was developed (NCRM). The categorization of this system was based on the number of adverse factors; NCRM 0 (0 adverse factors: N\(_0\), CRM\(-\)), NCRM 1 (1 adverse factor: N\(_0\), CRM\(+\) or N\(_1\), CRM\(-\)), NCRM 2 (2 adverse factors: N\(_2\), CRM\(-\) or N\(_1\), CRM\(+\)), NCRM 3 (3 adverse factors: N\(_2\), CRM\(+\)). The N1 status is counted as 1 adverse factor and N2 as 2 adverse factors. This staging system was compared with TNM and Jass’ staging\textsuperscript{16} with respect to the cancer-specific 5-year survival rate (Figure 1) and the distribution of patients. TNM and Jass’ staging could not be performed in 2 and 23 patients, respectively, because of missing data.

In the total population, the 5-year cancer-specific survival of different TNM stages ranged from 95% for TNM I to 58% in the TNM III group. For Jass’ staging these percentages ranged from 90% in stage-group I to 49% in stage-group IV. The NCRM staging showed an even larger variation in prognosis, from 92% in the best group (no adverse factors, N\(_0\), CRM\(-\)) to 36% in the worst group (3 adverse factors, N\(_2\), CRM\(+\)) (data not shown). In addition to a better discrimination for prognosis, the NCRM staging system also identifies more low-risk patients (Table 3). NCRM identifies 57% of patients with more than a 90% 5-year survival rate, TNM 33% and Jass’ 25%.
Finally, we tested the new system in the separate randomization arms of the trial, to evaluate whether the system is valid in both previously untreated patients and in patients with short-term preoperative radiotherapy treatment before surgery. In both arms the difference in prognosis was larger in the NCRM staging compared to both the TNM and Jass’ systems. The number of low-risk patients was high in both arms, 55% in the surgery only group and 58% in the preoperative radiotherapy group (Table 3).
Cox regression was used to compare the prognostic value of NCRM with the prognostic value of TNM. Starting with TNM, the addition of NCRM improves the model significantly ($\chi^2 = 50.5, p < 0.001$). The addition of TNM when starting with NCRM is also significant ($\chi^2 = 16.8, p < 0.001$), suggesting that NCRM is a better classifier, but that TNM has some additional predictive value in this data. The better performance of NCRM is confirmed using the percentage of variance explained: TNM 11.4% compared to NCRM 16.9%.

**Independent test group**

To validate our proposed classification system we applied the system to an independent test population. Figure 2 shows the survival curves for both classification systems in the independent test group. The survival rates for the total independent test group are depicted in Table 4. In concordance with our own population, the percentage of variance explained was higher in NCRM than TNM staging; 18.7% vs 13.6%, respectively. The analysis was repeated in the different randomization arms of the trial and both gave similar results.

**DISCUSSION**

In the diagnosis and treatment of cancer, staging is of utmost importance. Prognosis of patients can be predicted based on tumor stage and the choices of adjuvant therapy are largely dependent on these stages. The most commonly used staging system is the TNM. On basis of the data of the Dutch TME-study, we developed an improved staging system based on nodal status and circumferential margin involvement (the NCRM). Multivariate analysis of 1324 Dutch patients included in the TME trial showed that lymph node involvement was the most important prognostic factor for 5-year cancer-specific survival, followed by involvement of the circumferential margin. Combination of these two factors leads to a 4-stage system, reflecting the number of adverse factors.

Staging by the proposed NCRM system is transparent and easily applicable by pathologists. Inclusion of invasion depth as a third factor did not improve the staging (data not shown). Moreover, its inclusion would only make the system more complicated. The difference compared to other staging systems is the integration of a treatment-related factor (circumferential margin involvement). The replacement of tumor depth by CRM involvement is the most distinctive feature of NCRM staging when compared with TNM staging. Both CRM and nodal involvement are partly depended on the depth of tumor invasion, but provide additional information. This explains why tumor invasion depth has lesser prognostic impact on survival than the components of the NCRM system. TNM staging is based solely on tumor-related factors, while Jass’
Figure 2: Kaplan-Meier curves for cancer-specific survival in the independent test population. (A) TNM staging and (B) NCRM staging. For each category the percentage of patients is given.

staging also takes histology of tumor microenvironment into account.\textsuperscript{16} However, the factors that are indicative of tumor microenvironment (growth pattern and lymphoid reaction) were not significant in this study on multivariate analysis. Moreover, the application of Jass’ staging in this setting may be complicated by the effect of neoadjuvant therapy on lymphoid reactions.\textsuperscript{27} Finally, another shortcoming of Jass’ staging is the complexity of the system, which reduces its reproducibility due to subjective interobserver-variation.\textsuperscript{18}

An accurate estimation of prognosis for survival is a basic tool for the selection of patients for adjuvant therapy. The identification of high-risk patients to date has been difficult. Adjuvant chemotherapy has not resulted in a survival benefit in rectal cancer,\textsuperscript{19,20} probably due to the high local recurrence rates in those studies. For patients with colon cancer the effect of adjuvant chemotherapy is only proven in TNM III,\textsuperscript{21} although it has been suggested that also high-risk TNM II patients should be treated. Selection of these patients is not easy. The power of the NCRM is based on a more discriminative grouping of patients, with a more pronounced separation of cancerspecific outcomes, for the population as a whole as well as for the 2 randomization
groups tested separately. Thus, the system is most appropriate for the staging of resectable rectal tumors treated with short-term preoperative radiotherapy, followed by TME resection. Several studies confirmed preoperative therapy to be more effective than postoperative irradiation.\textsuperscript{22,23} As a result of this, preoperative radiotherapy in combination with surgery has become the preferred treatment for primary resectable rectum carcinoma in Europe and in many centers in North America, potentially making the NCRM broadly applicable.

For locally advanced rectal carcinoma, various long-term radiotherapy and radiochemotherapy regimens are used. These approaches result in tumor down-staging and downsizing, which may affect both circumferential margin- and lymph node involvement.\textsuperscript{7,19-24,26} In a study of 61 patients with locally advanced rectal carcinoma treated with long-term radiochemotherapy (45 Gy, bolus 5-fluorouracil) both circumferential margin involvement and lymph node metastases were the most important factors for cancer-specific survival in a multivariate model.\textsuperscript{27} The importance of the circumferential margin was confirmed in another study of 200 locally advanced rectal carcinoma patients treated with either long-term radiotherapy (44 Gy) or radiochemotherapy (with 5-fluorouracil).\textsuperscript{28} After preoperative hyperfractionated accelerated radiotherapy of 104 patients with locally advanced rectal cancer, 43% demonstrated tumor down-staging. None of the patients showed complete regression.\textsuperscript{29} In this study, both pathologic lymph node stage and CRM were independent prognostic factors for disease free survival. These results, in combination with the results in our independent test population, although relatively small, strongly suggest that the NCRM staging is also applicable to patients treated with long-term radio (chemo)therapy.

The current study implicates that a staging system based on both a tumor-related factor and a treatment-related factor is superior to TNM staging, which is solely based on tumor-related prognostic factors. We encourage further testing and validation of NCRM staging in large populations of patients with rectal cancer. The role of cancer treatment is of paramount importance for obtaining good long-term results. Tumor treatment nowadays is almost as important as tumor characteristics and this phenomenon should be considered in the design of a new generation of staging systems.
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Staging by combining tumor and treatment parameters
CHAPTER 3

CIRCUMFERENTIAL MARGIN INVOLVEMENT IS THE CRUCIAL PROGNOSTIC FACTOR AFTER MULTIMODALITY TREATMENT IN PATIENTS WITH LOCALLY ADVANCED RECTAL CARCINOMA

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Purpose
After preoperative (radio)chemotherapy (RCT) histological determinants for prognostification have changed. It is unclear which parameters, including assessment of tumor regression, are the best indicators for local recurrence and survival.

Patients and methods
A series of 201 patients with locally advanced rectal cancer (LARC) (cT3/T4, M0) presenting with an involved or at least threatened circumferential margin (CRM) on preoperative imaging (less than 2 mm) were evaluated using standard histopathological parameters and four different histological regression systems. All patients received neoadjuvant radiochemotherapy (RCT) or radiotherapy (RT). The prognostic value of all factors was tested with univariate survival analysis of time to local recurrence (LR) and overall survival (OS).

Results
LR occurred in only 8% of the patients with a free CRM compared to 43% in case of CRM involvement (p<0.0001). None of the four regression systems was associated with prognosis, not even when corrected for CRM status. However, we did observe a higher degree of tumor regression after RCT compared to RT (p<0.001). Absence of tumor regression was associated with increasing invasion depth and a positive CRM (p=0.02 and p=0.03 respectively).

Conclusion
Assessment of CRM involvement is the most important pathological parameter after RCT. Although tumor regression increases the chance on a free CRM, in cases with positive resection margins prognosis is poor irrespective of the degree of therapy induced regression.
INTRODUCTION

For patients with locally advanced rectum carcinoma (LARC), surgery alone is often not curative. In case of cT4 tumors or a threatened circumferential margin (CRM; less than 2 mm on preoperative imaging) in cT3 tumors, long-term neoadjuvant radio(chemo)therapy (RCT) is required. This will result in down-staging and increased local control. The histopathology of specimens obtained after this kind of preoperative therapy is markedly different compared to untreated cases. Various stages of histological tumor regression may be present, often resulting in changed morphology.

Histological changes after the RCT regime range from absence of any treatment effect to a complete response with no residual tumor identified. One of the first systems for grading histological regression focused on patients with esophageal carcinoma who were treated with RCT. Their results showed that after multivariate analysis, only grading of tumor regression was a significant predictor for disease-free survival. Subsequently, this system was modified by Dworak et al. for grading regression in the rectum. Currently, several different methodologies for measuring the degree of histological tumor regression after RCT in rectal cancer have been described but none has become universally accepted. Reproducibility seems to be a key factor.

The objective of our study is to evaluate which factors determine outcome in patients with LARC after RCT, focusing on the contribution of histological tumor regression grading and the CRM. Tumor regression after RCT was measured using four different methodologies and evaluated for prognostic impact with respect to overall survival (OS) and local recurrence (LR). CRM was evaluated according to Quirke et al. Additionally, prognostic implications of clinicopathological and histological parameters were determined.

METHODS

Patient selection
The patient population consisted of a consecutive series of patients with LARC with biopsy proven adenocarcinoma. All patients received multimodality treatment at the Catharina hospital between 1994 and 2005. Patients were referred from all over the Netherlands based on the assumption of the referring surgeon that a free CRM was unlikely to be obtained without neoadjuvant treatment. Until August 2005, 201 patients with stage cT4 or cT3 and a predicted CRM of less than 2 mm have been treated. Confirmation of the tumors proximity to the CRM and the absence of distant
metastasis (M0) on MRI were crucial for enrolment. Median follow-up was 22.8 months (range 0-124 months). Approval for the study was given by the local ethical committee of the Catharina hospital.

**Therapy**

Preoperatively, patients received different treatment regimens, considered state of the art at the time of treatment. Long-term radiotherapy (RT) \( (n=74) \) involved a total dose of 50.4 Gy in 1.8 Gy fractions, 5 times a week. Two RCT schedules have been used. The MAYO schedule, hereafter mentioned as interrupted schedule \( (n=102) \), comprises concurrent radiotherapy and chemotherapy (RCT): a total irradiation dose of 50.4 Gy, 1.8 Gy per fraction during 5 weeks synchronously with 5-FU \( (350 \text{ mg/m}^2) \) and leucovorin \( (20 \text{ mg/m}^2) \) in irradiation week 1 and 5. The radiation scheme of the continuous RCT regimen \( (n=25) \) compromises 45 Gy in fractions of 1.8 Gy during 5 weeks. On every radiation day 820 mg/m\(^2\) capecitabine was administered twice and 50 mg/m\(^2\) oxaliplatin was given at the first irradiation day of each week.

**Surgery**

The objective in both cT3 and cT4 tumors was to obtain a radical resection (negative CRM). Especially in cT4 tumors, the CRM encompassed surrounding structures: i.e. prostate vesicle, vaginal wall, pelvic floor, uterus, sacrum etc. The CRM was considered negative if the outer margin of the en bloc specimen was negative.

In case of all treatment schedules, surgery was performed 6 to 8 weeks after the last radiation date. All patients underwent resection by experienced and designated colorectal surgeons (HJTR, GAPN), who routinely perform total mesorectal excision (TME) surgery. The extended surgical procedures used were; abdominoperineal resection \( (n=98) \), low anterior resection \( (n=91) \), abdomino-transsacral resection \( (n=9) \) and exenteration \( (n=3) \). The TME principle was adhered to in all cases, even in extended resections 14.

**Histopathological assessment**

Surgical specimens were assessed according to the protocol of Quirke et al. 11,12. The most important issue is assessment of the CRM. In order to determine the CRM, the lateral resection margin of the fresh specimen was inked and subsequently the specimen was fixed in formalin for 48 hours. Blocks of the tumor in relation to the inked CRM were collected. Measurements of the margin were performed microscopically. A specimen with tumor ≤ 1 mm from the inked margin was considered as having a positive CRM. Classification of tumors was performed using the WHO guidelines; a tumor was considered mucinous when the proportion of the mucinous component was ≥ 50%. Tumors were graded according to histological differentiation into well, moderately and
poorly differentiated based on the poorest differentiated part of the tumor excluding
the invasive front. Growth patterns were assessed as circumscribed or infiltrating.
Evaluation of the tumor biopsies included assessment of tumor type and differentiation
grade.

**Histologic regression grading**
Histological therapy-induced tumor regression was assessed according to four different
grading systems described by Dworak, Scott, Bouzourene and Rödel. All four
regression systems semi-quantitatively assess the relative proportion of residual tumor
to stromal fibrosis. The following descriptions characterized the different regression
grades of the regression systems used: Dworak grade 0: no regression detectable,
grade 1: dominant tumor mass with obvious fibrosis and/or vasculopathy, grade 2:
dominantly fibrotic changes with few tumor cells or groups (easy to find), grade 3: very
few (difficult to find microscopically) tumor cells in fibrotic tissue with or without mucin
and grade 4: no tumor cells, only fibrotic mass or mucin. Scott minimal: less than 1/3
tumor regression, moderate: 1/3-2/3 tumor regression, good: more than 2/3 regression,
maximal: no primary tumor remaining. Bouzourene TRG 5: tumor shows no signs of
regression, TRG 4: residual tumor cells outgrowing the fibrosis, TRG 3: more tumor cells
than TRG2 but fibrosis still predominates, TRG 2: rare residual cancer cells scattered
throughout the fibrosis, TRG 1: absence of residual cancer and fibrosis extending
through the different layers of the rectal wall. Rödel 0: no regression or < 25% of tumor
mass, 1: 25% to >50% tumor regression, 2: complete regression.

When no tumor could be found macroscopically, sufficient tumor blocks were sampled
in order to establish a complete response. In the present series, 21 patients had a
complete response. The mean number of block samples collected from the fibrotic
area was 9 (median 7, range 3-22). Figure 1 shows representative examples of different
degrees of tumor regression. These microscopic images were digitalized using a Zeiss
AxioSkop 2 Plus microscope with a Sony 950P camera attached to it. Images were
digitized using 5x or 40x Plan-Neofluar objectives (Carl Zeiss MicroImaging). A-cellular
mucin was considered as absence of residual tumor. The degree of tumor regression
was determined semi quantitatively by two pathologists (JHJMvK and ITG) who were
blinded for patients’ clinical outcome. In addition, the amount of necrosis and the
presence of calcifications were scored as alternative parameters for regression.
Figure 1: Representative slides stained with H&E of different degrees of tumor regression observed after radio(chemo) therapy in patients with locally advanced rectal cancer (LARC). A: No sign of regressive changes, the fibrosis present is probably intrinsic to tumor development (original magnification: 50x). B: Marked fibrosis but large masses of vital tumor can still be observed (original magnification: 50x). C: Predominately fibrotic changes with smaller tumor masses (original magnification: 50x). D: Extensive tumor regression with few small clusters of tumor cells (arrow) scattered through the fibrotic area (original magnification: 50x). The boxed area in D is depicted with a higher magnification as an insertion in this panel. Three small clusters of tumor cells can be appreciated (original magnification: 400x).

**Statistical analysis**

Data were analyzed with the SPSS package (Statistical Product and Service Solutions 11.0 for Windows, SPSS inc., Chicago, Illinois, USA). Univariate survival analyses of time to death were performed using the Kaplan-Meier method with the time of surgery as the entry date. Differences in observed survival between groups were tested for statistical significance using log-ranks tests. Chi-Square tests were used to determine correlations between categorized variables. Multivariate analysis was performed using the Cox proportional hazards regression model (Backward elimination (conditional)). P-values of ≤ 0.05 were considered as statistically significant.
RESULTS

Pre-treatment patient characteristics
The majority of the patients was male (61%); median age was 63 years (range 35-86 years). Clinical T stage was cT4 in 59% and cT3 in 41% of the patients. Slightly more CRM involvement was present in cT4 tumors (25% versus 17%, \( p = 0.13 \)). There was no difference in outcome between cT3 and cT4 tumors (local recurrence 12% versus 19%, \( p = 0.31 \), metastases 22% versus 29%, \( p = 0.50 \), overall 5-year survival 58% versus 47%, \( p = 0.22 \)). Since there was no significant difference in outcome we combine both groups for further analysis.

Correlations between pre-treatment factors and regression
The 3-tier Rödel system was used to demonstrate correlations between the degree of regression and pre- and post-treatment factors (Table 1). The Rödel system consists of the lowest amount of categories and therefore avoids subgroups containing small numbers of patients. Furthermore, this system showed significant correlation within the framework of a randomized trial. 9

A strong association between treatment regimen and tumor regression was found (Table 1). The degree of tumor regression was significantly higher after RCT (12% Rödel 2) compared to RT (8% Rödel 2, \( p<0.001 \)). Regression was more pronounced in tumors showing poor differentiation in their pre-treatment biopsy (\( p=0.03 \)). Tumor regression was not influenced by preoperative cT stage.

Post-treatment clinicopathological and histological factors and prognosis
CRM involvement, lymph node status and tumor stage were strongly associated with both local recurrence (LR) and overall survival (OS) (Table 2). CRM involvement was the strongest predictor of LR (43% versus 8%, at 24 months, \( p< 0.001 \)) and OS (58% versus 80%, \( p= 0.004 \)) (Figure 2).

LR rates increased and OS rates decreased with the number of lymph nodes involved (\( p=0.001 \)). Mucinous histology and poor differentiation were both associated with poor prognosis. Although lymphangio invasion was not associated with LR, it did predict poor OS (40% versus 75%, \( p= 0.04 \)). All the analyses were repeated for the different treatment regimens, but this did not reveal different results. However, the groups are too small for fir conclusions per regimen.
<table>
<thead>
<tr>
<th>TUMOR TYPE</th>
<th>FACTOR</th>
<th>CATEGORY</th>
<th>RÖDEL 0</th>
<th>RÖDEL 1</th>
<th>RÖDEL 2</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>Therapy</td>
<td>Radiotherapy</td>
<td>53 (72%)</td>
<td>15 (20%)</td>
<td>6 (8%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radiochemo</td>
<td>48 (38%)</td>
<td>64 (50%)</td>
<td>15 (12%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clinical T stage</td>
<td>cT3</td>
<td>38 (46%)</td>
<td>32 (38%)</td>
<td>13 (16%)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cT4</td>
<td>63 (53%)</td>
<td>47 (40%)</td>
<td>8 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Differentiation grade</td>
<td>Good/Moderate</td>
<td>56 (58%)</td>
<td>31 (32%)</td>
<td>10 (10%)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor/Undifferentiated</td>
<td>20 (36%)</td>
<td>29 (52%)</td>
<td>7 (12%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ypT stage</td>
<td>ypT1, T2</td>
<td>5 (29%)</td>
<td>12 (71%)</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ypT3, T4</td>
<td>96 (59%)</td>
<td>66 (41%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ypN stage</td>
<td>ypNo</td>
<td>65 (50%)</td>
<td>49 (37%)</td>
<td>17 (13%)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ypN1</td>
<td>24 (51%)</td>
<td>19 (40%)</td>
<td>4 (9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ypTNM stage</td>
<td>ypN2</td>
<td>12 (52%)</td>
<td>11 (48%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>CRM</td>
<td>Negative</td>
<td>65 (50%)</td>
<td>49 (37%)</td>
<td>17 (13%)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>24 (51%)</td>
<td>19 (40%)</td>
<td>4 (9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type</td>
<td>Adenocarcinoma</td>
<td>75 (55%)</td>
<td>62 (45%)</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucinous</td>
<td>19 (33%)</td>
<td>17 (47%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Differentiation grade</td>
<td>Good/Moderate</td>
<td>62 (56%)</td>
<td>49 (44%)</td>
<td></td>
<td>0.40</td>
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<tr>
<td></td>
<td></td>
<td>Poor/Undifferentiated</td>
<td>39 (59%)</td>
<td>27 (41%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Growth pattern</td>
<td>Circumscribed</td>
<td>17 (58%)</td>
<td>8 (32%)</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td>83 (56%)</td>
<td>64 (44%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphoid reaction</td>
<td>None/Few</td>
<td>56 (55%)</td>
<td>45 (45%)</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate</td>
<td>32 (57%)</td>
<td>24 (43%)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Extensive</td>
<td>12 (80%)</td>
<td>3 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophilic infiltrate</td>
<td>None</td>
<td>71 (55%)</td>
<td>57 (44%)</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate/Extensive</td>
<td>29 (64%)</td>
<td>16 (36%)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Lymphangio invasion</td>
<td>No</td>
<td>94 (58%)</td>
<td>69 (42%)</td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>6 (54%)</td>
<td>5 (45%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calcification</td>
<td>No</td>
<td>76 (60%)</td>
<td>50 (40%)</td>
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<tr>
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<td></td>
<td>Yes</td>
<td>25 (54%)</td>
<td>24 (46%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor necrosis</td>
<td>No</td>
<td>24 (32%)</td>
<td>52 (68%)</td>
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<td>&lt;0.001</td>
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<td></td>
<td>Yes</td>
<td>76 (78%)</td>
<td>22 (22%)</td>
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</tr>
</tbody>
</table>

*: after a complete response (Rödel 2), post-treatment factors could not be assessed. Bold values indicate statistical significance. Abbreviations: ypT: pathological T stage, ypN: pathological N stage, ypTNM: pathological tumor stage, CRM: circumferential margin.
**TABLE 2. UNIVARIATE ANALYSIS OF POST-TREATMENT PATHOLOGICAL PARAMETERS IN RELATION TO LOCAL RECURRENCE AND OVERALL SURVIVAL**

<table>
<thead>
<tr>
<th>FACTOR TYPE</th>
<th>FACTOR</th>
<th>CATEGORY</th>
<th>n(%)</th>
<th>% LR at 24 months</th>
<th>P-VALUE</th>
<th>% alive at 24 months</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinico-pathologic</td>
<td>ypT stage</td>
<td>ypT0-ypT2</td>
<td>38 (19%)</td>
<td>3%</td>
<td>0.061</td>
<td>89%</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ypT3, ypT4</td>
<td>162 (81%)</td>
<td>19%</td>
<td>72%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ypN stage</td>
<td>ypTN0</td>
<td>131 (65%)</td>
<td>9%</td>
<td>0.001</td>
<td>81%</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ypTN1</td>
<td>47 (23%)</td>
<td>25%</td>
<td>69%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ypTN2</td>
<td>23 (12%)</td>
<td>46%</td>
<td>40%</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ypTNM</td>
<td>No residual tumor</td>
<td>14 (7%)</td>
<td>0%</td>
<td>0.022</td>
<td>100%</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stage I</td>
<td>10 (9%)</td>
<td>0%</td>
<td>81%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>Stage II</td>
<td>97 (45%)</td>
<td>13%</td>
<td>82%</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>Stage III</td>
<td>70 (35%)</td>
<td>29%</td>
<td>59%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CRM</td>
<td>Negative</td>
<td>158 (79%)</td>
<td>13%</td>
<td>0.001</td>
<td>80%</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>43 (21%)</td>
<td>43%</td>
<td>58%</td>
<td></td>
<td></td>
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<tr>
<td>Histologic</td>
<td>Type</td>
<td>Adenocarcinoma</td>
<td>137 (79%)</td>
<td>13%</td>
<td>0.04</td>
<td>75%</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
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<td>Mucinous</td>
<td>37 (21%)</td>
<td>32%</td>
<td>60%</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Differentiation grade</td>
<td>Good/ Moderate</td>
<td>111 (63%)</td>
<td>12%</td>
<td>0.04</td>
<td>80%</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor</td>
<td>66 (37%)</td>
<td>26%</td>
<td>61%</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Growth pattern</td>
<td>Circumscript</td>
<td>25 (14%)</td>
<td>17%</td>
<td>0.90</td>
<td>59%</td>
<td>0.35</td>
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<tr>
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<td></td>
<td>Diffuse</td>
<td>147 (86%)</td>
<td>18%</td>
<td>76%</td>
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<tr>
<td></td>
<td>Lymphoid reaction</td>
<td>None/ few</td>
<td>101 (58%)</td>
<td>22%</td>
<td>0.23</td>
<td>67%</td>
<td>0.19</td>
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<tr>
<td></td>
<td></td>
<td>Moderate</td>
<td>56 (32%)</td>
<td>15%</td>
<td>75%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extensive</td>
<td>16 (9%)</td>
<td>8%</td>
<td>94%</td>
<td></td>
<td></td>
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<tr>
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<td>Eosinophilic infiltrate</td>
<td>None</td>
<td>129 (74%)</td>
<td>19%</td>
<td>0.85</td>
<td>73%</td>
<td>0.33</td>
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<tr>
<td></td>
<td></td>
<td>Moderate/ extensive</td>
<td>45 (26%)</td>
<td>17%</td>
<td>77%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphangio invasion</td>
<td>None</td>
<td>163 (94%)</td>
<td>16%</td>
<td>0.51</td>
<td>75%</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>11 (6%)</td>
<td>30%</td>
<td>40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calcification</td>
<td>No</td>
<td>126 (71%)</td>
<td>19%</td>
<td>0.58</td>
<td>75%</td>
<td>0.56</td>
</tr>
<tr>
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<td></td>
<td>Yes</td>
<td>51 (29%)</td>
<td>13%</td>
<td>67%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor necrosis</td>
<td>No</td>
<td>76 (43%)</td>
<td>6%</td>
<td>0.02</td>
<td>68%</td>
<td>0.76</td>
</tr>
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<td></td>
<td></td>
<td>Yes</td>
<td>99 (57%)</td>
<td>22%</td>
<td>75%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patients with missing data were excluded from local recurrence and overall survival analysis. Bold values indicate statistical significance. Abbreviations: LR: local recurrence, ypT: pathological T stage, ypN: pathological N stage, ypTNM: pathological tumor stage.
Multivariate analysis of clinicopathological factors

Since histological factors could not be determined in patients with a complete response, multivariate analysis for LR and OS was performed for clinicopathological factors (category ypT stage, ypN and CRM) and the Rödel system only. A hazard ratio (HR) of 1 was attributed to the most favorable category. In case of LR, the CRM was the only factor significantly associated with this event (HR=4.44, confidence interval (CI): 1.83-10.81, p=0.001). With respect to OS, both N status and CRM were selected by the Cox regression model using conditional backward elimination: ypN1: HR=1.75, CI: 1.01-3.05, p=0.046, ypN2: HR=2.60, CI: 1.28-5.27, p=0.008 and CRM: HR=1.68, CI: 0.99-2.85, p=0.054.

Correlations between post-treatment factors and regression

Histological post-treatment factors could only be correlated to Rödel 0 and 1 since no tumor cells were left after complete tumor regression (Rödel 2). However, after complete tumor regression at the site of the primary tumor, positive lymph nodes, were still found in 4 (19%) out of 21 complete responders (Table 1). In two cases no lymph nodes were found. More extensive histological tumor regression was present in ypT1 and ypT2 tumors, as could be expected. As a consequence, an involved CRM was observed more than twice as often in patients with limited regression (Rödel 0) compared to patients with Rödel 1. (70% vs 30%, p=0.03). No significant correlations were found between regression and histological post-treatment factors. Tumor necrosis, which might be considered as an alternative parameter for regression, was inversely related to the degree of regression. In 78% of the tumors with minimal (less than 25% of the tumor mass) or no regressive changes, necrotic areas were observed. After more extensive regression these areas were identified in only 22 (22%) of tumor specimens.
Regression grading and prognosis

Surprisingly, none of the regression systems analyzed was found to be significantly associated with LR. In addition, no correlation with OS was found (Table 3). We repeated the analysis correcting for CRM status, since this factor was found the most potent predictor of prognosis. In the CRM negative cases (n = 158), again grading of tumor regression lacked prognostic implications for LR and OS.

| Table 3. Univariate Analysis of Regression Systems in Relation to Local Recurrence and Overall Survival |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| System   | Total Population (n=201) | CRM Negative Patients Only (n=158) | System   | Total Population (n=201) | CRM Negative Patients Only (n=158) | System   | Total Population (n=201) | CRM Negative Patients Only (n=158) |
| Dworak    |                                |                                |                                |                                |                                |                                |                                |                                |
| Grade 0   | 18 (9%)                        | 30%                            | 0.21                            | 0.13                            | 13 (8%)                      | 10%                            | 0.61                            | 0.31                            |
| Grade 1   | 83 (41%)                       | 18%                            | 75%                            | 59 (38%)                       | 7%                           | 85%                            |
| Grade 2   | 57 (28%)                       | 19%                            | 70%                            | 48 (30%)                       | 12%                          | 66%                            |
| Grade 3   | 22 (11%)                       | 0%                             | 75%                            | 17 (11%)                       | 0%                           | 78%                            |
| Grade 4   | 21 (11%)                       | 5%                             | 94%                            | 21 (13%)                       | 5%                           | 94%                            |
| Scott     |                                |                                |                                |                                |                                |                                |                                |                                |
| Minimal   | 48 (42%)                       | 19%                            | 0.26                            | 0.29                            | 59 (37%)                      | 0.17                           | 0.79                            | 0.15                            |
| Moderate  | 35 (18%)                       | 17%                            | 73%                            | 27 (17%)                       | 13%                          | 74%                            |
| Good      | 61 (30%)                       | 9%                             | 76%                            | 51 (33%)                       | 0%                           | 77%                            |
| Maximal   | 21 (10%)                       | 5%                             | 94%                            | 21 (13%)                       | 5%                           | 94%                            |
| Bouzourene|                                |                                |                                |                                |                                |                                |                                |                                |
| TRG4      | 17 (9%)                        | 31%                            | 0.19                            | 0.29                            | 12 (8%)                      | 11%                            | 0.53                            | 0.66                            |
| TRG3      | 58 (29%)                       | 19%                            | 77%                            | 47 (30%)                       | 13%                          | 76%                            |
| TRG2      | 23 (11%)                       | 0%                             | 66%                            | 20 (13%)                       | 0%                           | 69%                            |
| TRG1      | 21 (10%)                       | 5%                             | 94%                            | 21 (13%)                       | 5%                           | 69%                            |
| Rödel     |                                |                                |                                |                                |                                |                                |                                |                                |
| Minimal   | 101 (50%)                      | 20%                            | 0.44                            | 0.15                            | 71 (45%)                      | 8%                             | 0.95                            | 0.15                            |
| Moderate  | 79 (39%)                       | 14%                            | 64%                            | 66 (42%)                       | 9%                           |                                |
| Maximal   | 20 (11%)                       | 5%                             | 94%                            | 21 (13%)                       | 5%                           | 94%                            |

Abbreviations: TRG tumor regression grade, LR: local recurrence

Reproducibility of regression grading

In order to test reproducibility of our results and to determine the inter-observer variability, tumor regression was also assessed by a second pathologist in all cases (n=201). Analysis with the data obtained by the second pathologist confirmed our initial finding (data not shown). In order to express inter-observer variability, measurements of agreement were indicated in kappa values for each system (supplemental data). Kappa values for the regression system as a whole and for two successive categories
Chapter 3

Our study demonstrates the great importance of optimal surgery for patients with LARC who are treated with RCT. Although it has been suggested that neoadjuvant therapy could compensate for poor surgery, we demonstrate that radical excision (free circumferential margins) is essential for local control (Figure 2). The prognostic value of standard clinicopathological factors such as CRM, lymph node status and tumor stage is superior to grading therapy-induced tumor regression in patients with LARC (Table 1). This finding was also confirmed in a multivariate model. CRM involvement is a very important risk factor for local recurrence that is vastly influenced by treatment factors (neoadjuvant therapy and surgery). Evaluation of the CRM could therefore be considered as an early alternative endpoint for future randomized trials comparing different treatment regimens for patients with LARC \(^{17}\). The most advantageous treatment strategy for this specific subset of patients requires a multidisciplinary approach. This not only implies high quality surgery and neoadjuvant therapy but also optimal imaging in order to identify patients who will benefit from this strategy and accurate pathological assessment of CRM involvement \(^{11,12}\) for evaluating successfulness of the strategy.

The degree of tumor regression was found to be correlated with the neoadjuvant treatment regimen used. Tumor regression was found to be more extensive after RCT compared to long-term RT which is in accordance with literature \(^{18,19}\). We were not able to show any prognostic impact of regression scoring, irrespective of stratification for CRM involvement. However, tumor regression is important because the chance to obtain a negative CRM is increased after extensive tumor regression (Table 1).

The percentage of patients with a positive margin (21%) was relatively low taking into account that the inclusion criterion was a threatened CRM. This finding is in agreement with reports by Mawdsley et al and Glynne-Jones and co workers who found that 20% of the patients with LARC had a positive CRM after RCT \(^{17,20}\). LARC was defined by these authors as borderline resectable or irresectable disease, patients underwent curative surgery after neoadjuvant chemoradiation. Univariate analyses performed in the present study confirmed the importance of the CRM for both OS (p=0.004) and LR (p<0.001). Multivariate analysis of clinicopathological factors confirmed the importance
of CRM involvement for the prediction of local recurrence, (HR=4.44, CI: 1.83-10.81, p=0.001). Several other studies report similar results regarding the importance of the CRM as a predictor for outcome after neoadjuvant treatment. 17,21-24

Numerous studies investigated the prognostic value of tumor response after neoadjuvant therapy in rectal cancer, without consistent results. Similar to our study, no correlation was found in three different studies 19,25,26 with a total number of 385 patients. On the other hand, tumor regression was associated with LR (389 patients), 8,27-29, OS (247 patients), 8,30 or DFS (270 patients). 8,9,28,30

However, reproducibility of tumor regression assessment leaves room for improvement (supplemental data). Measurements on the variance between two successive categories within each system showed that kappa values improve as the amount of residual tumor decreases. Distinguishing absence (Figure 1A) from little regressive signs (Figure 1B) was reproduced poorly, probably because formation of fibrosis is also an intrinsic characteristic of tumor development. Discriminating intrinsic tumor fibrosis from therapy-induced fibrosis based on morphology is difficult. A complete tumor response, on the other hand, which is the only clearly definable degree of tumor regression largely depends on tissue processing and sampling which are often responsible for discrepancies in literature regarding the rate of complete responders 9,30. A possible way to standardize the criteria for a complete response could be as follows; sample 5 sites of the tumor area, if no tumor is present in these blocks the whole area suggestive for disease should be embedded in paraffin blocks. If still no tumor is present, H&E slides will be obtained from each block at three levels. If no tumor was found after this procedure, a complete response was established 31. The lack of clear definitions with respect to the morphological aspects of therapy-induced fibrosis and a complete tumor response explains both inter-observer and inter-study variance.

The term locally advanced is also not clearly defined. Definitions range from patients who received long-term neoadjuvant RT or RCT, to patients with positive lymph nodes, advanced cT3 or cT4 or patients with a threatened CRM. Three out of the nine studies investigating the prognostic value of grading tumor regression stated to have analyzed patients with LARC 8,25,29. The percentages of stage cT4 in these three reports range from 12% 25 to 32% 29 and are relatively low compared to the percentage of cT4 in the present population (59%). Moreover, none of the reports on LARC selected patients based on a threatened CRM (a predicted CRM on MRI of less than 2 mm). These unique pre-treatment characteristics distinguish the present population from other reports concerning patients with LARC. These differences in patient selection can also explain the inconsistency, regarding the prognostic implications of tumor regression, between the findings described in the present study and those described by others.
Our data indicate that tumor response to neoadjuvant long-term RT or RCT results in tumor shrinkage (arrow, Figure 3) rather than fragmentation of the tumor (dotted arrow, Figure 3). The scenario of tumor fragmentation implicates that the degree of tumor regression is not informative for depth of infiltration; e.g. vital tumor cells may still be scattered throughout the whole fibrotic area and reach the pre-treatment level of tumor invasion. Tumor fragmentation after neoadjuvant treatment still results in CRM involvement. However, our data suggest that tumor shrinkage is the main event after neoadjuvant therapy, resulting in negative CRM, which was obtained in almost 80% of patients with a clinically threatened margin. Moreover, our data revealed that patients with a negative CRM experienced significantly less local recurrence compared to patients with a positive CRM. This indicates that the fibrotic area that is depicted in grey in Figure 3B is sterile, pleading for the scenario of tumor shrinkage. However, if the CRM is involved, patient prognosis remains poor despite elaborate histological regression after neoadjuvant treatment (Figure 3C).

In case of OS, lymph node status revealed to have strong prognostic implications. Since treatment of patients with LARC consists of intensified local treatment aimed on the primary tumor, loco-regional tumor spread resulting in positive lymph nodes (and as a consequence decreased OS) is essentially not affected by this treatment. This was illustrated by our finding that positive lymph nodes can still be found after complete regression of the primary tumor mass (Table 1) which can explain why these patients can still develop metastasis.

In this study, which investigates a unique population of patients with LARC which had a threatened CRM and a high percentage of cT4, we have shown that assessment of the CRM is the most important pathological factor after RCT. Extensive tumor regression, resulting in tumor shrinkage, is essential for obtaining a free CRM, but incomplete resection implies a poor prognosis irrespective of the degree of these regressive changes.
Value of CRM in LARC

Figure 3: Schematic representation of the relation between the degrees of tumor regression and circumferential margin (CRM) involvement. Areas with vital tumor cells are depicted in black and fibrotic areas are depicted in grey. 

A: Pre-treatment situation: the tumor is locally advanced (ct4) and the CRM is threatened. The contours of this pre-treatment stage are also depicted in panel B, C and D.

After neoadjuvant long course radiotherapy (RT) or radiochemotherapy (RCT) two different scenarios concerning tumor regression are sketched (B, D). B illustrates the “tumor shrinkage” scenario in which the infiltration depth is less extensive than in the pre-treatment situation. D illustrates the scenario of “tumor fragmentation” which implies scattered tumor cells throughout the whole fibrotic area, reaching the initial infiltration depth. However, if the CRM is still positive after tumor shrinkage (panel C) patients’ outcome will still be poor irrespective of the degree of tumor regression.
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REFERENCE LIST


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Value of CRM in LARC
CHAPTER 4

LOSS OF MEMBRANOUS EP-CAM IN BUDDING COLORECTAL CARCINOMA CELLS

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Background
Tumor budding is a histological feature that reflects loss of adhesion of tumor cells and is associated with locoregional metastasis of colorectal carcinoma. Although nuclear localization of β-catenin is associated with tumor budding, the molecular mechanism remains largely elusive. In this study, we hypothesize that the epithelial cell adhesion molecule (Ep-CAM) is involved in tumor budding.

Patients and methods
In order to address this question we performed immunohistochemistry on Ep-CAM using 3 different antibodies (monoclonal antibodies Ber-ep4 and 311-1K1 and a polyclonal antibody) and a double staining on β-catenin and Ep-CAM. In addition, Ep-CAM mRNA was monitored with mRNA in situ hybridization. Subsequently, we determined the effect of Ep-CAM staining patterns on tumor spread in rectal cancer.

Results
In contrast to the tumor mass, budding cells of colorectal carcinoma displayed lack of membranous but highly increased cytoplasmic Ep-CAM staining and nuclear translocation of β-catenin. mRNA in situ hybridization suggested no differences in Ep-CAM expression between the invasive front and the tumor mass. Importantly, reduced Ep-CAM staining at the invasive margin of rectal tumor specimens (n=133) correlated significantly with tumor budding, tumor grade and an increased risk of local recurrence (p=0.001, p=0.04 and p=0.03 respectively).

Conclusion
These data demonstrate abnormal processing of Ep-CAM at the invasive margin of colorectal carcinomas. Our observations indicate that loss of membranous Ep-CAM is associated with nuclear β-catenin localization and suggest that this contributes to reduced cell-cell adhesions, increased migratory potential and tumor budding.
INTRODUCTION

Budding of dedifferentiated tumor cells at the invasive margin is well described in colorectal cancer. Tumor budding or sprouting is a histologic feature of loss of adhesion and is associated with locoregional tumor spread. These budding tumor cells have been described to exhibit nuclear β-catenin. In the nucleus, β-catenin can activate the transcription of genes associated with cell proliferation and differentiation. When β-catenin is bound to the cytoplasmic domain of E-cadherin, it enables E-cadherin to function as a cell-cell adhesion molecule and mediates the interplay of adherence junction molecules with the actin cytoskeleton. Aberrations of E-cadherin expression can lead to abundant cytoplasmic β-catenin and subsequent nuclear localization. Based on these well described interactions between β-catenin and E-cadherin, it is plausible that modulation of E-cadherin-mediated adhesion is involved in tumor budding. However, Masaki and co-workers did not find a correlation between nuclear β-catenin and changes of E-cadherin characteristics in budding colorectal tumor cells.

In addition to E-cadherin, the Epithelial Cell Adhesion Molecule (Ep-CAM, also known as Ber-EP4, CO17-1A, GA733 and EGP2) can also mediate cell-cell adhesion of epithelial cells. The adhesions between epithelial cells are facilitated by the extracellular domain of the protein. The smaller intracellular domain is known to interact with the actin-skeleton via α–actinin. Ep-CAM does not share significant homology with any of the four major families of adhesion molecules (cadherins, selectins, integrins and the immunoglobulins). Increased Ep-CAM expression is associated with enhanced proliferation and a lower differentiation grade of epithelial cells under non-pathological conditions. In the proliferative phase, Ep-CAM expression is associated with epithelial tissue remodeling. After cell proliferation, Ep-CAM expression declines and cellular differentiation initiates. An interesting example illustrating the correlations between Ep-CAM expression and cell proliferation in normal tissue is the hair follicle where Ep-CAM is only expressed by cells in the highly proliferative zone. Furthermore, differential Ep-CAM expression is observed during embryonic development of different tissues such as pancreas and lung. In these perspectives Ep-CAM can thus be regarded as a morphoregulatory protein.

Among neoplastic tissues, Ep-CAM is abundantly expressed in tumors of epithelial origin, e.g. lung, breast, prostate, renal cell and colorectal carcinoma. Ep-CAM is present in high amounts in normal colorectal tissue but its expression increases even further in polyps and carcinomas. In tumor cells, increased Ep-CAM expression promotes cell-cell adhesion. Ectopic expression the murine Ep-CAM ortologue (mEGP) by murine colorectal carcinoma cells increased cell-cell adhesion, attenuated tumor cell...
invasion in Matrigel, and decreased tumor incidence and metastasis when inoculated into the spleen of mice. Thus these data suggest that Ep-CAM expression antagonizes tumor growth and metastasis. Because tumor budding is well described in colorectal cancer and is associated with poor prognosis, we hypothesized that altered Ep-CAM characteristics in rectal cancer may correlate with tumor budding and poor disease outcome. To this end, we have performed a detailed analysis of intra-tumoral Ep-CAM staining patterns using three different antibodies, and correlated these patterns with local and distant recurrence rates in patients with rectal cancer. By using two monoclonal antibodies with different epitopes on the Ep-CAM ectodomain we could closely monitor changes in epitope availability of this domain. The combination of these two monoclonal antibodies with a polyclonal antibody, which recognizes the whole Ep-CAM protein (Figure 1), enabled us to study sub-cellular distributions of Ep-CAM and possible modifications of the protein such as cleavage or aberrant localization.

**Materials and Methods**

**Immunohistochemistry**

Immunohistochemical staining of Ep-CAM was performed on paraffin-embedded tissue sections using monoclonal antibodies Ber-EP4 (1:100, Dako, Glostrup, Denmark) and 311-1k1 (1:50, Pickcell laboratories, Leiden, the Netherlands, 1:50). The localization...
Loss of membranous Ep-CAM in budding colorectal carcinoma cells

of their epitopes is depicted in Figure 1. In case of β-catenin, immunohistochemical staining was performed with the Clone 14 monoclonal antibody (1:6000, Transduction Laboratories, Lexington, USA). Staining was performed using the avidin-biotin peroxidase complex in case of visualization with 3, 3′-diaminobenzidine hydrochloride solution (DAB) and the avidin-biotin alkaline phosphatase complex for staining with fast blue. Paraffin sections were de-waxed and re-hydrated. All reactions were performed at room temperature, unless stated otherwise. Endogenous peroxidase activity was blocked by incubation in phosphate-buffered saline (PBS) containing 3% H$_2$O$_2$ for 30 min. After rinsing with PBS, antigen retrieval for Ber-EP4 staining consisted of incubation with 1% pronase at 37 °C for 10 min. Retrieval for 311-1K1 and β-catenin staining involved microwave boiling in a 10 mM sodium citrate buffer (pH 6.0) for 10 min. After boiling, the slides were allowed to cool down for at least 30 min. After rinsing with PBS, slides were pre-treated with 20% normal horse serum for 10 min. to reduce non-specific staining. All sera and antibodies were dissolved in PBS with 1% BSA. Subsequently, slides were incubated in a humidity chamber with the primary antibody at 4 °C for 16-20 h. This long incubation time resulted in saturation of the reaction and therefore optimal staining. Staining of the DAB substrate was intensified with a 0.5% copper sulfate solution, in case of double staining this step was omitted. Slides were counterstained with Hematoxylin solution or Nuclear Fast Red for 1 min, dehydrated and enclosed with Permount (Fisher Chemicals, New Jersey, USA).

A complete loss of Ber-EP4 staining was never observed and no distinction was made between the various degrees of decrease which were very subtle. Therefore, staining patterns of the Ber-EP4 antibody were categorized as follows: (i) no decrease of staining, (ii) decrease of staining at the front, or (iii) decreased staining throughout the tumor.

Immunofluorescence

Immunofluorescence double stainings were performed with Ber-EP4 and a chicken polyclonal anti-Ep-CAM antibody (kindly provided by Dr. S. Litvinov) and with Ber-EP4 and a monoclonal anti cytokeratin antibody, Cam 5.2 (1:40, Becton Dickinson, Franklin Lakes, USA). Briefly after dewaxing and re-hydration, slides were rinsed with PBS and incubated in this solution at 4 °C for at least 16 h to reduce auto-fluorescence. In case of Ber-EP4 and CAM 5.2 double staining antigen retrieval involved incubation with 1% pronase at 37 °C for 10 min. Antigen retrieval for the Ber-EP4 and Ep-CAM polyclonal antibody double staining consisted of microwave boiling in a 10 mM sodium citrate buffer (pH 6.0) for 10 min. After cooling down and rinsing with PBS, Slides were incubated with 20% normal goat serum for 10 min. Slides were incubated with the primary antibody Ber-EP4 at 4 °C overnight. After rinsing, slides were incubated with goat anti-mouse IgG-conjugated Alexa 488/594 (1:200, Invitrogen Molecular Probes, Eugene, USA) for 30 min. After rinsing, slides were incubated with 20% goat serum or 100% normal
goat serum to reduce non-specific binding of the polyclonal antibody. Subsequently, slides were incubated with the second primary antibody of choice at 4 °C overnight. The polyclonal antibody was detected with goat anti chicken IgG-conjugated Alexa 594 (1:200, Invitrogen Molecular Probes) for 30 min. CAM 5.2 binding was visualized with goat anti-mouse IgG2a-conjugated Alexa 488 (1:200, Invitrogen Molecular Probes) for 30 min. After washing in PBS, slides were stained with 4′-6-diamidino-2-phenylindole (DAPI) for 30 sec. Sections were enclosed in fluorteck (Euro-Diagnostica, Arnhem, the Netherlands). For analysis a fluorescent microscopy (Leica, Solms, Germany) was used. Images were captured, using a 5X, 10X, 20X or 40X objective and were prepared with Adobe Photoshop version 7.0.

**Ep-CAM mRNA in situ hybridization**

A 420 bp human cDNA fragment was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) according to standard procedures. Briefly, total RNA isolated from the CaCo2 colon carcinoma cell line was isolated with Trizol (Gibco BRL) according to the manufacturer’s protocol and 0.5 µg was reverse-transcribed at 42° C for 1 hour using SuperScript II (Promega) and oligo dT16-18 primer. Ep-CAM cDNA was amplified using specific primers (5′-CTGGCCGTAAAAGCTTTGT and 5′-CCTTCTCTAGTGTTGCGCAAT) and were based on the reported sequence of human Ep-CAM (gi: 10439469). Amplification consisted of pre-incubation at 95°C for 5 minutes before adding Taq polymerase and then 30 cycles at 95°C for 1 minute, 55°C for 30 seconds and 72°C for 30 seconds. A PCR product of the predicted size was cloned into the pGEM-T easy vector (Promega, Madison, USA) and sequence was verified. The plasmid containing the partial cDNA of Ep-CAM was linearized by digestion with PstI or SphI and antisense and sense riboprobes were synthesized using T7 and SP6 RNA polymerase, respectively, and dioxygenin-labelled rUTP (Roche, Basel, Switzerland) according to standard procedures. mRNA in situ hybridization of paraffin-embedded tissue sections was performed as previously described 16.

**Patient selection**

In order to study the effect of Ep-CAM distribution in colorectal tumors on local and distant recurrence, we used data obtained from the radiotherapy (RT) + Total Mesorectal Excision (TME) trial. The TME trial was initiated in The Netherlands and included 1530 patients from January 1996 until December 1999 17,18. This prospectively randomized trial evaluated TME surgery with or without preoperative radiotherapy (5x5 Gy). To be eligible, patients had to have histologically confirmed rectal adenocarcinoma, without evidence of distant metastasis. Patients with previous or coexisting cancer and those who had previously undergone large-bowel surgery, chemotherapy or radiotherapy were excluded. The TME trial was approved by the medical ethics committees of all participating hospitals and after informed consent had been obtained, selected patients
were randomized and assigned to either radiotherapy (5 Gy on each of five days) followed by TME or to TME alone. Radiotherapeutical, surgical and pathological procedures were standardized and subjected to strict quality control. Outcome measures included local and distant recurrences confirmed by radiographic imaging and/or histological diagnosis. Since local recurrence rates were very low after TME surgery, an artificial selection of 160 patients was made to study the role of biological markers in both local and distant recurrence. 40 stage II and 40 stage III patients without local recurrence or distant metastasis, 40 with distant metastasis and without local recurrence and 40 patients with local recurrence and without distant metastasis were selected. Former results obtained by studying this population indicated sufficient statistical power in this population. The selection implies that local- and distant recurrence percentages cannot be extrapolated towards the total population studied. Patients were selected from both randomization arms to exclude possible therapy related effects. From these 160 patients tumor samples were available for Ep-CAM immunohistochemistry of 133 patients. The median follow-up was 41.7 months.

Pathology procedures
Tumor staging was performed by the use of the tumor-node-metastasis (TNM) classification. Growth pattern assessment was performed according to Jass. A circumferential margin of 1 mm or less was considered positive. Histological differentiation grade was classified as undifferentiated, poorly differentiated, moderately and well. The grading decision was based on the least differentiated area. Tumor budding at the invasive margin was assessed as described by Ueno et al. In case of 117 tumors (88%) a representative HE staining of the tumor area assessed with Ber-EP4 could be obtained. After choosing a field where budding was most intensive, the number of budding foci was counted using a 25X microscope objective. A budding focus was defined as a single isolated tumor cell or a cluster of tumor cells composed of fewer than 5 cells. Subsequently, these data were categorized as follows: I: 0-4 budding foci, II: 5-9 budding foci, III: 10-19 budding foci and IV: more than 20 budding foci.

Statistical analysis
Ep-CAM staining patterns of patients included in the Dutch TME trial were correlated with local, distant and overall recurrence using Kaplan-Meier curves and log-rank testing. Associations between Ep-CAM patterns and histopathological parameters were analyzed by Chi-Square testing. P values of <0.05 (two tailed) were considered as statistically significant.
RESULTS

Loss of the Ber-EP4 epitope in budding tumor cells

Normal colon mucosa was intensely stained with Ber-EP4 and 311-1K1 monoclonal antibodies (Figure 2 A, B). In colorectal tumors, decreased staining was predominately observed in budding tumor cells at the invasive margin. Additionally, we found focally decreased staining with these antibodies within the tumor mass (Figure 2 C, D, E, and F). In order to identify potential Ber-EP4-negative tumor cells, we used an immunofluorescence (IF) double staining with Ber-EP4 and the tumor marker CAM 5.2 (cytokeratin 7 and 8). With this method, a pattern of decreased Ber-EP4 staining on isolated infiltrating tumor cells was observed (Figure 3 A, B, C). To demonstrate a true correlation between localization of β-catenin and the presence or absence of the Ber-EP4 epitope, we performed double stainings. Double staining of Ber-EP4 and β-catenin showed that tumor cells with decreased Ber-EP4 immunoreactivity frequently displayed nuclear translocation of β-catenin (Figure 4A, B).

These data suggested either an inhibition of Ep-CAM mRNA expression or abnormal processing of Ep-CAM at the invasive front. Ep-CAM mRNA in situ hybridization and Ber-EP4 immunohistochemistry on consecutive sections demonstrated that mRNA expression in cells displaying decreased Ber-EP4 staining was not reduced (Figure 5). Therefore, these results suggested that the focal loss of Ber-EP4 and 311-1K1 immunoreactivity was probably caused by abnormal processing of Ep-CAM.

Figure 2: Representative Ep-CAM staining patterns in normal mucosa of the colon and colorectal adenocarcinoma with monoclonal antibodies Ber-EP4 and 311-1K1. A, Normal mucosa stained with Ber-EP4 (original magnification: 50X). B, Normal mucosa stained with 311-1K1 (original magnification: 100X). C, Decreased staining with Ber-EP4 in sprouting tumor cells, arrow (original magnification: 200X). D, Decreased staining of tumor mass with the 311-1K1 antibody, arrow (original magnification: 50X). E and F, serial sections with clusters of tumor cells stained with Ber-EP4 (E) and 311-1K1 (F) (original magnification: 50X). Loss of immunoreactivity of isolated tumor cells and small clusters was observed with both 311-1K1 and with Ber-EP4 antibodies (arrowheads).
Loss of membranous Ep-CAM in budding colorectal carcinoma cells


→ color figures
Cytoplasmic patterns of Ep-CAM immunoreactivity in budding tumor cells

We subsequently addressed whether loss of membranous immunoreactivity could also be detected with a polyclonal anti-Ep-CAM antibody. Double staining with Ber-EP4 and the polyclonal antibody showed that a decreased Ber-EP4 staining was accompanied by cytoplasmic staining of Ep-CAM as visualized by the polyclonal antibody (Figure 6 D, F). This was predominantly observed in budding tumor cells or clusters. In normal mucosa, both antibodies showed a membranous staining pattern of Ep-CAM with equal staining intensity (Figure 6 A-C). These data demonstrated that loss of membranous Ep-CAM immunoreactivity coincided with increased cytoplasmic staining.
Clinical relevance of heterogeneous staining patterns

In order to analyze the prognostic value of decreased Ber-EP4 immunoreactivity in budding tumor cells, the relation between Ber-EP4 staining patterns and local, distant and overall recurrence was assessed by using data from the TME trial in which patients were randomized for TME surgery only or radiotherapy followed by TME surgery. No correlations were found between the surgery only and the irradiated group with respect to the immunohistochemical staining patterns. Furthermore, no correlations were found between Ep-CAM staining patterns and lymph node involvement, tumor depth, circumferential margin involvement and tumor stage (Table 1).
Differentiation grade and presence or absence of membranous Ep-CAM at the invasive margin was found to be significantly correlated (Table 1). Tumors with selective loss of membranous Ep-CAM at the invasive margin were scored as poorly differentiated or undifferentiated in 35% of the cases in contrast to 20% of tumors with no loss of membranous Ep-CAM ($p=0.04$). Differentiation grading alone failed to show significant correlations to local-, distant- or overall recurrence (data not shown), indicating that loss of membranous Ep-CAM expression in budding cells of rectal carcinoma is of higher prognostic value than tumor differentiation grade. In addition, a significant correlation was found between loss of membranous Ep-CAM and the extent of tumor budding (Table 1, $p=0.001$). Loss of membranous Ep-CAM was associated with a higher extent of tumor budding. This correlation between tumor budding and loss of membranous Ep-CAM is emphasized by the finding that tumor budding was also significantly correlated with tumor grade. A higher degree of budding was correlated with a lower degree of differentiation ($p=0.048$, $\chi^2$ testing).
Loss of membranous Ep-CAM in budding colorectal carcinoma cells

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<th>TABLE 1. EP-CAM PATTERNS IN RELATION TO CLINICOPATHOLOGICAL FACTORS</th>
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Abbreviations, RT: radiotherapy, *: P-value calculated with Mann Whitney test. **Bold** values indicate statistical significance.

Tumors (n=133) were stained and scored as (i) no decrease of Ep-CAM staining (70/133, 53%), (ii) decrease of staining at the tumor front (39/133, 29%), or (iii) decrease of staining throughout the whole tumor (24/133, 18%). Tumors with a decreased Ber-EP4 staining throughout the whole tumor were found to be a distinct group. This subpopulation of tumors behaved differently with respect to distant recurrence from tumors with decreased Ber-EP4 staining restricted to the tumor front. A loss of membranous Ep-CAM throughout the tumor was found to be correlated with an elevated risk of local recurrence alone compared to patients with no decrease of membranous Ep-CAM (p=0.05). Moreover, in our opinion, this sub-population is not suitable for studying the process of tumor budding at the invasive margin. Because this sub-population has prognostic implications which are distinct from tumors with decreased Ber-EP4 staining exclusively observed at the front it could interfere with the prognostic implications of the latter group. Because of this reason the effect of the Ep-CAM characteristics at the tumor front was conducted in 109 (39+70) cases. Decreased staining intensity of Ber-EP4 at the invasive front was always accompanied by a focally infiltrating growth pattern. Loss of membranous staining at the infiltrative margin correlated with a significantly higher risk of local recurrence (p=0.03, Figure 7) compared to tumors with homogenous Ep-CAM patterns. A decrease of Ep-CAM staining at the front indicated...
an elevated risk on distant recurrence, but this finding was not statistically significant (p=0.06). For overall recurrence, loss of membranous Ep-CAM strongly correlated with a significant higher risk of this event (p=0.01).

**Figure 7:** Kaplan-Meier curves presenting the correlation between different Ep-CAM staining patterns and local recurrence (A), distant recurrence (B) and overall recurrence (C), (N=109). Black line: selective decrease of Ber-EP4 staining at the invasive margin, grey line: no decrease of Ber-EP4 immunoreactivity at the tumor front.

**DISCUSSION**

In this study, we demonstrate a focal loss of membranous Ep-CAM immunoreactivity at the invasive margin predominantly in sprouting tumor cells, which was frequently accompanied by nuclear β-catenin translocation. These findings strongly suggest that the morphoregulator Ep-CAM is involved in budding of rectal carcinoma.

Analysis of Ep-CAM mRNA expression in rectal carcinoma did not reveal strong
loss of membranous Ep-CAM in budding colorectal carcinoma cells

differences in levels of transcription indicating that Ep-CAM at the invasive margin
evidences abnormal post-translational processing. It has to be emphasized that budding
cells are not deprived from Ep-CAM. Post-translational modifications did not affect
Ep-CAM expression, i.e. the polyclonal antibody showed that it was still present, but
altered its cellular localization. Therefore, infiltrating cells possibly retain the features
that contribute to high levels of Ep-CAM, e.g. enhanced proliferation and loss of
differentiation. Additionally, lack of extracellular Ep-CAM can enhance the migratory
capacity by attenuating cell-cell adhesion.

The aberrant localization of Ep-CAM could contribute to tumor budding. During
embryonic development of the pancreas, the highest levels of Ep-CAM expression are
found in islet-like cell clusters budding from the ductal tree, suggesting that increased
expression contributes to budding under non-pathological conditions. In contrast,
we found post-translational modification of Ep-CAM in budding tumor cells of rectal
cancers in which Ep-CAM was already abundantly expressed. Assessment of the extent
of tumor budding showed that loss of membranous Ep-CAM was significantly correlated
to a higher extent of tumor budding. Furthermore, tumors with loss of membranous
Ep-CAM at the invasive margin are more often graded as poorly or undifferentiated.
Also, the clinical implications of tumor budding, i.e. increased locoregional spread are
reflected by the loss of membranous Ep-CAM staining since it correlated with increased
local recurrence following surgical removal of the tumor. Tumor cells at the invasive
front that exhibit loss of extracellular Ep-CAM have increased migratory potential and
can spread through the bowel wall more easily. This is also reflected by the percentage
of involved margins; 31% and 23% for tumors with and without loss of extracellular
Ep-CAM at the tumor front respectively (Table 1). Increased local tumor spread due to
loss of extracellular Ep-CAM could account for non-curative resections and therefore
increased local recurrence.

Loss of membranous Ep-CAM (Table 1) and tumor budding do not correlate with
TNM-criteria. Although we do not have a proven explanation, it might be that tumor
budding is a biological phenomenon that can occur at various moments during tumor
development and is described to occur at various T stages including T1 and T2
Thus, tumor budding reflects interaction on the level of the tumor microenvironment
and is independent of the T stage itself. A significant correlation between loss of
membranous Ep-CAM and lymph node (Table 1) and distant metastasis was also not
observed (Figure 7). Invasion due to loss of adhesion, although related to metastasis is
a distinct feature during tumor progression. In order to metastasize tumor cells require
more characteristics than increased invasive potential such as extracellular matrix
remodelling, induction of angiogenesis, and modulation of cell-cell and cell-matrix
adhesive properties.
Infiltrating, sprouting tumor cells are discriminative from the tumor mass with respect to modifications of the Ep-CAM protein and thus suggests a role for this protein in the formation of tumor budding. Therefore, cytoplasmic Ep-CAM localization could alter its morphoregulatory capacity as compared to its membranous localization, and that sustains tumor budding.

Loss of membranous Ep-CAM can modulate changes of the cytoskeleton and hence change cell morphology. The extracellular domain of Ep-CAM with its two EGF-like repeats in a cystine rich domain followed by a cystin poor domain is similar to the organization of the extracellular domains of the lin21/Notch family. These proteins are involved in inter-cellular signaling and cell cell-interactions that are important for differentiation and segregation. It has been demonstrated that the cytoplasmic domain of Ep-CAM interacts with the actin-cytoskeleton. Recently, it was demonstrated that high cytoplasmic expression of actinin-4, a modulator of the cytoskeleton, is predominantly observed in budding cells of colorectal carcinoma. Furthermore, high levels of actinin-4 are associated with increased cell motility. Although it remains elusive whether there is a causal relation between cytoplasmic Ep-CAM and actinin-4 expression, it is tempting to speculate that both proteins are closely involved in tumor budding.

It remains elusive how Ep-CAM retains its cytoplasmic localization in budding tumor cells. The observed decrease in immunoreactivity of two monoclonal antibodies binding to the extracellular epitopes and a cytoplasmic staining pattern with the polyclonal antibody suggests internalization of Ep-CAM remnants after proteolytic modifications. Internalization of Ep-CAM has been reported previously by Litvinov and co-workers. Our finding that loss of membranous Ep-CAM frequently concurs with nuclear β-catenin expression (Figure 4) makes a scenario of proteolytic Ep-CAM modifications plausible. The Lef/Tcf transcription factors, which are activated after β-catenin translocation, have been described to modulate the expression of different matrix metalloproteinases (MMP’s) such as MMP-1 and MMP-7. Since proteolysis of cell-cell adhesion molecules is a common feature in cancer, cleavage of Ep-CAM could be a downstream effect of nuclear translocation of β-catenin. This view is supported by a recent study by Hörkkö and co/workers who postulate that nuclear accumulation of β-catenin is a requirement for tumor budding but other factors, which are more related to sprouting, also play role.

Alternatively, loss of membranous Ep-CAM in budding cells could be explained by splice variants of Ep-CAM. However, Balzar et al conducted an extensive analysis of Ep-CAM mRNAs in a large number of carcinoma cell lines and did not reveal any variations in Ep-CAM mRNA splicing. Furthermore, alternative glycosylation of the Ep-CAM
Loss of membranous Ep-CAM in budding colorectal carcinoma cells

ectodomain can shield of Ber-EP4 and 311-1K1 epitopes. This in combination with impaired translocation to the membrane could also explain our findings. We have not addressed this possibility in our study.

Because high amounts of Ep-CAM are present on the membranes of many tumor types, it is an attractive target for immunotherapy. Since the 90’s, a number of trials investigated the therapeutic value of postoperative treatment with the 17-1A Ep-CAM antibody (Edrecolomab) \(^{37-39}\). This antibody also binds to EGF domain I \(^{40}\) and its epitope is in close proximity to Ber-EP4 (Figure 1). The results of these studies are conflicting, attributing positive, no or adverse effects of Edrecolomab therapy on survival and disease free survival of patients with colorectal cancer. The findings presented in this study may explain these conflicting results. A subpopulation of tumor cells which were located at the invasive margin is undetectable for the Edrecolomab antibody. These cells lacked large amounts of extracellular Ep-CAM on their surfaces, which not only conceals them from the antibody, but also increased their migration capacity due to attenuated Ep-CAM-mediated cell-cell adhesion.
REFERENCE LIST

Loss of membranous Ep-CAM in budding colorectal carcinoma cells


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Loss of membranous Ep-CAM in budding colorectal carcinoma cells
CHAPTER 5

SIGNET RING CELL DIFFERENTIATION IN MUCINOUS CARCINOMA

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Background
Approximately 10% of all colorectal carcinomas are mucinous carcinomas, characterized by extracellular mucin. Occasionally, mucin accumulates intracellular in these tumors, causing signet ring cell differentiation. We hypothesize that signet ring cells arise from a separate genetic pathway. In this study the molecular background of signet ring cell differentiation is investigated by analysing genetic changes, changes in the expression of adhesion molecules, and mucin content. Furthermore, its clinical relevance is addressed.

Patients and methods
Cell lines of colorectal tumors with non-mucinous (AC), mucinous (MC) and signet ring cell phenotype (MCSRC) were used for Multiplex Ligation-dependent Probe Amplification to detect deletions and amplifications in specific oncogenes and tumor suppressor genes. Furthermore, the expressions of E-cadherin, β-catenin, ITF (intestinal trefoil factor) and MUC2 in signet ring cells were studied by immunohistochemistry, immunofluorescence, and mRNA in situ hybridization. Results were validated using a large cohort of colorectal carcinomas from which clinicopathological data were available.

Results
Specific amplifications and deletions in cell lines of AC, MC and MCSRC were detected. Bcl-2 was amplified in MCSRC and MC cell lines, but not in AC cell lines. Bcl-2 FISH analysis confirmed this in patient material.
Signet ring cells have a decreased expression of adhesion molecules (E-cadherin, β-catenin) and are strongly positive for ITF and MUC2, two peptides which are normally only produced by goblet cells. RNA in situ hybridization confirmed the production of ITF. Carcinomas with signet ring cell differentiation present at a higher T stage (16% versus 3.5%, p<0.001) and are more frequently node positive (77% vs. 39-44%; p<0.001) compared to adenocarcinomas and adenocarcinomas with mucinous differentiation. Prognosis is significantly worse.

Conclusion
The presence of signet ring cells in carcinomas with mucinous differentiation correlates with increased T-stage and poor prognosis. These cells, characterized by ITF and MUC2 production, showed a disruption of the E-cadherin/β-catenin complex, as well as an amplification of Bcl-2.
INTRODUCTION

The most common type of colorectal malignancies is adenocarcinoma n.o.s. (AC). Approximately 10% of all colorectal carcinomas are mucinous carcinomas, characterized by large amounts of extracellular mucin. Sometimes mucin accumulates intracellular as well, resulting in signet ring cell morphology. We call these tumors mucinous carcinoma with signet ring cells (MCSRC). This tumor type has to be distinguished from signet ring cell carcinoma, in which there is no extracellular mucin present. More than 96% of the signet ring cell carcinomas arise in the stomach. Signet ring cell carcinoma of the colorectal area is an uncommon, yet distinctive, type of malignancy present in 0.7-2.6% of all primary colorectal carcinomas.

Signet ring cells are usually presented as single cells or in loose clusters, which implicates a disruption in the cell-cell adhesion. This could explain their aggressive behavior with regard to invasion and metastasis. E-cadherin and β-catenin are important molecules that mediate the cell-cell adhesion, which might be altered in signet ring cells and therefore further investigated in this study.

Another characteristic of signet ring cells is their abundant cytoplasmic mucin, which might result from increased mucin production or mucin uptake from the environment. The latter might be a possibility when signet ring cells develop in adenocarcinomas with mucinous differentiation (MC), in analogy with the formation of muciphages in mucin producing tumors. Both Intestinal Trefoil Factor (ITF or TFF3) and various MUCs are the main components of mucin. Normally, ITF and some types of mucin (e.g. MUC2) are only produced by goblet cells. In this study, we compared mucin content of goblet cells and signet ring cells.

Development of malignancies is partly dependent on genomic deletions and amplifications. Each type of malignancy supposedly has its own pathway of genetic alterations leading to different phenotypes. Differences in the genetic background of tumors are often reflected by differences in clinical behavior and response to therapy. Identification of such genetic marker with prognostic or therapeutic implications in a molecular diagnostic setting can be of great additional value.

Zhang et al. describe a separate genetic pathway for MC, explaining their biological behavior. However, the genetic pathway involved in the development of signet ring cells has not been studied before. We hypothesize that signet ring cells arise from a separate genetic pathway. In this study the development of signet ring cell differentiation in MC is further investigated by examining the clinical relevance, the role of genetic alterations,
adhesion molecules and characteristics of mucin. In vitro results were confirmed using patient material from a well characterized clinical trial.

**MATERIALS AND METHODS**

**Tumor samples**

*Patients*

Patients were selected from the Total Mesorectal Excision (TME) study, a large multicenter trial in the Netherlands, in which 1530 patients with rectal cancer were included from January 1996 until December 1999. Pathological data from this trial are well documented and standardized. In this randomized trial patients were assigned to TME surgery with preoperative radiotherapy or TME surgery alone; however, therapy was not of importance in this study. Final histology review identified 296 cases with mucinous differentiation (MC, mucinous component is more than 1%), one case as primary colorectal signet ring cell carcinoma, and 31 cases as MC with signet ring cells (percentages of signet ring cells vary between 10-40%, no invasive signet ring cell component).

**Cell lines and cell culture**

Cell lines were used for DNA isolation and subsequently MLPA. Colon carcinoma cell lines with a non-mucinous phenotype (AC) were used: HT-29, Caco-2, SW-480, Colo-320, T84. The LoVo cell line consists of signet ring cells (SRC). Colon carcinoma cell lines with a mucinous phenotype (MC) were Ls174T (ATCC CL-188) and 5585-S (kindly provided by Dr. P.T.M. Moerkerk, University of Maastricht, the Netherlands). In the latter, signet ring cells were observed as well. These cells were grown in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) containing 10% heat-inactivated fetal calf serum and 0.1% gentamicin.

**Multiplex ligation probe amplification**

*DNA isolation*

DNA isolation of all cell lines and blood samples was performed using the DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to the manual of the manufacturer. The concentration and purity of DNA was measured by the GeneQuant UV spectrophotometer (Pharmacia LKB Biochram Ltd, Cambridge, England).

**Multiplex Ligation-dependent Probe Amplification**

MLPA was performed using the P005 salsa human chromosomal aberrations test kit (MRC-Holland, Amsterdam, the Netherlands), which contains target sequences of 41 genes that are often deleted or amplified in various tumors. Supplemented buffer
solutions were used from the kit. Procedures were described before\textsuperscript{19}. Data were analysed with Genotyper software (Applied Biosystems). To calculate the probe ratio, peak values identified by the Genescan software were imported in Excel files for further process. The probe ratio was then calculated by dividing the peak value of each probe amplification product by the total peak value of all 41 probes. This ‘relative peak area’ was then divided by the ‘mean relative peak area’ of the probe within all normal tissue samples (blood samples of healthy men and women). Thresholds to detect gains and losses were set at 1.2 and 0.8 respectively\textsuperscript{20}.

### Immunohistochemistry and in situ hybridization

#### Immunohistochemistry

Immunohistochemistry was performed using the avidin-biotin peroxidase complex method. A rabbit anti-human ITF polyclonal antibody (kindly provided by A. Giraud, University of Melbourne, Australia, 1:3000), a mouse anti-human E-cadherin monoclonal (clone 36, BD Transduction Laboratories, USA, 1:500), a mouse anti-human β-catenin (BD Transduction Laboratories, USA, 1:200), and a mouse anti-human MUC2 monoclonal antibody (Santa Cruz, California, USA, 1:300) were used. Pretreatment was performed with sodium citrate buffer (10mM). Staining intensities were graded as negative (no staining), weak (light brown), moderate (brown) and intense (dark brown). All cases were scored by two independent observers.

#### Immuno-fluorescence double-staining

Immuno-fluorescence double-staining was performed with an extracellular (HECD-1, monoclonal, 1:100, Takara Bio Inc, Japan) and transmembranal (clone 36, monoclonal, BD Transduction Laboratories, USA) epitope of E-cadherine, and with ITF and β-catenin. Procedures were performed as described above, with the secondary antibodies goat anti-mouse IgG conjugated with alexa 594 (1:200; Molecular Probes, Inc; Eugene, USA) and donkey anti-rabbit IgG conjugated with alexa 594 (1:200; Molecular Probes, Inc; Eugene, USA) respectively. After the application of the second primary antibody, the second secondary antibody (goat anti-mouse IgG 2a conjugated with alexa 488 (1:200; Molecular Probes, Inc; Eugene, USA) was applied. A fluorescence microscope equipped with 490/20 and 575/30-filters (Leica; Solms; Germany) was used.

#### ITF mRNA In situ Hybridization

A 221 bp sequence for human Intestinal Trefoil Factor was subcloned into Bluescript II SK (kindly donated by R. Poulsom, Histopathology Unit, Cancer Research UK, London, UK). Plasmid DNA was linearized with PstI (sense) and XhoI (α-sense) and transcribed with T7 and T3 RNA polymerase respectively\textsuperscript{21}. In situ hybridizations procedure was performed as described previously.\textsuperscript{22}.
**Bcl-2 FISH**

An IgH/Bcl-2 FISH probe mix (Vysis Inc.) was used to visualize Bcl-2 gene amplification. One μl Vysis probe was dissolved in 7 μl LSI hybridization buffer and 2 μl distilled water. Sections were denatured 10 minutes at 80°C, hybridized at 37°C overnight. Subsequently, nuclei were stained with DAPI (0.2 mg/ml, 0.5 minutes) and analyzed with a fluorescence microscope as described above. All cases were scored by two independent observers.

**Statistical analysis of clinical data**

Statistical analyses were performed using the TME database and cases were analyzed with SPSS statistical software. An ANOVA test was used to compare means and Kruskal-Wallis tests were used to compare quantitative and ordered variables. Chi-square tests were used to compare proportions; analysis of distant recurrence and survival was carried out by the Kaplan-Meier method, and the evaluation of differences between the groups was performed with the log-rank test. P-values < 0.05 were considered significant.

**RESULTS**

In order to establish the importance of signet ring cell differentiation in carcinomas with mucinous differentiation we started analyzing genetic aberrations in this type of cells, using cell lines as a model. Our findings were validated on patient material, with special attention for adhesion molecules, based on both our current results as well as on literature results. After establishing the nature of these cells, we confirmed their clinical importance by using data from a clinical trial.

**DNA copy number changes**

DNA copy number changes were examined using MLPA analyzing 41 different loci genes to compare the different tumor types. Peak values of cell lines with characteristics of AC (HT-29, Caco-2, SW480, Colo320, T48), MC (Ls174T) and MCSRC (Lovo, 5585S) were identified by Genescan and probe ratios of the different cell lines were compared (Figure 1). Almost all genes showed an aberration in at least one cell line. Deletions (probe ratio of < 0.8) were more frequently observed than amplifications (probe ratio of > 1.2).
Figure 1: Illustration of amplifications and deletions of 41 genes (divided into four graphs). Normal range is between 0.8 and 1.2 (dotted line). Some cell lines showed an amplification of more than 2 in certain genes (*). Genes described in results (MYC, CDH1, Bcl2) are marked. → color figures
Bcl-2 amplification was observed in MCSRC and MC (LoVo, Ls174T, 5585S) in contrast to the deletion observed in AC cell lines. This difference was confirmed in patient material using Bcl-2 FISH analysis. Only 8% (2/25) of the AC showed an amplification of the Bcl-2 gene, against 44% (7/16) of the MC and 38% (6/16) of MCSRC (p=0.017). Furthermore, high copy amplifications (probe ratio of >2) of MYC in almost all AC cell lines were detected, whereas no amplifications were present in the MC and MCSRC.

**E-cadherin expression in signet ring cells**

It has been reported that E-cadherin and β-catenin expression is reduced or absent in colorectal signet ring cells. Our MLPA analysis on cell lines did not reveal consistent genomic aberrations of the CDH1 gene that encodes E-cadherin. Therefore, immunohistochemical staining for E-cadherin and β-catenin was performed on patient material. Both transmembranous and extracellular E-cadherin were markedly decreased in signet ring cells. In cases with an intact transmembrane part of E-cadherin, the extracellular epitope was not available (Figure 2). Staining of membranous β-catenin was variable in signet ring cells, but most cases showed a decrease in membranous staining of β-catenin. These findings suggest a disruption of the E-cadherin/β-catenin complex.

![Illustration of E-cadherin by immunofluorescence staining. The nuclei are represented by the blue colour. Subtype clone 36 (transmembrane) is represented by the green colour, subtype HECD-1 (extracellular) is represented by the red colour. The yellow colour of the normal mucosa (A) indicates that both epitopes are still present. (B) A signet ring cell at high magnification (100x), the transmembranal epitope is still available, while the extracellular epitope was partly not detected.](image)

→ color figures
Nuclear β-catenin and high expression of Intestinal Trefoil Factor

Disruption of E-cadherin function might be caused by ITF, a component of mucin, by specific tyrosine phosphorylation of β-catenin. Therefore, the possible correlation between β-catenin and ITF in signet ring cells was subsequently investigated (Figure 3). In the normal mucosa (Figure 3A) β-catenin is confined to the membrane and the goblet cells are filled with ITF. ITF is sometimes secreted into the lumen (arrowheads). In signet ring cells membranous staining of β-catenin correlated with weak cytoplasmic ITF staining (Figure 3B), while nuclear staining for β-catenin was observed in cases with intense staining of ITF. These data demonstrate a relation between high levels of ITF and nuclear translocation of β-catenin.

Figure 3: Immunofluorescence illustration of a normal mucosa (A) with goblet cells (arrows) secreting ITF (red, arrowheads) into the lumen; (B) signet ring cell showing a strong membranous and cytoplasmic staining of β-catenin (green) and weak cytoplasmic staining of ITF; and (C) a signet ring cell showing a weak membranous staining of β-catenin and strong cytoplasmic staining of ITF, while β-catenin has moved to the nucleus (D).

→ color figures
Distribution of Intestinal Trefoil Factor and Mucin-2

The origin of ITF in signet ring cells is not clear; in analogy with muciphages mucin with ITF might be absorbed from the environment. On the other hand, ITF might be produced by signet ring cells, in analogy with goblet cells. Indeed, goblet-like cells surrounding the mucinous lakes in MC also show an intense staining of ITF (data not shown). The staining pattern in signet ring cells is highly variable (Figure 4A-B). mRNA in situ hybridization of ITF showed a positive staining in signet ring cells (Figure 4C), suggesting that signet ring cells can produce their own ITF, like goblet cells.

In order to further evaluate the analogy between signet ring cells and goblet cells MUC2 expression was analyzed. Goblet cells in the normal mucosa showed an intense staining of the secreted MUC2 (Figure 4E), just as the goblet-like cells around the mucin lakes in MC. A weak staining of MUC2 in the cytoplasm and intense staining in the cell membrane was observed in clustered signet ring cells. Solitary signet ring cells showed a weak or moderate distribution of MUC2 (Figure 4F). Thus, although signet ring cells and goblet cells have a distinct morphology, they show similar features with respect to MUC2 and ITF expression.

Figure 4: ITF distribution in clustered signet ring cells (A) and solitary signet ring cells (B). Signet ring cells were positive for ITF mRNA (C, dark purple) and negative using the sense probe (D, negative control), indicating that signet ring cells can produce their own ITF. Expression of MUC2 in normal mucosa (E) and solitary signet ring cells (F). → color figures
Clinicopathological factors
In order to address the clinical relevance of MCSRC, we have correlated the presence of signet ring cells with prognosis. The total number of patients included in the statistical analysis was 1462 (incomplete data of 68 patients); most tumors were AC (RT+TME: n=545, TME: n=589). The tumors with mucinous differentiation (mucinous component is more than 1%) were divided into tumors without signet ring cells (MC; RT+TME: 164, TME: 132) and with signet ring cells (MCSRC; RT+TME: 20, TME: 11). There was one signet ring cell carcinoma without mucinous differentiation.

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>CATEGORY</th>
<th>AC N = 1134</th>
<th>AC N = 296</th>
<th>MCSRC N = 31</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localization (distance from the anal verge)</td>
<td>&lt;5.0 cm</td>
<td>28.7%</td>
<td>35.8%</td>
<td>48.4%</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>5 - 9.9 cm</td>
<td>41.9%</td>
<td>37.2%</td>
<td>25.8%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 10 cm</td>
<td>29.3%</td>
<td>27.0%</td>
<td>25.8%</td>
<td></td>
</tr>
<tr>
<td>Invasion depth</td>
<td>T1</td>
<td>5.9%</td>
<td>3.0%</td>
<td>0%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>33.0%</td>
<td>32.1%</td>
<td>16.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>57.3%</td>
<td>59.8%</td>
<td>67.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>2.9%</td>
<td>4.7%</td>
<td>16.3%</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>N0</td>
<td>60.7%</td>
<td>55.9%</td>
<td>22.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>22.2%</td>
<td>23.4%</td>
<td>16.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>11.7%</td>
<td>12.9%</td>
<td>41.9%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>5.4%</td>
<td>7.8%</td>
<td>19.4%</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>≤ 2.0 cm</td>
<td>9.0%</td>
<td>3.8%</td>
<td>6.5%</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>2 - 5 cm</td>
<td>68.5%</td>
<td>71.4%</td>
<td>54.8%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 5 cm</td>
<td>22.5%</td>
<td>24.8%</td>
<td>38.7%</td>
<td></td>
</tr>
</tbody>
</table>

Clinicopathological factors were not different between the randomization arms (data not shown). Therefore, both groups were analyzed together (Table 1). MCSRC presents at a later T stage (T3: 67.7% and T4: 16.1%) compared to both AC (T4: 2.9%) and MC (T4: 4.7%, p < 0.001, Table 1). The number of node positive patients was significantly higher in MCSRC than in AC and MC (77.4% vs. 39.3% and 44.1% respectively; p < 0.001).

The mucinous component can be located deep, superficial or total (spread through the whole surface area). Whereas in the majority of the MCSRC the mucinous spread was total (80.6%), in the MC the distribution was in 67.2% limited to the deep areas (p < 0.001). MCSRC have a larger mucinous component than MC (p<0.001, Table 2).
Patients with MCSRC have a poor prognosis (Figure 5). Distant recurrence rate was 67% after 5 year, compared with 28% (AC) and 34% (MC, \( p = 0.0017 \)). Survival was decreased, 45% versus 64% and 56% respectively (\( p = 0.016 \)). When we compared only T3/4 tumors, the difference was still there (42%, 40% versus 26%), although not longer significant (\( p = 0.30 \)). Survival of patients without lymph node metastases is not affected by tumor type (52%, 59%, 60% respectively, \( p = 0.26 \)). However, in the lymph node positive group survival was significantly worse in patients with signet ring cells (33%, 23% versus 18%, \( p = 0.002 \)). Prognosis was determined in the non-irradiated group of the trial. Patients with short-term radiotherapy show the same results. Thus, the presence of signet ring cells in mucinous carcinomas correlates with increased invasion depth and presence of lymph node metastases. The presence of signet ring cells is a N-stage independent prognostic marker for increased recurrence rate and decreased survival.

### Table 2. Mucinous Component and Distribution for the Various Tumor Types

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>CATEGORY</th>
<th>AC</th>
<th>MC</th>
<th>MC</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucinous Component</td>
<td>0%</td>
<td>1134 (100%)</td>
<td>0%</td>
<td>0%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0-20%</td>
<td>129 (43.6%)</td>
<td>34 (11.8%)</td>
<td>35 (12.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20-40%</td>
<td>35 (11.8%)</td>
<td>34 (11.5%)</td>
<td>4 (12.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40-60%</td>
<td>31 (10.5%)</td>
<td>34 (11.5%)</td>
<td>2 (6.45%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60-80%</td>
<td>67 (22.6%)</td>
<td>2 (6.45%)</td>
<td>4 (12.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80-100%</td>
<td></td>
<td>20 (64.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td>No mucinous component</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Superficial</td>
<td>8 (2.7%)</td>
<td>1 (3.2%)</td>
<td>1 (3.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deep</td>
<td>199 (67.2%)</td>
<td>5 (16.1%)</td>
<td>5 (16.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>20 (3.2%)</td>
<td>25 (80.6%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: \( \chi^2 \)-test, #: Kruskal-Wallis test. Bold values indicate statistical significance.  

Figure 5: (A) Distant recurrence in months since surgery and (B) survival in months since surgery
DISCUSSION

Signet ring cell differentiation in colorectal carcinomas is a rare phenomenon. In the present study the incidence of primary signet ring cell carcinoma was 0.06%, while the incidence of MCSRC was 2%, which confirms other observations. Formation of signet ring cells is associated with a poor prognosis. However, the mechanisms underlying the formation of signet ring cells are poorly understood. We applied various strategies to detect the possible mechanisms and to understand the poor prognosis of these patients.

Clinical analysis revealed that MCSRC were most frequently diagnosed when the disease was advanced, which is consistent with other reports. As a consequence, distant recurrence was more frequently observed and survival was decreased.

Differences in biological behavior of tumors might be a reflection of their development along different genetic pathways. To detect differences in genetic aberrations between MCSRC, MC and AC we applied a screening assay on cell lines, to be tested on patient material. We found an amplification of Bcl2 in MCSRC, whereas a deletion is more common in AC. Verification in patient tissue confirmed this observation. Since Bcl-2 is a suppressor of apoptosis, amplification of this gene might account for more aggressive growth. Based on literature, MYC amplification was expected in MC, but our cell line analyses revealed only amplification in AC, but not in MC and MCSRC.

In analogy with hereditary signet ring cell gastric carcinoma, E-cadherin is expected to be absent in signet ring cells. Indeed, a deletion was observed in the MCSRC cell line, which was subsequently verified in patient material and is in accordance with other studies. Immunofluorescence showed that the extracellular part of the E-cadherin molecule in signet ring cells was not detected. The transmembranal part of the molecule was less affected. These alterations in E-cadherin expression could implicate a disruption of the adhesion complex. In addition, the membranous localization of β-catenin was slightly reduced, whereas nuclear expression was present in signet ring cells. By reduced cell-cell adhesion, signet ring cells have the opportunity to lose contact with the surrounding structure and spread diffusely through the whole body. It can be suggested that the aggressive biological behavior of MCSRC is partly contributed by a decreased expression of E-cadherin and β-catenin.

Tyrosine phosphorylation of β-catenin results in the disruption of the E-cadherin function and altered migratory activity of tumor cells might be caused by ITF. ITF is a trefoil peptide which is specifically produced by goblet cells, but might show uptake...
by surrounding cells. The role of ITF in prognosis of colorectal carcinomas is a point of discussion. In present series goblet-like cells in MC situated around the mucin lakes showed strong staining patterns for both ITF and MUC2. Signet ring cells showed variable, though positive ITF staining patterns. Similar patterns were observed with MUC2 staining. Weiss et al. reported that MUC2 (as well as ITF) is goblet cell specific, and expression of MUC2 mRNA was preserved in MC (goblet cell phenotype), while expression in AC (enterocytic phenotype) was decreased. Signet ring cells have a goblet cell phenotype, suggesting that it is possible that they can produce their own ITF and MUC2. Indeed, ITF mRNA expression was present in signet ring cells.

In conclusion, in the current study we demonstrate that the presence of signet ring cells in MC correlates with increased T-stage and poor prognosis. These cells, characterized by ITF and MUC2 production, showed a disruption of the E-cadherin/β-catenin complex, as well as an amplification of Bcl-2.
REFERENCE LIST


18 Verstijnen CP, Arends JW, Moerkerk PT, et al. The establishment and characterization...


Chapter 6

COX-2 expression in rectal cancer is of prognostic significance in patients receiving preoperative radiotherapy

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Purpose
To determine the impact of cyclooxygenase-2 (Cox-2) expression on clinical behavior in irradiated and non-irradiated rectal carcinomas.

Patients and methods
Tumor samples were collected from 1231 patients of the Dutch TME trial in which rectal cancer patients were treated with standardized surgery, and randomized for preoperative short-term (5 times 5Gy) radiotherapy or no preoperative radiotherapy. Tissue micro arrays were constructed from primary tumor material and Cox-2 expression was assessed by immunohistochemistry. Tumor cell apoptosis was determined by M30 immunostaining.

Results
A high level of Cox-2 expression after radiotherapy was associated with low levels of tumor cell apoptosis ($p=0.001$). Cox-2 expression had no significant impact on patient survival or tumor recurrence in non-irradiated tumors. However, in patients receiving preoperative radiotherapy, high level of Cox-2 expression was associated with higher incidence of distant recurrences ($p=0.003$, HR: 1.7, CI: 1.2-2.5), and shorter disease free survival ($p=0.002$, HR: 1.5 CI: 1.2-2.0) and overall survival ($p=0.009$, HR: 1.8, C.I.: 1.1-2.5), independent of patient age, tumor stage, tumor location or the presence of tumor cells in the circumferential resection margin.

Conclusion
A high level of Cox-2 expression following preoperative radiotherapy in resection specimens is associated with apoptosis resistance, high distant recurrence rates and a poor prognosis in rectal cancer.
INTRODUCTION

In recent years the role of a key enzyme in prostaglandin synthesis, cyclooxygenase-2 (Cox-2) has been appreciated in cancer development and progression. Cox-2 is responsible for the conversion of arachidonic acid to prostaglandins and other eicosanoids. In addition to its well-known role in inflammatory reactions, Cox-2 plays a role in tumor progression, angiogenesis, metastasis and abrogation of the anti-tumor immune response\(^1\)\(^-\)\(^4\). Cox-2 prevents apoptosis by generation of anti-apoptotic PGE\(_2\)\(^5\) and PGI\(_2\)\(^6\) and by removal of the pro-apoptotic substrate arachidonic acid\(^7\). PGE\(_2\) induces transformations that result in increased Bcl-2 expression, and prolong the cell cycle G\(_1\) phase with increased cyclin D\(_1\) expression\(^8\).

Numerous epidemiological studies have indicated that the use of NSAIDs and Cox-2 inhibitors is associated with a significant decreased incidence and mortality rate in colorectal cancer\(^9\)\(^-\)\(^12\). In addition, selective Cox-2 inhibitors have been shown to decrease Cox-2 expression and Cox-2 activity in gastrointestinal malignancies\(^13\). The clinical impact of Cox-2 expression has been evaluated in a large number of studies in colorectal cancer but results have not been consistent\(^9\)\(^,\)\(^14,\)\(^15\). Considering the distinct differences in tumor biology\(^16\), treatment, recurrence rates and metastatic behavior, it is regrettable that most studies make no distinction between rectal and colon cancer.

The purpose of the current study was to obtain a conclusive answer of the clinical relevance and prognostic value of immunohistochemically-determined Cox-2 expression in rectal cancer and to investigate the effects of radiation therapy on Cox-2 expression and subsequent biological and clinical behavior. The investigated patients were included in the Dutch TME trial, a prospective multicenter trial, and were randomized between standardized preoperative radiotherapy treatment followed by TME surgery or TME surgery alone\(^17\).

METHODS

Study population

Patients were obtained from the Dutch TME trial, a large multicenter trial in which 1861 patients were included from January 1996 until December 1999. Patients with a resectable carcinoma of the rectum were included in this international multicenter clinical trial and were subsequently randomized for radiotherapy (5x5 Gray) followed by TME surgery or for TME surgery alone without preoperative radiotherapy\(^17\). Radiotherapeutical, surgical and pathological procedures were standardized and
quality-controlled. Patients who complied with the eligibility criteria of the TME trial with sufficient paraffin-embedded tumor material were selected for this study. Archival tumor material was collected from the 1530 Dutch patients that were included in the trial. Tumor material was available from 1231 patients. For the evaluation of Cox-2 expression, patients were only included if at least 2 of the 3 included punches on the tissue microarray (TMA) could be evaluated, leaving 1038 eligible stage I-III rectal cancer patients for analyses of clinical impact of Cox-2 expression.

**Tissue microarray preparation**

Tissue micro arrays from formalin-fixed, paraffin-embedded tumors included in the Dutch TME trial were constructed with a custom-built precision tissue arrayer (Beecher Instruments, Silver Spring, MD) using a 2-mm-diameter punch as described previously.

**Immunohistochemistry**

For the quantification of Cox-2 expression, 4-µm sections of the TMAs were stained with Cox-2-specific mouse anti-human monoclonal antibodies (clone CX229, Cayman Chemical Co., Ann Arbor, Michigan, USA). The immunohistochemical procedures were described in detail elsewhere. Antigen retrieval was performed by boiling the sections in 10mM citrate buffer (pH 6.0) for 10 min. Sections were incubated overnight at room temperature with antibodies against human Cox-2 (1:100). Specificity of the antibodies was confirmed in this study by staining randomly selected rectal cancer specimens with and without pre-absorption of the primary antibody with human Cox-2 antibody-blocking peptides (10μl/mL, Cayman Chemical) for 1 hour at room temperature before the staining procedure. All tumor specimens were stained simultaneously to avoid inter-assay variation. Cox-2 immunostaining was assessed by two independent observers (P.H. and M.J.E.M.G.) in a blinded manner.

For high-throughput analysis of the TMAs, the scoring criteria proposed by Buskes et al. were used: A score of 0 indicates no staining; score 1, weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of the cancer cells); score 2, moderate to strong granular cytoplasmic staining in 10%-90% of the tumor cells; and score 3, more than 90% of the tumor cells stained with strong intensity. The stained 3 TMA punches taken from each tumor were scored independently. The median score of the punches was used for analysis. In case of disagreement a consensus score was obtained. In the present study Cox-2 scores 0, 1 and 2 were defined as Cox-2 low, a score of 3 was defined as Cox-2 high.

Apoptosis levels had previously been characterized in this series of patients by immunohistochemical analysis of M30 expression. Data on Cox-2 expression and apoptosis was available in 1024 patients.
Statistical analyses
All analyses were performed with SPSS statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). Paired samples t-test, Mann-Whitney U, Kruskall-Wallis, and Spearmans’ Rho tests were used to compare continuous variables. The χ²-test was used to compare categorical variables. Patient survival was estimated according to the Kaplan-Meier method and compared using the log-rank test. The entry date for the survival analyses was the time of surgery of the primary tumor. Events for time to local recurrence, distant recurrence, disease-free and overall survival were defined as follows; time from of surgery to: time of local disease relapse, distant disease relapse, time of disease relapse or death and time of death respectively. Cox’ regression analyses were used to calculate Hazard Ratios (HR) with 95% confidence intervals (CI). Variables with a p-value of ≤0.10 in the univariate analyses were subjected to a multivariate analysis. Inter-observer variability was calculated by κ statistic, as described by Landis and Koch: κ-values of 0.2 to 0.4 indicate “fair”, of 0.4 to 0.6 “moderate”, and of > 0.6 “excellent” results.

RESULTS

Cox-2 protein expression in rectal cancer TMAs
The immunohistochemical Cox-2 staining pattern exhibited a brown diffuse granular cytoplasmic staining (Figure 1). No staining of Cox-2 was observed in 5 tumors (0.5%) (Figure 1A). A weak diffuse, moderate or strong staining was observed in respectively 114 (11.0%), 602 (58.0%) and 317 (30.5%) of the tumors (Figure 1B-D)

The inter-observer κ-value score for evaluation of Cox-2 expression was 0.62, indicating minimal inter-observer variation. Ten randomly selected rectal cancer specimens were stained with Cox-2 antibodies with or without blocking peptide. All tumor cell signals were blocked by this control procedure in all specimens.

Cox-2 expression and clinicopathological parameters
Clinical data and conventional prognosis factors (TNM stage, age, histology, localization) of the patients in the current study have been published previously. Cox-2 expression did not significantly differ between irradiated and non-irradiated tumors (p=0.27, Table 1) and were distributed evenly in non-irradiated and irradiated patients with regard to various clinical and pathological parameters as age, gender, tumor size, depth of invasion, lymph node involvement, TNM stage, type of surgery, circumferential margin and distance from anal verge. All p-values were not significant.
Figure 1: Representative stainings of Cox-2 expression in tissue microarray cores from the 1231 rectal cancer specimens evaluated in this study. Figure 1A: Cox-2 negative tumor (score 0). Figure 1B: weak diffuse cytoplasmic staining (score 1). Figure 1C: moderate to strong granular cytoplasmic staining (score 2). Figure 1D: strong intensity of the staining (score 3).  → color figures

(data not shown). A poor grade of differentiation was borderline significantly associated with high Cox-2 expression levels in non-irradiated tumors (p=0.06). High levels of Cox-2 expression were more often observed in adenocarcinomas (as compared to tumors of the mucinous type) in irradiated and non-irradiated tumors (p=0.05/p=0.04).

<table>
<thead>
<tr>
<th>COX-2 EXPRESSION</th>
<th>TME</th>
<th>RT + TME</th>
<th>TOTAL</th>
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<tr>
<td>0</td>
<td>3 (0.6%)</td>
<td>2 (0.4%)</td>
<td>5 (0.5%)</td>
</tr>
<tr>
<td>1</td>
<td>51 (9.7%)</td>
<td>63 (12.4%)</td>
<td>114 (11%)</td>
</tr>
<tr>
<td>2</td>
<td>300 (56.8%)</td>
<td>302 (59.2%)</td>
<td>602 (58%)</td>
</tr>
<tr>
<td>3</td>
<td>174 (33.9%)</td>
<td>143 (28.0%)</td>
<td>317 (30.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>528</td>
<td>510</td>
<td>1038</td>
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</table>

Abbreviations: TME: total mesorectal excision, RT: radiotherapy
Cox-2 expression in relation to radiotherapy and apoptosis

Cox-2 expression was not associated with apoptosis in resection specimens of non-irradiated rectal cancer tumors (p=0.13), but was significantly associated with decreased levels of apoptosis in irradiated tumors (p=0.001, Mann Whitney test). As can be seen in Figure 2, the analysis remained significant when Cox-2 scores were dichotomized as score 0-2 (Cox-2 low) versus score 3 (Cox-2 high) (p=0.001, Mann Whitney test).

The median time period from completion of radiotherapy to surgery was 4 days (Inter Quartile Range: 3-6 days). No significant differences were observed between the levels of Cox-2 expression with regard to the median time between radiotherapy and surgery (p=0.06, Kruskall-Wallis test).

Cox-2 expression in relation to radiotherapy and tumor prognosis

Subsequently, we analyzed the impact of Cox-2 expression on tumor recurrence and patient survival. Figure 3A-C shows the impact of Cox-2 expression in non-irradiated tumors on local recurrence rates, overall survival and disease free survival.

Cox-2 expression did not have an impact on local recurrence (Figure 3A, p=0.44), distant recurrences (Figure 4, p=0.77), overall survival (OS) (Figure 3B, p=0.61) or disease free survival (DFS) (Figure 3C, p=0.57) in non-irradiated rectal cancer specimens. As can be seen in Figure 4, after radiotherapy, tumors with high levels of Cox-2 expression showed a significantly higher rate of distant recurrences (p=0.005), but this was not observed in non-irradiated tumors. Figure 5A-C shows tumors with high levels of Cox-2 expression after radiotherapy to be associated with poor DFS (p=0.004) and OS (p=0.006), but not with local recurrence rates (p=0.92).
Figure 3: Kaplan-Meier survival estimates by dichotomized Cox-2 tumor epithelial staining in non-irradiated (RT-) rectal tumors for local recurrence (A), overall survival (B) and disease free survival (C). Grey lines denote low levels of Cox-2 and black lines high levels of Cox-2 expression.

Figure 4: Kaplan-Meier survival estimates by dichotomized Cox-2 tumor epithelial staining in irradiated and non-irradiated rectal tumors. Distant recurrence rates estimates by Cox-2 tumor epithelial staining in irradiated and non-irradiated patients. Cox-2 expression does not have an impact on distant recurrences in non-irradiated tumors (gray lines, p=0.77), but significantly impacts distant recurrences in irradiated tumors (black lines, p=0.005).

Figure 5: Kaplan-Meier survival estimates by dichotomized Cox-2 tumor epithelial staining in irradiated (RT+) rectal tumors for local recurrence (A), overall survival (B) and disease free survival (C). Grey lines denote low levels of Cox-2 and black lines high levels of Cox-2 expression. High Cox-2 expression is a poor prognostic factor for disease free (p=0.004) and overall survival in irradiated rectal cancer patients (p=0.006).
Univariate and Multivariate analyses in irradiated patients

Univariate Cox regression analyses were performed to identify prognostic factors for OS in irradiated patients. Advanced patient age (HR: 1.03, C.I.: 1.01-1.05, p<0.0001), advanced pathological stage (HR: 1.75, C.I.: 1.47-2.03, p<0.0001), tumor-positive circumferential resection margins (HR: 2.46, C.I.: 1.82-3.33, p<0.0001), distal location of the tumor (HR: 1.46, C.I.: 1.01-2.06, p=0.05) and high Cox-2 expression (HR: 1.48, C.I.: 1.11-1.96, p=0.006) proved to be significant in the univariate analyses and were subjected to Cox multivariate analysis (Table 2). Patient age above the median, advanced pathological stage, tumor-positive circumferential resection margins and high Cox-2 expression (HR: 1.46, C.I.: 1.10-1.94, p=0.009) retained their strength as independent prognostic factors for OS (Table 2). In addition, Cox-2 proved to be an independent prognostic factor for high distant recurrence rates (p=0.003, HR: 1.7, C.I.: 1.2-2.5) and DFS (p=0.002, HR: 1.8, C.I.: 1.2-2.5).

<table>
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<tr>
<th>FACTOR</th>
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<th>HAZARD RATIO</th>
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<th>P-VALUE</th>
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<td>Age</td>
<td>Below median</td>
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<tr>
<td></td>
<td>Above median</td>
<td>1.03</td>
<td></td>
<td></td>
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<tr>
<td>TNM Stage</td>
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<td>1.22 - 2.74</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>II</td>
<td>1.83</td>
<td>1.96 - 4.22</td>
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<tr>
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<td>III</td>
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<td></td>
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<tr>
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<td>1.41 - 2.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>1.94</td>
<td></td>
<td></td>
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<tr>
<td>Distance of tumor from</td>
<td>10.1 - 15 cm</td>
<td>1</td>
<td>1.03 - 2.13</td>
<td>0.07</td>
</tr>
<tr>
<td>the anal verge</td>
<td>5.1 - 10 cm</td>
<td>1.48</td>
<td>1.01 - 2.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤ 5 cm</td>
<td>1.44</td>
<td></td>
<td></td>
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<tr>
<td>Cox-2 expression</td>
<td>Low</td>
<td>1</td>
<td>1.10 - 1.94</td>
<td>0.009</td>
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<tr>
<td></td>
<td>High</td>
<td>1.46</td>
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</table>

A variable was included in the multivariate analysis if the p-value in the univariate analysis was less than 0.10. Patients with missing data were excluded from the analysis. **Bold** values indicate statistical significance. Abbrevations, CI: confidence interval, CRM: circumferential margin.

**DISCUSSION**

The major observation in the current study is that increased Cox-2 expression in irradiated rectal cancer specimens is associated with reduced levels of apoptosis and poor prognosis. This indicates that Cox-2 expression can be used to identify a cohort of patients with a poor prognosis after radiotherapy.
In several forms of cancer, radiation exposure is associated with an increase in eicosanoid production. Within hours after radiation, increased levels of prostaglandin’s and thromboxanes are detectable in most tissues and increased levels may persist for several days or weeks. In the current study high Cox-2 expressions after radiotherapy were associated with apoptosis resistance and can therefore decrease the levels of radiotherapy-induced apoptosis.

Anti-apoptotic proteins of the Bcl-2 family are able to suppress radiation-induced cell death. Cox-2 is known to induce Bcl-2 expression and is associated with apoptosis resistance. De Bruin et al. demonstrated by immunohistochemical evaluation of M30 that intrinsic apoptosis is a prognostic factor for local recurrence in rectal cancer. However, radiotherapy-induced apoptosis was not of prognostic value. Since the current study found a prognostic impact of Cox-2 in irradiated patients only, whereas apoptotic rates were only prognostic in non-irradiated cases, our findings can not provide a mechanistic explanation of our observations in relation with tumor cell apoptosis.

A possible explanation for the clinical behavior of tumors with high levels of Cox-2 expression after radiotherapy lies in the fact that Cox-2 is an immediate early response gene. The interval between the short-term radiotherapy and surgery could be sufficient for a change in Cox-2 activity and subsequent prostaglandin production to influence the clinical behavior of the tumor. Elevated Cox-2 expression has shown to lead to alterations in the invasive and metastatic potential of cancer cells. Cox-2 expression and prostaglandin production induce cell-surface glycosyltransferases and type-I sialyl Lewis antigens, leading to enhanced tumor cell adhesion to endothelial cells and animal studies reported that Cox-2 inhibition prevented the formation of distant metastases. Moreover, the immunosuppressive effect of increased prostaglandin production may allow circulating tumor cells to escape the host anti tumor response and metastasize. However, it is not very likely that these events will take place during the short interval between completion of radiation and surgery.

It has been established in several animal models and clinical studies that Cox-2 inhibitors synergize with radiotherapy and can be administered safely. Cox-2 inhibitors could prevent the adverse effects of elevated Cox-2 levels and subsequent increased prostaglandin production that can occur during radiotherapy. It is tempting to speculate that the addition of Cox-2 inhibitors to preoperative radiotherapy may help to reduce distant recurrences and improve patient survival.

In the current study, using patients from a trial that evaluated TME surgery with or without preoperative radiotherapy, Cox-2 expression did not have any impact on
local recurrence rates or prognosis in non-irradiated tumors. We have not studied pre-treatment biopsies but our results regarding non-irradiated tumors indicate that evaluation of Cox-2 expression in non-irradiated rectal cancer specimens or pre-radiation biopsies is not a useful discriminator for response to therapy or prognosis. The prognostic value of Cox-2 expression has extensively been investigated in retrospective studies with colorectal cancer specimens \(^{14,15}\) (and reviewed in \(^9\)), but the independent prognostic value of Cox-2 expression remains unclear. The disagreement on the prognostic value of Cox-2 in colorectal cancer in previous studies might be due to the apparent lack of prognostic value of Cox-2 expression in non-irradiated rectal cancer as seen in the current study, hereby confounding the results in studies that compile rectal and colon patients. The low numbers of Cox-2 negative tumors in the current study (<1%) as compared to the 10-30% negative tumors reported in studies evaluating Cox-2 expression in colorectal cancer specimens \(^{35}\) suggest a biological difference in tumors originating from the proximal or distal large bowel. Whether this is due to a larger number of mismatch repair defective tumors (which show reduced Cox-2 expression \(^{36,37}\) in right-sided tumors \(^{38,39}\) or other factors is beyond the scope of the current study. However, the apparent differences in tumor biology do confound the evaluation of the clinical relevance of Cox-2 expression in the large bowel and underscore the need for Cox-2 assessment in well-defined, standardized and uniformly treated patient groups as was performed in the present study.

In conclusion, in the current study we showed that high levels of Cox-2 after radiotherapy are associated with diminished apoptosis and high distant recurrence rates. Our data indicate that evaluation of Cox-2 expression after radiotherapy can be used to identify patients with a poor prognosis. These results suggest that the addition of Cox-2 inhibitors to pre-radiotherapy may help to reduce distant recurrences and improve patient survival.
REFERENCE LIST


data in the central databases of multicenter randomized trials need to be based on pathology reports and controlled by trained quality managers.


Iacopetta B. Are there two sides to colorectal cancer? *Int J Cancer* 2002;101:403-408.

CHAPTER 7

THYMIDYLATE SYNTHASE GENOTYPING IS MORE PREDICTIVE FOR THERAPY RESPONSE THAN IMMUNOHISTOCHEMISTRY IN PATIENTS WITH COLON CANCER

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Adriaan J.C. van den Brule

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Background
Thymidylate synthase (TS) is a potentially valuable marker for therapy response since it is the molecular target of 5-fluorouracil (5-FU). TS can be analyzed at the DNA (gene polymorphisms and amplification) and protein level (immunohistochemistry). This study investigated the predictive role of TS at the DNA and protein levels in patients with N+ colon cancer (n=38).

Patients and methods
Tumor and normal tissues were genotyped using PCR for variable number of tandem repeats (VNTR), a single nucleotide polymorphism (SNP) in the 3R allele and a 6-bp deletion (1494del6) in the TS gene. Tumor tissues were additionally analyzed for loss of heterozygosity (VNTR polymorphism). A newly developed real time PCR assay was used to detect the presence of TS gene amplifications in tumor tissues.

Results
VNTR analysis in normal tissue was significantly associated with distant tumor recurrence (8% for 2R/2R versus 52% for patients carrying a 3R allele, p=0.038) and cancer specific survival (p=0.021). IHC was not found to be significantly associated with patients’ outcome. No correlations between TS gene polymorphisms and IHC were found. However, TS gene amplification was correlated with a strong IHC staining intensity.

Conclusion
This study indicates that DNA based analysis is more predictive for patient outcome than TS IHC.
INTRODUCTION

A substantial number of patients with colon cancer who received adjuvant 5-fluorouracil (5-FU) -based therapy will not benefit. Therefore, predictive markers are needed in order to discriminate between responsive and non-responsive patients. Thymidylate synthase (TS) is a central enzyme in DNA synthesis and is a potentially valuable marker since it is the molecular target of 5-FU. TS protein expression is affected by three different functional polymorphisms in the untranslated regions (UTR’s) of the gene. Sensitivity to 5-FU based therapy might be largely influenced by the intra-cellular levels of the TS protein.

TS protein levels can be studied directly by western blotting, enzyme activity assays, ELISA and immunohistochemistry (IHC). The mainstream method is IHC because it is a relatively cheap, widely implemented technique that enables studying protein expression in situ. At the DNA level, TS protein expression is affected by different underlying functional polymorphisms as shown by several functional studies. These polymorphisms are: a variable number of tandem repeats (VNTR) containing two (2R) or three (3R) repeats of 28-bp, a single nucleotide polymorphism (SNP) of a G to C substitution in the 3R allele in the 5’UTR and a 6-bp deletion at nucleotide 1494 in the 3’UTR (1494del6). Recently, a SNP of a G to C substitution in the first repeat of the 2R allele has also been found (hereafter referred to as the 2RC allele). TS mRNA with three repeats has greater translation efficiency than mRNA with two repeats. Individuals with a 3R/3R genotype will, in theory, have higher TS protein levels than individuals homozygous for the 2R allele. Furthermore, the SNP has been described to decreased translation efficiency of the 3R allele to the level of the 2R allele. The 1494del6 polymorphism was found to be associated with decreased mRNA stability and lower TS protein levels.

Although TS expression can be studied using either IHC or genotyping, it is still unclear which methodology is clinically most valuable, as recently indicated in the ASCO 2006 update for recommendations for the use of tumor markers in gastrointestinal cancer. In addition, the experimental procedures for both methods remain unclear. In the case of IHC it is undecided which antibody should be used and on what tissue type (tumor, normal or metastatic) it should be performed. These differences in technical approach, the semi-quantitative nature of IHC analysis, observer dependence and incomplete standardization for IHC assessment could account for the significant heterogeneity between studies investigating the predictive role of TS with IHC. In the adjuvant and advanced settings, a predictive role of TS IHC was found by some studies but enfeebled by others, which was also confirmed by the meta-analysis conducted...
by Popat et al. With respect to TS genotyping, it is still unclear which polymorphism(s) should be analyzed and whether the germline or somatic genotype should be investigated. That is, TS genotype in tumor tissue can deviate due to chromosomal aberrations such as loss of heterozygosity (LOH) and gene amplification.

This study aimed to perform a detailed analysis of the predictive values of TS at both the protein level (IHC) and DNA level (genotyping) in patients with colon cancer. In order to achieve this, TS was thoroughly evaluated with these two methodologies in both tumor and normal tissue in a homogenous patient population of patients with N+ colon adenocarcinoma who received postoperative 5-FU based chemotherapy.

**PATIENTS AND METHODS**

**Patient population**
Thirty-eight patients treated in the Catharina hospital in Eindhoven, the Netherlands were investigated in this study. Patients diagnosed with an adenocarcinoma in the colon between 1995 and 2002 and staged as any T, N+, Mo (stage III) were selected for this retrospective study. Patient and tumor characteristics are depicted in Table 1. This specific patient population was studied because all patients received adjuvant chemotherapy and the absence of metastasis at time of diagnosis enabled us to study the distant recurrence free interval as a clinical parameter. All patients received postoperative 5-FU based chemotherapy according to the National treatment guidelines. Chemotherapy consisted of leucovorin (20 mg/m²) iv bolus followed by 5-FU (370-425 mg/m²) iv bolus. Both drugs were administered on days 1 to 5 of each cycle (28 days), patients received 6 cycles. For five patients metastatic tissue from liver (n=2, obtained 13 and 27 months after primary surgery), ovarium (n=1, collected 48 months after primary surgery) and peritoneum (n=2, collected 17 and 7 months after primary surgery) was also available.

Patient data were obtained from the Comprehensive Cancer Centre South (Eindhoven, Cancer Registry). Trained registrars recorded the following tumor characteristics: tumor grade (well/ moderately differentiated versus poorly or undifferentiated tumors), postoperative tumor depth (T1/T2, T3, T4) and lymph node involvement (N1, N2). Clinical factors such as adjuvant chemotherapy, year of diagnosis and, if applicable, dates of death were also recorded. Additional data on recurrence dates were retrieved from patient records.
DNA isolation

Genomic DNA was obtained from archival formalin-fixed paraffin-embedded normal and tumor tissues. In the case of tumor specimens, enrichment was performed by macro dissection of tumor areas with more than 50% tumor cells. DNA was extracted by overnight incubation with proteinase K (Merck, Darmstadt, Germany) at 56°C followed by boiling for 5 min. Subsequently, DNA was purified using the high pure PCR template preparation kit (HPPTP kit, Roche Diagnostics, Mannheim, Germany).

VNTR analysis

The VNTR region was amplified by a PCR. The primers and PCR conditions used were previously described by Kawakami et al and were optimized for our laboratory settings. Briefly, primers: 6-FAM 5'-GCGGAAGGGTCCCTGCCA-3' and 5' TCCGAGCCGGCCACAGGCAT-3', 1 unit of AmpliTaq Gold Polymerase, PCR Gold buffer

<table>
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*in case of scale variables the median and range values are depicted.
(Applied Biosystems, Foster City, California, USA), 1.5 mM MgCl\textsubscript{2} and 5% DMSO (Merck, Darmstadt, Germany). Primers were labeled to facilitate, if necessary, more detailed fragment analysis using the ABI Prism 310 Genetic Analyzer. PCR conditions were as follows: pre-heating: 5 min. at 95 °C; 40 cycles: 45 seconds at 95°C, 45 seconds at 58°C, 45 seconds at 72°C and 5 min. at 72°C. The presence of the 3R (135 bp) or 2R (107 bp) repeats was evidenced after electrophoresis on a 2% agarose gel and ethidium bromide staining. In the case of heterozygous patients, both PCR products were observed.

**SNP analysis**
Digestion of the VNTR PCR products with HaeIII during 1 hour at 37°C (New England Biolabs, Ipswich, United Kingdom) was used to detect the SNP of a G to a C substitution in the 3R allele\textsuperscript{8}. In the case of a SNP, an additional 94 bp fragment was observed after gel electrophoresis on a 3% agarose gel and ethidium bromide staining. In order to study the SNP in the first repeat of the 2R allele which has been recently described\textsuperscript{13,14}, fragments after digestion were additionally analyzed with the ABI Prism 310 Genetic Analyzer.

**1494del6 analysis**
The TS 6-bp deletion polymorphism at nucleotide 1494 was assessed using PCR and primers 6-FAM 5'-CAAATCTGAGGGAGCTGAGT -3’ and 5'-CAGATAAGTG GCAGTACAGA-3’\textsuperscript{30}, 1 unit of AmpliTaq Gold Polymerase, PCR Gold buffer (Applied Biosystems) and 2.5 mM MgCl\textsubscript{2} for amplification. PCR conditions were similar to the ones described for the VNTR analysis. PCR products were analyzed with the ABI Prism 310 Genetic Analyzer in order to distinguish between the +6-bp and -6-bp alleles.

**Allelic imbalance**
*Loss of heterozygosity*
Loss of heterozygosity (LOH) was analyzed in patients heterozygous for the VNTR polymorphism (2R/3RG, 2R/3RC) (n=16). LOH was determined by assessment of the peak surfaces ratios acquired by ABI Prism 310 Genetic Analyzer. After analysis in normal tissue the peak surface of the 2R allele was divided by the surface of the 3R allele (ratio in normal tissue). This number was divided by the ratio between the 2R and 3R allele found in tumor tissue. An obtained LOH ratio of 0.76-1.00 or 1.00-1.30 indicated retention of both alleles. A LOH ratio of ≤ 0.59 (loss 3R) or ≥ 1.70 (loss 2R) indicated LOH. Intermediate ratios (0.60-0.75 and 1.3-1.69) were considered as inconclusive.

**TS gene amplification**
TS gene amplification was determined by a newly developed multiplex quantitative real time PCR using a LightCycler 2.0 apparatus (Roche Molecular Biochemicals, Mannheim, Germany) and Taqman probes. TS gene copy number was normalized to the reference β-
globin gene. For both genes, specific Taqman probes (Applied Biosystems) were newly designed to anneal to region to be amplified as a duplex PCR assay by using the LightCycler ProbeDesign software. Two differently labeled Taqman probes enabled simultaneous quantification of the TS and reference gene; respectively FAM-5’-AGGCCATTACTTTGCCATAATTGTACGACC-3’ and VIC-5’-AGTCTGGCCTACTGCCCTGTGG-3’. In case of the TS gene, PCR primers with the following sequences were used: 5’-GCTTTGGGAAAGGTCTGG-3’ and 5’-CGGACATGAGGACAGAATTAC-3’. These primers amplified a portion of the TS gene resulting in a fragment of 99-bp. PCR primers used for the reference gene (β-globin), 5’-ACACAACTGTGTTCAC TAGC-3’ and 5’-CAACTTCATCCACGTTACCC-3’ resulting in a fragment of 110-bp were previously described. The PCR reaction for both genes was performed using a LC FastStart DNA Mastermix Plus Hybridization Probes kit (Roche). PCR conditions were 10 min. 95°C; 45 cycles of 10 seconds at 95°C, 10 seconds at 55°C, 10 seconds at 72°C. The normalized amplification rate (relative quantification) was calculated with the Light Cycler Relative Quantification Software (Roche). A TS / β-globin ratio of > 1.5 was considered positive for TS gene amplification. This amplification ratio was calculated by dividing the ∆Cp of the target gene by the ∆Cp of the reference gene.

**Immunohistochemistry**

Immunohistochemical staining of TS was performed on 4 µm sections obtained from paraffin-embedded normal and tumor tissues using the TS-106 antibody (1:1000, DakoCytomation, Glostrup, Denmark, kindly provided by DakoCytomation, the Netherlands). This clone is commonly used to investigate TS with IHC, as reviewed by Popat et al. Staining was performed using the PowerVision plus method (ImmunoVision, Brisbane, California, USA) and visualization occurred with 3,3’-diaminobenzide hydrochloride solution (DAB). Briefly, paraffin sections were de-waxed and re-hydrated. Endogenous peroxidase activity was blocked by incubation for 30 min. in aquadest containing 3% H₂O₂ (Fisher Chemicals, Fair Lawn, New Jersey, USA) and 0.1% sodium azide (Fisher Chemicals). Antigen retrieval consisted of boiling in an EDTA buffer (pH 9.0). Subsequently, slides were allowed to cool down for at least 30 min. Before applying the antiserum, slides were pre-treated with 0.5% casein (Sigma-Aldrich, St. Louis, Missouri, USA) and 0.1% sodium azide in phosphate-buffered saline (PBS) for at least 10 min. To reduce non-specific staining, antibodies were dissolved in PBS with 2% goat serum (Gibco, Invitrogen Corporation Carlsbad, California USA) and 0.1% sodium azide. Next, slides were incubated in a humidity chamber with the primary antibody for 20 hours (overnight) at 4 °C. Staining was developed with PowerDAB (ImmunoVision) during 5 min. Slides were counterstained with hematoxylin solution, dehydrated and enclosed with Permount (Fisher Chemicals). All reactions were performed at room temperature, unless stated otherwise. All slides were stained simultaneously in order...
to reduce inter-sample variability. Test slides of normal and cancer tissue revealed that (immature) lymphocytes were intensely stained and that normal epithelial cells were primarily stained in the basal area of the colonic crypts. These observations were in agreement with the performance characteristics of the antibody as mentioned in the DakoCytomation datasheet.

TS staining intensity and localization were evaluated in matched tumor and normal tissues by two observers, one being a pathologist (ITG), who were not acquainted with patients’ clinical outcome. Slides were evaluated with respect to several IHC parameters by both observers at the same time in order to achieve a consensus score immediately. Staining intensity was categorized as follows: (0) no staining, (1) weak staining, (2) moderate staining and (3) intense staining (Figure 1). Immature lymphocytes displayed a consistently high TS staining intensity (3) and were therefore used as an internal reference point. In addition to the overall staining intensity (the intensity that was observed in more than 50% of tumor cells), the strongest staining intensity and percentage of cells stained with this intensity were also assessed. Furthermore, the staining localization was scored as nuclear (N) or nuclear and cytoplasmatic (N+C). Finally, the ratio between the overall intensity observed in the tumor and normal epithelia was assessed. A ratio of 0 indicated equal staining intensities. When the normal tissue showed a stronger intensity than the tumor tissue, the ratio was −. In the case of reverse observations the ratio was scored as +.

Assignment to TS categories
Patients were categorized into low or high TS producers based on their genotypes (Table 2) and immunohistochemically assessed protein levels. Dichotomization into low and high TS producers was performed for both tumor and normal tissue. Heterozygous individuals were categorized as high producers following a dominant model. The 3RC allele was considered to encode for amounts of TS proteins equal to a 2R allele. Therefore, the 3RC/3RC and 2R/3RC genotypes were categorized as low TS producers. In case of IHC, TS levels were dichotomized based on the chromogen intensity as follows: low TS producers: an overall staining intensity of 0-1, high TS producers: an overall staining intensity 2-3 as described by several other studies.19,22,32,33

Statistical analysis
Relations between various parameters were analyzed using the Chi-square and One-way ANOVA method. The correlation of the TS amplification assay which was performed in duplicate was determined using the Pearson correlation. Survival analyses of time to cancer related death or the occurrence of distant metastasis were performed using the Kaplan-Meier method, with the time of surgery as entry date. Patients who experienced were excluded from survival analysis (n=4) since these patients probably already had
metastasis at time of diagnosis that were undetected. These patients had 2R/2R (n=2) and 2R/3R (n=2) genotypes and will be categorized as low (2) and high (2) TS producers, respectively, after dichotomization.

Differences in observed survival between groups were tested for statistical significance using log-rank tests. A p-value of < 0.05 was considered statistically significant.
RESULTS

Distribution of TS polymorphisms
VNTR analysis and subsequent digestion resulted in clear visible bands after gel electrophoresis (Figure 2A, B). The presence of a 6 bp deletion in the 3’UTR region could be easily analyzed after detailed fragment analysis with the ABI Prism Genetic Analyzer (Figure 2C).

Figure 2: Representative images of TS PCR products after gel electrophoresis. A: Variable number of tandem repeat (VNTR) analysis, B: Subsequent digestion of the amplified VNTR region. The appearance of a fragment of 94 bp indicates a base pair substitution of a C to a G. In panel C: PCR products after analysis with the ABI Prism 310 Genetic Analyzer are depicted. M=marker.

The distribution and categorization of the three different polymorphisms is depicted in Table 2. The majority (42%) of the patients was found to be heterozygous for the VNTR polymorphism. Although 4 up to 9 repeats in the VNTR region have been described, no alleles with more than three tandem repeats were observed. Seventeen of the 33 3R alleles (52%) revealed to have a G to C substitution. Combining the VNTR polymorphism
with the SNP analysis resulted in the following genotype distribution: 32% 2R/2R, 3% 2RC/2R, 21% 2R/3RC, 21% 2R/3RG, 10% 3RC/3RC, 10% 3RG/3RG and 3% 3RG/3RC (Table 2). The majority (58%) of the patients had no 6-bp deletion in the 3’UTR region. VNTR with subsequent SNP analysis were performed in both tumor and normal tissue. Genotypes were similar in both groups except for heterozygous individuals exhibiting LOH.

### TABLE 2: DISTRIBUTION OF THE TS POLYMORPHISMS

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<tr>
<th>POLYMORPHISM</th>
<th>GENOTYPE</th>
<th>FREQUENCY</th>
<th>PREDICTED LEVEL OF PROTEIN EXPRESSION</th>
</tr>
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</tr>
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<td>2R/3R</td>
<td>16 (42%)</td>
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</tr>
<tr>
<td></td>
<td>3R/3R</td>
<td>9 (24%)</td>
<td>High</td>
</tr>
<tr>
<td>VNTR+SNP*</td>
<td>2R/2R</td>
<td>12 (32%)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2RC/2R</td>
<td>1 (3%)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2R/3RG</td>
<td>8 (21%)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2R/3RC</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>3RC/3RG</td>
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</tr>
<tr>
<td></td>
<td>3RC/3RC</td>
<td>4 (10%)</td>
<td>Low</td>
</tr>
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<td>VNTR+SNP+LOH#</td>
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<td>6 (37%)</td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>+bp/-6bp</td>
<td>15 (39%)</td>
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</tr>
<tr>
<td></td>
<td>-6bp/-6bp</td>
<td>1 (3%)</td>
<td>Low</td>
</tr>
</tbody>
</table>

*A* dominant model, #only analyzed in tumor tissue of patients heterozygous for the VNTR polymorphism (n=16). Abbreviations: R: retention, IR: intermediate ratio, wt: wild type

### Allelic imbalance

*Loss of heterozygosity*

LOH was found in tumor specimens in eight of sixteen (50%) patients heterozygous for the VNTR polymorphism. Of the remaining eight patients, retention and intermediate ratios were observed in four patients each (Table 2). Additional LOH analysis affected the tumor genotype in 21% of the total patient population. Loss of the 3R allele was most frequently observed (75%). Heterozygous tumors with loss of the 3R allele were categorized as low TS producers and tumors with loss of the 2R allele remained high TS producers.
TS gene amplification
In this study a new real time PCR was developed to determine TS gene amplification. This was done by comparing the amount of PCR products of the β-globin reference gene with the TS gene. For this it is crucial that the efficiency, as measured by the curve slopes, of both PCR reactions is comparable. Our data showed that in control DNA (15 ng/μg human genomic DNA, LightCycler control kit DNA, Roche Applied Science) the efficiency of the TS and β-globin amplification gene was equal; also after analyzing a tenfold dilution-series. The curve shape and slope as observed after analysis of the control DNA were confirmed in formalin-fixed paraffin-embedded tissue. Serial dilutions of DNA isolated from tumor samples showed similar amplification curves for the TS and β-globin gene (Figure 3A). TS gene amplification could be determined by this newly developed assay in 37 of the 38 patients (Table 3). Amplification was performed in duplicate. The correlation between these two measurements was highly significant (p<0.001, correlation coefficient = 0.927, Pearson correlation) indicating

Figure 3: Amplification curves observed after the newly developed real time PCR assay, performed on DNA obtained from formalin-fixed paraffin-embedded tissue. A dilution series (panel A) and an example of TS gene amplification (panel B) are depicted. Panel A: amplification curves of the TS gene (solid line) and β-globin gene (dotted line), *: undiluted, **: 10X diluted, ***: 100X diluted. The Δ indicated the difference of Cp (crossing point) values between the TS and reference gene for each dilution. The Cp value is defined as the cycle at which the fluorescent signal exceeds the threshold value as determined by the automatic fit point module. Panel B: S: sample (tumor DNA), R: reference (normal DNA), Δ: difference in Cp values between the TS and β-globin gene. The amplification ratio was calculated by dividing the ΔCp of the target gene by the ΔCp of the reference gene.
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<th>GN</th>
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<th>IHC N</th>
<th>IHC T*</th>
<th>Mean amp ratio PT*</th>
<th>IHC M</th>
<th>Mean amp ratio M</th>
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<td>TS 35</td>
<td>2/3G</td>
<td>2/3G</td>
<td>-6bp/+6bp</td>
<td>2</td>
<td>1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS 36</td>
<td>3C/3C</td>
<td>3C/3C</td>
<td>+6bp/+6bp</td>
<td>1</td>
<td>0</td>
<td>1.1</td>
<td>2</td>
<td>0.88</td>
</tr>
<tr>
<td>TS 37</td>
<td>3G/3C</td>
<td>3G/3C</td>
<td>-6bp/+6bp</td>
<td>1</td>
<td>0</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS 38</td>
<td>2/2</td>
<td>2/2</td>
<td>+6bp/+6bp</td>
<td>2</td>
<td>1</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: G: genotype, T: tumor, N: normal, PT: primary tumor, M: metastasis, amp ratio: amplification ratio of the TS gene * the mean amplification ratio was found to be correlated to the IHC intensity as observed in the majority of the tumor cells (p<0.001). This correlation is also depicted in Figure 5.
that the amplification assay was highly reproducible. A mean amplification ratio of \( >1.5 \) was found in 2 out of 37 tumors (5%) (Figure 3B). In addition, one out of five (20%) metastasis had a mean ratio of \( >1.5 \). The primary tumor (TS03) of the metastasis with TS gene amplification did not demonstrate amplification (mean ratio 1.0).

**Immunohistochemical assessment of TS**

Respectively 11%, 47%, 37% and 5% of the tumors displayed an overall staining intensity of 0, 1, 2 and 3 (Table 4). Overall TS staining intensity in the normal mucosa could be evaluated in 36 out of 38 patients; normal tissue could not be found in one patient, in the case of another patient IHC of normal tissue could not be assessed due to an artefact. Staining was predominately localized in the nucleus (tumor tissue: 71%, normal tissue 89%).

<table>
<thead>
<tr>
<th>IHC SCORE</th>
<th>NORMAL</th>
<th>TISSUE TUMOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2 (6%)</td>
<td>4 (12%)</td>
</tr>
<tr>
<td>1</td>
<td>8 (22%)</td>
<td>18 (47%)</td>
</tr>
<tr>
<td>2</td>
<td>23 (64%)</td>
<td>14 (37%)</td>
</tr>
<tr>
<td>3</td>
<td>3 (8%)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Localisation</td>
<td>n: 32 (89%)</td>
<td>n: 27% (71%)</td>
</tr>
<tr>
<td></td>
<td>n+c: 4 (11%)</td>
<td>n+c: 11 (29%)</td>
</tr>
</tbody>
</table>

**Prognostic significance**

The different IHC parameters (overall and strongest staining intensities, staining localisation and the ratio of staining between normal and tumor cells) failed to demonstrate a significant association with distant recurrence (DR) and cancer specific survival (CSS). TS dichotomization based on the VNTR analysis in normal tissue (as summarized in Table 2) was found to be of prognostic significance for DR \( (p=0.038) \) and CSS (5-year survival proportions for TS low: 88 % and TS high: 37%, \( p=0.021 \)) (Figure 4A, B). As illustrated in Figure 4C, additional SNP analysis of DNA extracted from normal tissue did not add prognostic information. This was also observed for DR.

Although it was decided on forehand to exclude 4 patients who experienced a metastasis during the administration of chemotherapy (i.e. within six months after surgery) because these patients would probably have stage IV disease at time of diagnosis instead of stage III disease, we also performed survival analysis on the total population based on clinical practice at time of diagnosis (CSS: TS low: 74%, TS high: 34%, \( p=0.076 \); DR: TS low: 33%, TS high: 60%, \( p=0.148 \)). In contrary to the IHC parameters, a strong trend
towards a significant difference between TS low and high producers, especially with respect to CCS, could still be observed after analysis of the total population, confirming that TS genotyping is a better prognostic marker than IHC. Most likely the outcome in stage IV disease is rather a product of both the intrinsic tumor biology and sensitivity to 5FU and it would be interesting to further study this in a larger study population.

**Figure 4: Kaplan-Meier survival curves of A: distant recurrence (DR) and B cancer specific survival (CSS). TS low: solid line, TS high: dotted line. Patients developing metastasis within 6 months were excluded from analysis. C: Kaplan-Meier survival curve demonstrating the effect of SNP analysis in normal tissue. Patients with 3RC/3RC or 2R/3RC genotype (striped line) behave differently from patients with the 2R/2R genotype (solid line).**

**Correlation between TS genotype and IHC**

No correlation was found between the predicted protein levels (high, low) based on TS polymorphisms and overall or strongest TS staining observed after IHC. This correlation was lacking for both tumor and normal tissues. Combining the strongest staining intensity with the percentage of maximal stained cells also did not result in a significant correlation with TS genotype. The other IHC parameters (staining localization, percentage of cells with strongest staining and staining ratio between normal and tumor tissue) also failed to show a correlation with TS genotype. However, the overall staining intensity was significantly correlated ($p=0.001$, One-Way ANOVA) with the mean amplification ratio (Figure 5). This $p$ value was still significant after correcting for multiple comparisons ($p<0.0083$ in case of 4 groups). This relationship was mostly pronounced in tumors with gene amplification because these tumors were intensely stained in specific subpopulations of tumor cells as depicted in Figure 1H. The observed staining pattern of this tumor is very heterogeneous; some tumor cells are completely negative and others display an intensely stained nucleus.
Figure 5: Scatter plot depicting the correlation between TS gene amplification in the primary tumor and TS staining intensity as observed in the majority of the tumor cells. T=tumor.

DISCUSSION

This study shows that the intrinsic variable number of tandem repeats (VNTR) was the only polymorphism of the TS gene demonstrating a significant association with distant recurrence (DR) and cancer specific survival (CCS) (Figure 4A, B). Assessment of the other two polymorphisms; the single nucleotide polymorphism (SNP) and the 3’UTR deletion (1494del6) did not add prognostic information. The different TS IHC parameters were also not predictive for patients’ outcome.

The clinical implications of VNTR analysis with respect to the favorable outcome of patients with a 2R/2R genotype in the adjuvant setting was similar to that of several other studies \(^{11,36,37}\). In addition, the lack of prognostic information of VNTR combined with SNP analysis is in accordance with the findings of Dotor et al \(^{39}\). This was clearly
illustrated in Figure 4C; patients who had low predicted protein levels due to a SNP in the 3R allele had a worse prognosis than patients with a 2R/2R genotype. However, others found that the 3R/3R genotype was associated with a better outcome which is in contrast with our findings. This observation was confirmed by Jakobsen et al investigating patients with disseminated disease. However, nothing can be said about the differences in 5-FU sensitivity alone because the outcome is affected by both the intrinsic tumor biology and sensitivity to 5-FU.

In contrast to TS genotyping, this study demonstrated that IHC is not predictive for therapy response. A predictive role of TS IHC, in the adjuvant and advanced settings, was found in some studies but enfeebled by others. This supports that TS IHC is prone to inter-laboratory variation and is not adequate for quantification of protein levels. Differences in tissue fixation, observer dependant assessment of staining patterns, severe heterogeneity in staining intensities between cells and most importantly, antibody binding may all cause an inaccurate reflection of the protein content of the specimen.

In the present study, 52% of the 3R alleles revealed to have a G to C substitution. The frequency of this SNP varies between ethnic groups and has been described to be 56% for Caucasians which is in agreement with our findings. Even though SNP analysis did not add prognostic information, it would have been desirable to study LOH in patients with three repeats heterozygous for the SNP because it can affect the predicted protein levels. i.e., 3RC/3RG individuals with loss of the 3RG allele would have low predicted protein levels. However, with current techniques, LOH analysis in 3RC/3RG individuals was found to be too challenging from a technical point of view. In the future, other techniques like primer extension might facilitate this analysis and is presently under investigation. In addition, we identified the recently identified SNP in the fist tandem repeat of the 2R allele in one patient who was homozygous for the VNTR but heterozygous for the SNP in the 2R allele (2R/2RC). The frequency of the 2RC allele has been described to be 4.2% to 1.5%. In our population, the frequency of the 2RC allele was found to be 1.3% which is consistent with previous findings.

Although several in vivo and in vitro studies indicated that TS genotype and TS protein levels are correlated, the present study indicated that this correlation could not be confirmed with IHC. Patients with a 3R/3R genotype did not show higher TS staining intensities than patients with a 2R/2R genotype. This finding might explain the discrepancies in literature concerning the predictive value of TS as indicated by the ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. Dotor and co-workers also observed that the three polymorphisms were not associated with IHC staining intensities (evaluated in 129 tumors). Besides the technical
considerations of IHC, several other mechanisms could account for the finding in the present study that IHC did not confirm the TS protein levels predicted by genotyping. Firstly, a variety of post-translational mechanisms such as protein glycosylation, folding and protein-protein interactions can influence epitope-availability of monoclonal antibodies directed against TS. Secondly, the antibody used largely affects the staining pattern. Indeed, even using antibodies of the same clone (TS-106) but from different companies (Abcam versus DakoCytomation) resulted in poor reproducibility (data not shown).

This is the first study reporting an association between TS gene amplification and IHC (Figure 5). This observation makes it tempting to speculate that the variations in TS protein levels caused by the TS polymorphisms are very subtle and can therefore not be clearly visualized with IHC. TS gene amplification, on the other hand, can increase the concentration of TS proteins to such an extent that IHC staining patterns will be more pronounced in those specific tumor cells. Figure 1H depicts a staining pattern which can be best characterized as “dotted” with some tumor cells displaying an intense nuclear staining and others lacking staining. It was also observed that these intensely stained cells often displayed mitotic spindle figures indicating cell proliferation. Since amplification analysis was performed on DNA extracted from a selected tumor area containing >50% tumor cells and tumors are known to be very heterogeneous, the intensely stained cells would probably have an amplification ratio of for example 5 or 6 to compensate for those with no amplification. This hypothesis could be further investigated by using fluorescent in situ hybridization (FISH) since this technique can visualize differences in copy numbers between individual tumor cells.

TS gene amplification is not frequently observed in primary tumors in contrast to tissue exposed to 5-FU based therapy. This is consistent with our results since we found that 5% (2/37) of the primary tumors demonstrated amplification versus 20% (1/5) of the metastatic tissues. This percentage is in accordance with Wang et al. who reports TS gene amplification in 23% of liver metastasis that have been exposed to 5-FU. The single metastasis with TS gene amplification also demonstrated a strong (3) IHC staining intensity, which is in agreement with our finding that gene amplification is correlated with IHC staining intensity. However, due to small patient numbers firm conclusions cannot be drawn. Treatment with 5-FU might provoke selection of tumor cells with TS gene amplification. Loss of heterozygosity (LOH), which can also result in allelic imbalance, was frequently reported at the site of the TS gene. LOH frequencies of 63% up to 73% were found in patients with a 2R/3R genotype. These findings were confirmed by the present study which reported LOH in eight of sixteen (50%) patients heterozygous for the VNTR polymorphism. Although, we have performed macro dissection for purposes of tumor enrichment, the percentage of LOH reported
could be underestimated if substantial numbers of normal cells are still present.

Although the number of patients in the present population is relatively small (38 patients), significant differences regarding CSS and DR based on VNTR analysis in germline DNA were obtained. Studying these germ line DNA alternations has several advantages. Analysis can be performed on readily available tissue such as peripheral blood and does not require harvesting of tumor tissue. Moreover, genomic polymorphisms are stable providing certain robustness in favor of DNA based assays. To validate the data obtained by this study, TS genotyping will be further investigated in a large group of well defined patients with colorectal cancer to study the predictive value for response to 5-FU.
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29 Kawakami K, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human


TS genotyping versus IHC
PREOPERATIVE RADIOCHEMOTHERAPY IS SUCCESSFUL ALSO IN PATIENTS WITH LOCALLY ADVANCED RECTAL CANCER WHO HAVE INTRINSICALLY HIGH APOPTOTIC TUMOURS

In press, Annals of Oncology
**Background**
Not all patients with locally advanced rectal cancer (LARC) respond equally to neo-adjuvant radiochemotherapy (RCT). Patients with highly apoptotic less advanced rectal cancers do not benefit from short-term radiotherapy. This study investigates whether this is also the case in the setting of RCT for LARC.

**Patients and methods**
Tissue micro arrays (TMA) were constructed of biopsy and resection specimens of 201 LARC patients. Apoptosis (M30) and several apoptosis regulating proteins (p53, Bcl-2, Bax, cyclooxygenase-2 and maspin) were studied with immunohistochemistry. Subsequently, predictive values for local recurrence (LR), overall survival (OS) and histological tumour regression were analyzed.

**Results**
Apoptotic levels, quantified as the number of apoptotic cells/mm² tumour epithelium, were higher in post-therapy tissues compared to biopsies (p<0.001). Biopsies from cT4 tumours demonstrated significantly higher levels of apoptosis than cT3 tumours (p=0.020). Therapy-induced apoptosis was higher when the interval between the last day of irradiation and surgery increased (p<0.001, cc=0.355). Pre- and post-therapy apoptosis, p53, Bcl-2, Bax and Cox-2 were not associated with LR, OS or tumour regression. Intense pre-therapy cytoplasmic staining of maspin indicated a higher risk on LR (p=0.009) only.

**Conclusion**
Combined RCT is also successful in highly apoptotic tumours and is therefore independent of intrinsic apoptosis.
INTRODUCTION

Patients with locally advanced rectal cancer (LARC) are at high risk of local failure. Therefore, surgery alone is often not curative and neoadjuvant radio(chemo)therapy (RCT) is required in order to improve local control and achieve a radical resection. Tumour response to RCT varies considerably and various stages of histological tumour regression can be observed in the surgical resection specimens. Markers that can predict therapy outcome would permit adaptation of the therapeutic strategy and improve treatment of LARC; e.g. poor responders could be offered a different therapy regimen or could be operated much sooner with more extensive surgery.

In order to prevent local recurrences and improve local control, patients with mobile rectal cancer (T1-T3) currently receive short-term neoadjuvant radiotherapy as standardized treatment for rectal cancer in the Netherlands. However, not all patients benefit equally; some might experience side effects. Therefore, we would like to predict tumour response. De Bruin et al showed that levels of apoptosis (assessed with M30) can attribute to the selection of patients with a high risk on local failure, since radiotherapy was effective in patients with low levels of intrinsic apoptosis, but patients who had high levels of intrinsic apoptosis in their primary tumour and did not receive radiotherapy had the same risk on developing a local recurrence as patients that did receive radiotherapy. It is however unclear what the prognostic implications of intrinsic and therapy-induced apoptosis are in patients with LARC in the neoadjuvant setting.

Radiotherapy and chemotherapy can both induce apoptosis in malignancies of the gastrointestinal tract. However, the complexity of the apoptotic pathway enables tumour cells to escape from apoptosis inducing therapy resistance and affecting patient’s outcome. At the cellular level, the regulation of apoptosis depends on a very complex balance between pro- and anti-apoptotic proteins. Amongst them are p53, Bcl-2, Bax, cyclooxygenase-2 (Cox-2) and mamma serine protease inhibitor (maspin). The extensively described oncogene p53 is commonly inactivated in colorectal cancer and is a cell cycle regulator and a potent inducer of apoptosis as a response to DNA damage. Bcl-2 and Bax act as antagonists and are members of the same family of proteins. While Bcl-2 is an important inhibitor of apoptosis, over expression of Bax induces programmed cell death. During irradiation, Cox-2 has been described to act as a survival factor by inhibiting apoptosis. Maspin is a multifaceted protein that has been described to promote apoptosis.

In the present study we evaluated the prognostic value of intrinsic and induced
apoptosis (M30) and 5 different apoptosis regulating proteins: p53, Bcl-2, Bax Cox-2 and maspin, in patients with LARC who were treated with RCT. Local recurrence and overall survival were the main outcome parameters. In addition, the association between these markers and histological tumour regression was investigated.

PATIENTS AND METHODS

Patient selection
The patient population consisted of a consecutive series of 201 LARC patients with biopsy proven adenocarcinoma as described previously. All patients received multimodality treatment at the Catharina hospital between 1994 and 2005. Treatment decision was based on the best regimen according to the national guidelines of that moment in time.

Histological assessment of therapy-induced tumour regression
Histological therapy-induced tumour regression was assessed according the regression grading system described by Rödel et al. The degree of tumor was estimated and subdivided into the following categories: 0: no regression or < 25% of tumour mass, 1: 25% to >50% tumour regression, 2: complete regression. The degree of tumour regression was determined semi quantitatively by an experienced pathologist (JHMvK) who was unfamiliar with patients’ clinical outcome. The investigations of the clinical value of therapy-induced tumor regression assessment in this patients population has been extensively described in a previous study.

Tissue microarray construction
Tissue micro arrays (TMA) were constructed from formalin-fixed, paraffin-embedded biopsy tissues (three 0.6 mm punches) and resection specimens (three 2 mm punches) using an Alphelys TMA booster (Westburg, Leusden, the Netherlands). Three punches have been previously described to be sufficient to overcome tumour heterogeneity. Moreover, most studies validating the use of TMA for studying markers with IHC advise to use tree to four punches of 0.6 mm. We used an equivalent of 10 tumor punches of 0.6 mm which is well above the advised number of 0.6 mm punches. In case normal tissue was available, one punch was also taken from this area in order to construct a TMA containing normal tissues only. Preferably, normal tissue adjacent to the tumour this tissue was used (55%). If this was not present, normal tissue was obtained from another paraffin block (45%).

Immunohistochemistry
Immunohistochemical stainings were performed on 4 µm TMA sections using the
PowerVision plus method (ImmunoVision, Brisbane, California, USA). Visualization involved incubation with PowerDAB (ImmunoVision) for 5 min. Methods for antigen retrieval and the dilutions of the antibodies used can be found in Table 1. Slides were incubated in a humidity chamber with the primary antibody for 20 hours (overnight) at 4 °C. Negative controls involved incubation with PBS with 2% goat serum and 0.1% sodium azide only. Slides were counterstained with hematoxylin. Staining patterns of p53, Bcl-2, Bax Cox-2 and maspin were all evaluated semi-quantitatively (Table 1) by two independent observers who were unfamiliar with patients’ outcome. In case of disagreement, a consensus score was obtained which was used for further analysis.

<table>
<thead>
<tr>
<th>ANTIBODY ( company)</th>
<th>CLONE</th>
<th>DILUTION</th>
<th>ANTIGEN RETRIEVAL</th>
<th>IHC ASSESSMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>M30 CytoDEATH (Roch, Roche Applied science, Indianapolis, USA)</td>
<td>M30 specific for human cytokeratine 18</td>
<td>1:500</td>
<td>Incubation with a 10mM sodium citrate buffer (pH6.0) for 30 min at 98°C</td>
<td>Number of apoptotic cells per mm² tumour epithelial was quantified</td>
</tr>
<tr>
<td>P53 (BioGenex, San Ramon, USA)</td>
<td>BP 53-12</td>
<td>1:10,000</td>
<td></td>
<td>Intensity of nuclear staining (0-3) and the proportion of negative cells: 10-30%, 30-60%, or 60-90%*</td>
</tr>
<tr>
<td>Bcl-2 Oncoprotein (Dako, Glostrup, Denmark)</td>
<td>124</td>
<td>1:500</td>
<td></td>
<td>Intensity of cytoplasmic staining (0-3)*</td>
</tr>
<tr>
<td>Bax (Santa Cruz Biotechnology, Santa Cruz, USA)</td>
<td>B-9</td>
<td>1:1000</td>
<td></td>
<td>Intensity of cytoplasmic staining (0-3)*</td>
</tr>
<tr>
<td>Cox-2 (Cayman Chemicals, Ann Arbor, USA)</td>
<td>CX229</td>
<td>1:100</td>
<td></td>
<td>Combination of intensity and proportion of cytoplasmic staining*[^12,18]</td>
</tr>
<tr>
<td>Maspin (Becton Dickinson Pharmingen, Franklin Lakes, USA)</td>
<td>G167-70</td>
<td>1:20,000</td>
<td></td>
<td>Intensity of cytoplasmic and nuclear staining (0-3)*</td>
</tr>
</tbody>
</table>

* Staining patterns were assessed by two independent observers. Abbreviations: IHC: immunohistochemistry
Quantification of apoptosis
In case of M30 immunohistochemistry, the number of apoptotic tumour cells per mm² tumour epithelium was assessed for each punch. In order to establish the area of tumour epithelium for each punch, TMA were stained with the pancytokeratin MAK-6 (1:10, protease antigen retrieval, Zymed Laboratories, Invitrogen, Carlsbad, USA). Microscopic images measuring 0.74 mm² were digitized using an RGB CCD camera (AxioCam MRc, Zeiss, Germany), resulting in a specimen level pixel size of 1.3x1.3um². Quantitative measurements of the tumour area or area of normal epithelium were performed using a digital image analysis system (KS400, Carl Zeiss, Germany). Punches of 0.6mm diameter were analyzed using a 10X objective and 2 mm punches using an objective of 2.5X. Digitized images were corrected for unequal illumination using a stored image of an empty microscopic field. The immuno-positive area (in mm²) was calculated automatically. Subsequently, the number of apoptotic cells was manually counted to calculate the apoptotic level defined as the number of apoptotic cells per mm² tumour epithelium. Values that exceeded the standard deviation two times were considered outliers and were therefore excluded from further analysis.

Statistics
Data were analyzed with the SPSS package (Statistical Product and Service Solutions 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA). Inter-observer variability was calculated by κ statistic as described by Cohen. κ-values of 0.2 to 0.4 indicate “fair”, 0.4 to 0.6 “moderate”, and values of > 0.6 “excellent” agreements. Correlations between apoptosis and the interval between the last day of irradiation and surgery were analyzed according to the Spearman rank correlation test. Univariate survival analyses of time to death or local recurrence were performed using the Kaplan-Meier method and log-rank testing with the time of surgery as the entry date. Patient outcome parameters were local recurrence (LR), and overall survival (OS). P-values of ≤ 0.05 were considered as statistically significant.

Multiple variable analyses involved Cox’s proportional hazards regression (enter method), logistic regression (enter method) and building of decision trees using the DTREG software for predictive modeling and forecasting (www.dtreg.com). Single tree models for classification using the Gini splitting algorithm in which the variables were equally weighted were constructed. Pruning and validation of the tree model was performed with the V-Fold cross validation in order to determine the statistically optimal tree size.
RESULTS

Patient and tumour characteristics
Patient, treatment and tumour characteristics are summarized in Table 2. The majority (59%) of the patient population initially presented with a tumour invading into other adjacent organs or structures (cT4). Complete histological tumour regression was observed in 21 (11%) patients. Poor (Rödel 0) and moderate (Rödel 1) tumour regression grades were observed in 50% and 39% patients, respectively.

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>CATEGORY</th>
<th>n(%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, (range)</td>
<td></td>
<td>63, (35-8)</td>
</tr>
<tr>
<td>Sex</td>
<td>male</td>
<td>122 (61%)</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>79 (39%)</td>
</tr>
<tr>
<td>cT-stage</td>
<td>cT3</td>
<td>83 (41%)</td>
</tr>
<tr>
<td></td>
<td>cT4</td>
<td>118 (59%)</td>
</tr>
<tr>
<td>Neoadjuvant therapy</td>
<td>RT</td>
<td>74 (37%)</td>
</tr>
<tr>
<td></td>
<td>RCT</td>
<td>102 (51%)</td>
</tr>
<tr>
<td></td>
<td>interrupted</td>
<td>25 (12%)</td>
</tr>
<tr>
<td></td>
<td>continuos</td>
<td></td>
</tr>
<tr>
<td>Median interval: last day RT-surgery</td>
<td>7.8* weeks</td>
<td></td>
</tr>
<tr>
<td>CRM</td>
<td>positive</td>
<td>43 (21%)</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>158 (79%)</td>
</tr>
<tr>
<td>ypT-stage</td>
<td>ypT0</td>
<td>20 (10%)</td>
</tr>
<tr>
<td></td>
<td>ypTis</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td></td>
<td>ypT1</td>
<td>2 (1%)</td>
</tr>
<tr>
<td></td>
<td>ypT2</td>
<td>15 (7%)</td>
</tr>
<tr>
<td></td>
<td>ypT3</td>
<td>132 (66%)</td>
</tr>
<tr>
<td></td>
<td>ypT4</td>
<td>30 (15%)</td>
</tr>
<tr>
<td></td>
<td>n.a.</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>ypN-stage</td>
<td>No</td>
<td>131 (65%)</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>47 (24%)</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>23 (11%)</td>
</tr>
<tr>
<td>Histologic regression</td>
<td>0</td>
<td>101 (50%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>79 (39%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21 (11%)</td>
</tr>
<tr>
<td>Pre-treatment apoptosis</td>
<td></td>
<td>0*</td>
</tr>
<tr>
<td>(mean of cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-treatment apoptosis</td>
<td></td>
<td>10.39%</td>
</tr>
<tr>
<td>(mean of cells)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RT: radiotherapy, RCT: radiochemotherapy, CRM: circumferential margin, n.a.: not assessed, *: not a number but the mean or median is given.
Apoptosis; prognostic significance and correlations with clinicopathological factors

Representative staining patterns observed after M30 IHC are depicted in Figure 1A. The apoptotic levels in neither the post-therapy resection specimens nor the pre-treatment biopsies were associated with (LR) or (OS). The amount of apoptotic cells was significantly higher in post-treatment resection specimens compared to pre-treatment biopsies (median 10.4 vs. 0, p=<=0.001, Wilcoxon test). M30 IHC was also performed on TMA containing normal tissues. This revealed that the amount of apoptosis in normal epithelium was significantly lower in patients who received the capecitabine containing continuous schedule (7.2 apoptotic cells per mm$^2$ epithelium) compared to patients who received the interrupted RCT (15.3 apoptotic cells per mm$^2$ epithelium) schedule (p=0.018). Apoptotic levels did not differ between normal tissues obtained adjacent to the tumour or from another paraffin block (p=0.163). The amount of apoptotic tumour cells did not significantly differ between the different therapy regiments (Table 3).

| TABLE 3. CORRELATION BETWEEN APOPTOSIS AND CLINICOPATHOLOGICAL FACTORS |
|---|---|---|---|---|---|
| FACTOR | CATEGORY | PRE-TREATMENT APOPTOSIS (MEDIAN/MEAN) | P-VALUE | PRE-TREATMENT APOPTOSIS (MEDIAN/MEAN) | P-VALUE |
| cT-stage | cT3 | 0/5.89 | 0.020$^a$ | 11.37/21.12 | 0.57$^a$ |
| | cT4 | 3.65/11.71 | | 9.78/17.95 | |
| Neoadjuvant therapy | RT: interrupted | 0/9.51 | 0.12$^b$ | 11.45/19.23 | 0.97$^b$ |
| | RT: continuous | 2.47/9.43 | | 9.68/20.11 | |
| | | 6.03/2.47 | | 10.71/18.08 | |
| Interval: last day RT-surgery | p=0.34$^a$, cc=0.084 | | p=0.001$^c$, cc=0.328 | |
| ypT-stage | ypT0, ypT1, ypT2, ypT3, ypT4 | 0/8.40 | 0.93$^a$ | 9.28/21.39 | 0.75$^a$ |
| | | 0/9.52 | | 10.39/18.84 | |
| ypN-stage | No | 0/9.45 | 0.23$^b$ | 9.48/19.91 | 0.87$^b$ |
| | N1 | 3.32/10.62 | | 17.14/18.79 | |
| | N2 | 0/5.56 | | 11.45/16.33 | |
| CRM | positive | 0/9.34 | 0.96 | 11.45/18.47 | 0.811 |
| | negative | 0/9.28 | | 9.48/19.50 | |
| Histologic regression | 0 | 0/8.94 | 0.041$^b$ | 12.40/22.31 | 0.003$^a$ |
| | 1 | 5.42/10.80 | | 3.94/10.68 | |
| | 2 | 1.24/5.94 | | n.a. | |

Associations between apoptosis and the different clinicopathological factors can be found in Table 3. Tumours with a clinical T4 stage (cT4) were found to have higher levels of intrinsic apoptosis than cT3 tumours ($p=0.020$). In addition, M30 IHC revealed that a longer interval between last day of RT and surgery resulted in an increased number of apoptotic cells ($p=0.001$, Pearson correlation coefficient $=0.328$), indicating a large effect of this parameter on the post-treatment apoptosis. Apoptotic levels were found to be significantly higher in resection specimens with limited tumour regression (Rödel 0) than in specimens with 25% to >50% tumour regression (Rödel 0, $p=0.003$). Intrinsic apoptosis was not predictive for histological tumour regression since comparable levels of apoptosis were observed in patients with a poor and complete response (Table 3).

**p53, Bcl-2, Bax, Cox-2 and maspin; prognostic significance**
Representative staining patterns after staining for p53, Bcl-2, Bax, Cox-2 and maspin are depicted in panel B, C, D, E and F respectively of Figure 1. As indicated in Table 1, IHC staining patterns for p53, Bcl-2, Bax, Cox-2 and maspin were assessed by two independent observers. In case of the resection specimens, the calculated mean kappa values of the three tumour containing punches were found to be 0.61, 0.79, 0.79, 0.71 and 0.78 respectively for p53, Bcl-2, Bax, Cox-2 and maspin. For pre-treatment biopsies these values were respectively 0.71, 0.85, 0.85, 0.58 and 0.86. These kappa values mainly indicate excellent degrees of agreement. Negative IHC controls showed no staining.

Staining patterns and intensities of p53, bcl-2, Bax, Cox-2 and maspin that were assessed in the biopsies and resection specimens did not correlate with histological tumour regression. Pre-and post-therapy levels of p53, Bcl-2, Bax and Cox-2 did not influence LR or OS rates. Stratification for the different treatment regiments resulted in similar findings.
A strong cytoplasmic staining of maspin in the biopsy tissues was associated with a higher risk on developing a local recurrence (Figure 2). However, this association was not observed after separate analysis of each treatment regimen.

Multiple variable analysis for prediction of patients prognosis and therapy response
Investigation on the associations between apoptosis regulating proteins and apoptotic levels showed that increased nuclear staining of maspin in tumour biopsies were associated with increased levels of apoptosis in biopsy specimens ($p=0.019$). Increased Cox-2 staining intensities in the resection specimens were significantly associated with increased levels of apoptosis ($p=0.028$). However, this is not in consistency with the anti apoptotic effect of Cox-2 that has been described in literature $^8,18$. 
Figure 1: examples of immunohisto-chemical staining patterns observed after staining with M30 CytoDEATH (A), p53 (B), Bcl-2 (C), Bax (D), Cox-2 (E) and maspin (F). The arrows in panel A depict examples of apoptotic tumour cells that demonstrate intense staining with the M30 antibody. Original magnifications B-F: 200X, A: 400X.

→ color figures
Cox’s proportional hazards regression indicated that different combinations of apoptosis and the different apoptosis regulating proteins were not predictive for LR or OS. In order to study whether combinations of apoptosis and the different apoptosis regulating proteins are predictive for tumour regression, logistic regression was performed and decision tree models were built. In the case of both logistic regression analysis and the CART analysis histological tumour regression was dichotomized in two different ways; Rödel 0 and 1 versus Rödel 2 and Rödel 0 versus Rödel 1 and 2. This analysis revealed that none of the variables or variable combinations was predictive for tumour regression.

**DISCUSSION**

Pre-therapy (intrinsic) or post-therapy (induced) levels of apoptosis, p53, Bcl-2, Bax and Cox-2 in tumour cells were not found to be associated with local recurrence (LR) or overall survival (OS) in patients with LARC. Maspin was found to be correlated to LR only (Figure 2). These data implicate that success of neoadjuvant RCT is independent of these factors. In addition, we found that apoptosis of tumour cells was not predictive for the degree of tumour response in these patients.

De Bruin et al also studied the prognostic value of apoptosis in TMA with M30 IHC which is similar to our study design. In concordance with that study we also found that the post-therapy apoptotic levels did not influence patients’ prognosis. However, the study reported by Bruin et al was a randomized trial in which the surgery only
arm acted as a control, while we did have access to pre-treatment biopsies. Tannapfel et al investigated intrinsic and therapy-induced levels of apoptosis in LARC patients who received RCT and did also not find a correlation between apoptosis and LR, OS or tumour regression after RCT which is consistent with our findings. However, other studies found that intrinsic levels of apoptosis were related to the degree of tumour regression and patients’ outcome after long-term neoadjuvant RCT. These differences in literature indicate that the prognostic implications of apoptosis in patients with rectal cancer are not established and need further research in order to determine the clinical usefulness. Since radiotherapy is the main therapy used in the present population, it would also be interesting to study the clinical of apoptosis in the setting of systemic treatment with chemotherapy.

Our finding that apoptotic levels were significantly increased in the surgical resection specimens is in consistency with other reports. Based on the apoptosis inducing potential of radiotherapy and chemotherapy this was not an unexpected finding. Analysis of apoptotic levels in normal epithelial cells yielded the surprising result that the number of apoptotic cells significantly decreased in case of the continuous RCT schedule in comparison to the interrupted RCT schedule. This might be explained by the use of capecitabine in the continuous schedule which is, in contrast to 5-FU, converted into the active metabolite in cells with a higher metabolism delivering the 5-FU predominately to the tumour cells. Another, interesting finding was that when the interval between the last day of irradiation and surgery was longer, the post-therapy apoptotic levels increased. It is tempting to speculate that this observation could partially explain the positive effect of a prolonged interval between radio(chemo)therapy and surgery as indicated by a French trial. Several studies have shown that long course radiotherapy with concurrent 5 fluorouracil (5-FU) based chemotherapy contributed to tumour downstaging and increased local control. However, the optimal doses of radiotherapy and chemotherapy of this multimodality therapy and the type of 5-FU administration and combination with other cytotoxic agents can still be improved.

Our study design has several limitations. Firstly, investigations on post-therapy resection specimens have the general limitation that no tumour can be assessed after a complete response, especially after neoadjuvant RCT. An added problem of TMA evaluation is that after a high degree of tumour regression tumour sampling can be problematic. This can obscure the evaluation of the IHC staining patterns in the post-therapy specimens. Secondly, the complex interactions between the different apoptosis regulating proteins interfere with the analysis of each individual marker. However, we tried to overcome this problem by adding a multiplevariable statistical approach. Numerous reports studying the predictive and prognostic role of apoptosis
controlling proteins can be found. However, the clinical implications of these markers remain elusive due to conflicting data. Our data shows that the regulating proteins did not have prognostic implications and, more importantly, the downstream effect of these markers (i.e. apoptosis of tumour cells) was also clinically irrelevant. This indicates that the discussion about the individual contributions of the regulation proteins is less relevant.

Finally, evaluation of apoptosis with the M30 CytoDEATH antibody detects only apoptotic events in tumour cells or normal epithelial cells; caspase independent apoptosis, mitotic catastrophe and apoptosis of stroma cells, caspase independent apoptosis are not detected. Other reports for example used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphatase-biotin nick-end-labelling (TUNEL) method or measured caspase activity. Evaluation of caspase-3 activity, measuring caspase dependant apoptosis in tumour- and stroma cells, in patients with rectal cancer indicated that low caspase activity in biopsies decreased the risk on LR. These findings could indicate that apoptosis in stroma cells could be of substantial importance as previously described by others. However, fresh frozen tissues are required for this assays which were unfortunately not available in the present patient population.

In the present study we investigated the effect of apoptosis and 5 apoptosis regulating proteins on prognosis and tumour regression in patients with LARC. We studied the effect of apoptosis in patient with LARC by quantifying M30 positive tumor cells and semi-quantitatively analysing p53, Bcl-2, Bax, Cox-2 and maspin. Subsequently we analysed these data in both pre-and post-therapy tumour and normal tissues in a multiple variable fashion in a well characterized population of 201 patients with LARC. The data show that apoptosis of tumour cells does not predict local control or tumour regression in LARC patients. This indicates that, in contrary to short-term RT, the success of long-term RCT does not depend on this parameter since RCT is also effective in tumours with high intrinsic levels of apoptosis. This could suggest that the multimodality RCT regiment can adequately manage heterogeneity with respect to intrinsic levels of apoptosis.
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Chapter 8


Apoptosis in LARC
SUMMARY/SAMENVATTING
BACKGROUND

The incidence of colorectal cancer is rising in Western countries. In the Netherlands, 10000 patients are diagnosed with colorectal cancer each year. Patients' prognosis relies on a number of tumor and treatment-related factors. Tumor stage, type and grade are among the first group. The circumferential margin (CRM), defined as the smallest distance between the plane of resection and tumor invasion through the bowel wall, is a treatment-related prognostic factor. The majority of colorectal cancer patients undergo surgery combined with neoadjuvant (pre operative) or adjuvant (post operative) therapy. Depending on the tumor sites (colon or rectum), stage, estimation of the CRM and other high risk factors, this (neo)adjuvant therapy consists of radiotherapy, chemotherapy or a combination of radio and chemotherapy.

Therapy-related tumor response also influences prognosis and can therefore be considered as a treatment related prognostic factor. The genetic profile of the tumor and the expression of proteins like cyclooxygenase-2 (Cox-2) and thymidylate synthase (TS) can influence the extent of response. This thesis describes several different pathological and biological aspects of colorectal cancer treatment. In order to achieve this, three different patient populations have been studied:

1. Patients enrolled in the Total Mesorectal Excision (TME) trial. These patients were diagnosed with a mobile (T1-T3) rectum carcinoma and were randomized for either standardized TME surgery alone or short term radiotherapy (5X5 Gy) followed by a TME.
2. Patients with a locally advanced rectum carcinoma (LARC). These patients were diagnosed with an advanced, fixed rectal carcinoma (late T3-T4) and received long term radio(chemo)therapy.
3. Patients with stage III colon carcinoma who received adjuvant 5-Fluorouracil (5-FU) based chemotherapy.

SUMMARY

Chapter 1 describes the effects of the changed treatment approach of rectal cancer on the prediction of patients' prognosis. The introduction of Total Mesorectal Excision (TME) surgery and enhanced neoadjuvant therapies have improved patients' prognosis drastically, as has been shown by several prospective randomized trials. However, tumor staging did not change and is still based on tumor-related prognostic factors. Modern staging should at least be partially based on treatment-related factors. In the
current era of neoadjuvant therapy, the value of the circumferential margin (CRM) is more important for the staging of rectal tumors than classic tumor-related prognostic factors such as T stage (tumor invasion depth).

The analyses of the TME trial indicating that the CRM should be included in modern staging of rectal cancer are described in Chapter 2. The classic Tumor Node Metastasis (TNM) staging system is based solely on tumor-related prognostic factors. Univariate and multivariable analysis of overall survival demonstrate that NCRM staging, based on the CRM lymph node (N) stage, leads to a better prediction of patients’ survival than the conventional TNM system. These findings were validated in an independent data set of patients who participated in another prospective randomized trial. This chapter concludes that combining of treatment with tumor-related prognostic factors provides more information on patients’ prognosis than staging solely based on tumor-related factors.

Chapter 3 deals with the prognostic implications of the CRM in a population of patients with locally advanced rectal cancer (LARC). All LARC patients presented with a threatened CRM (less than 2mm on magnetic resonance imaging (MRI)) and received radio(chemo)therapy (RCT) in order to improve local control. This chapter investigates which factors are the best indicators for survival or local recurrence after the intense RCT regimen. Histopathological factors and four different systems for assessing tumor regression are evaluated. The data show that the CRM is the most important prognostic factor after RCT. The prognostic implications of tumor regression are dependent on the CRM but after a higher degree of tumor regression, a free CRM can be obtained more easily. Tumor regression improves local control provided that a free CRM can be obtained.

After describing the different pathological aspects of rectal cancer treatment in Chapter 1-3, several biological aspects will discussed in chapters 4-7. Chapter 4 describes the epithelial cell adhesion molecule (Ep-CAM) at the invasive front of colorectal cancers. Immunohistochemical experiments using three different antibodies directed against different epitopes of Ep-CAM link the loss of extracellular Ep-CAM with an increased migratory potential and tumor budding. Investigations of patients of the TME trial show that loss of extracellular Ep-CAM is significantly related to increased local recurrence rates and tumor budding at the invasive front. Additional results suggest that cleavage of Ep-CAM under influence of β-catenin causes internalisation of the protein remnants.

Chapter 5 studies the clinical relevance and underlying biological mechanisms of signet ring cell differentiation in mucinous rectal carcinomas. The presence of signet ring cells
in mucinous tumors is relatively rare, and its molecular background poorly understood. Several cell lines with different phenotypes are studied with Multiplex Ligation-dependent Probe Amplification (MPLA); this revealed that the Bcl-2 gene is amplified in cell lines with a mucinous phenotype and not in cell lines with a non-mucinous phenotype. Immunohistochemistry shows that signet ring cells are strongly positive for ITF and MUC2 and have declined levels of E-cadherin and β-catenin. Investigations on the clinical relevance showed that signet ring cell differentiation is associated with a poor prognosis.

The prognostic and value of cyclooxygenase-2 (Cox-2) in rectal carcinomas is described in Chapter 6. Cox-2 is involved in the production of prostaglandins and plays an important role in tumor development. Immunohistochemical assessment of Cox-2 is performed on tumor tissues obtained from patients of the TME trial. Irradiated patients demonstrate higher levels of Cox-2 in their tumors than patients who did not receive radiotherapy. Tumors with increased Cox-2 levels have significantly lower levels of apoptosis than patients with less Cox-2 expression. Furthermore, increased Cox-2 levels are associated with a poor overall and distant metastasis free survival. However, this only applies for patients who received short term radiotherapy. This chapter ends with the proposal that the administration of Cox-2 inhibitors during radiotherapy could improve patients’ prognosis.

Chapter 7 studies the predictive value of thymidylate synthase (TS) in stage III colon cancer at the DNA and protein level. TS is the target enzyme of 5-Fluorouracil (5-FU) which is the major component of the adjuvant chemotherapy administered to this patient population. Three functional polymorphisms and amplification of the TS gene are investigated with PCR. Immunohistochemistry was used to study TS at the protein level. The results demonstrate that the variable number of tandem repeats (VNTR) polymorphism in normal tissue is predictive for distant recurrence free survival and cancer specific survival. Patients homozygous for the double repeat (2R/2R) are found to have a favourable prognosis. Evaluations at the protein level are not associated with patients’ outcome. This chapter concludes that analysis of TS at the DNA level has a higher predictive value than analysis at the protein level.

Chapter 8 describes the prognostic value of apoptosis and five apoptosis regulating proteins (p53, Bcl-2, Bax, Cox-2 and maspin) in LARC patients who received RCT. Immunohistochemistry was performed on pre-therapy and post-therapy tissues. Apoptosis is investigated with the M30 antibody. Apoptosis or the related proteins (p53, Bcl-2, Bax and Cox-2) are not related with survival or local recurrence in this population. A strong cytoplasmatic staining pattern of maspin in pre-treatment biopsies significantly correlates with an increased risk on local recurrence but is not associated with survival. These
data lead to the conclusion that success of RCT in patients with LARC is independent of the intrinsic levels of apoptosis or the apoptosis regulating proteins studied.

CONCLUSIONS AND FUTURE RESEARCH

In this thesis several different studies related to treatment of colorectal cancer are described. Investigations of the pathological aspects were performed on patients with rectal cancer. These studies indicate that the CRM is an important prognostic factor in patients with mobile rectal cancer and with a more advanced rectal tumor (LARC). The CRM should therefore be part of modern staging of rectal cancer. The value of tumor staging based on the CRM needs to be validated in future randomized trials before this can be incorporated into clinical practice.

The biological aspects that are described in this thesis indicate that adhesion molecules such as Ep-CAM, E-Cadherin and β-catenin play a role in respectively tumor budding and signet ring cell development in mucinous rectal cancers. Investigations on Ep-CAM (chapter 4) indicate that cleavage of this protein occurs. Additional studies with for example cell lines could further elucidate this and could shed some light on the temporal aspects of this process. Studies on Cox-2 (chapter 6) suggest that the administration of Cox-2 inhibitors during short term radiotherapy could have an advantageous effect on survival and distant recurrence free survival of patients with rectal carcinoma. In order to further investigate this, a prospective randomized study should be conducted. However, the administration of selective Cox-2 inhibitors is nowadays controversial since the publication of the cardiovascular side effects of Rofecoxib.

A currently important research field aims to identify prognostic biomarkers or biomarkers predictive for therapy response. Numerous studies have tried to identify these markers in tumor tissue but the number of tissue-based markers that is currently used for colorectal cancer in clinical practice is disappointingly low. Investigations on TS, microsatellite instability (MSI), p53, K-ras and deleted in colon cancer (DCC) revealed that there is insufficient evidence to recommend routine use of these markers as predictive or prognostic tools in colorectal cancer. The studies described in this thesis have investigated the predictive potential of TS (chapter 7) and apoptosis (chapter 8) for patients with colon cancer and LARC respectively. The predictive value of apoptosis seems to be modest; tumor response to RCT is independent of this parameter. The VNTR polymorphism of the TS gene appears to be a promising marker but since this conclusion is based on analyses in a small number of patients, it would be highly desirable to include this analysis in future clinical trial.
ACHTERGROND

De incidentie van dikkedarm- (colon) en endeldarm- (rectum) kanker in de Westerse landen is stijgende. In Nederland worden ieder jaar ongeveer 10.000 patiënten gediagnosticeerd met colorectale kanker. De prognose na deze diagnose hangt af van een aantal tumor- en behandelingsgerelateerde factoren. Onder de eerst genoemde groep vallen het tumor stadium, type en de differentiatie graad. De circumferentiele marge (CRM), de kleinste afstand tussen het chirurgische snijvlak en de tumor ingroei in de darmwand, is een voorbeeld van een behandelingsgerelateerde factor. De behandeling van colorectale tumoren bestaat voor de meerderheid van de patiënten uit chirurgie in combinatie met adjuvante (na de operatie) en neoadjuvante (voor de operatie) therapie. Deze (neo)adjuvante therapie kan bestaan uit radiotherapie, chemotherapie of een combinatie van radio- en chemotherapie afhankelijk van de tumor lokalisatie (colon of rectum), het tumor stadium, de inschatting van de CRM en andere risico factoren.

De reactie van de tumor op deze behandeling heeft vervolgens ook weer invloed op de prognose en kan derhalve beschouwd worden als een behandelingsgerelateerde factor. In welke mate een tumor reageert op de toegediende therapie kan onder andere afhangen van het genetische profiel van de tumor en van de expressie van eiwitten zoals bijvoorbeeld cyclooxygenase-2 (Cox-2) en thymidylaat synthase (TS). Dit proefschrift beschrijft verschillende pathologische en biologische aspecten die een rol spelen bij de behandeling van colorectale tumoren. Daartoe zijn verschillende patiënten groepen bestudeerd:

1. Patiënten die geïncludeerd zijn in de RT+TME trial. Deze patiënten hebben een mobiel (T1-vroeg T3) rectum carcinoom en zijn gerandomiseerd voor wel of geen kortdurende neoadjuvante radiotherapie (RT) gevolgd door gestandaardiseerde “Totale Mesorectale Excisie” (TME) chirurgie.
2. Patiënten met een lokaal voortgeschreden rectumcarcinoom (LARC). De tumoren van deze patiënten populatie waren op het moment van diagnose in een vergevorderd stadium (late T3 en T4). Al deze patiënten hebben derhalve neoadjuvante langdurige radio(chemo)therapie ontvangen.
3. Patiënten met een stadium III colon carcinoom die allen adjuvante 5-Fluorouracil (5-FU) gebaseerde chemotherapie hebben gehad.

SAMENVATTING

Hoofdstuk 1 bediscussieert de invloed van de ontwikkelingen in de behandeling van het rectum carcinoom op de voorspelling van de prognose. Veranderingen in de behan-
deling van het rectum zoals de introductie van de “Totale Mesorectale Excisie” (TME) chirurgische techniek en de introductie en verbetering van neoadjuvante behandelingen hebben de prognose van patiënten met een rectum carcinoom sterk verbeterd, zoals de laatste jaren is aangetoond in vele prospectieve gerandomiseerde trials. Echter de stadiëring van tumoren is niet wezenlijk veranderd en berust nog steeds op enkel tumor gerelateerde factoren. Moderne stadiëring zou namelijk ook deels moeten berusten op de behandelinggerelateerde prognostische factoren. In dit tijdperk van neoadjuvante behandeling is de waarde van de CRM voor de stadiëring van rectum tumoren belangrijker dan klassieke tumor gerelateerde factoren zoals het T stadium die de mate van tumor invasie aangeeft.

In hoofdstuk 2 staan de analyses uit de TME trial beschreven die aantonen dat de CRM een belangrijke rol zou moeten spelen in de stadiëring van het rectum carcinoom. Het stadiëren van rectum tumoren berust op het zogenaamde “Tumor Node Metastasis” (TNM) systeem. Dit systeem is opgebouwd uit tumorgereleerde prognostische factoren. Univariabele en multivariabele analyses met betrekking tot de overleving van patiënten uit de TME trial tonen aan dat de combinatie van het lymfeklier (N) stadium en de CRM, resulterend in de NCRM classificatie, leidt tot een betere voorspelling van de overleving dan het klassieke TNM systeem. Dit geldt voor zowel de patiënten die niet bestraald zijn als de patiënten die wel RT hebben ontvangen (5X5 Gy). Deze bevinding werd gevalideerd in een onafhankelijke data set bestaande uit patiënten die hadden deelgenomen aan een andere prospectief gerandomiseerde trial. Dit hoofdstuk sluit af met de conclusie dat de combinatie van een tumorgerelateerde met een behandelingsgerelateerde factor meer prognostische informatie geeft dan een classificatie die enkel berust op tumor gerelateerde factoren.

In hoofdstuk 3 wordt de prognostische waarde van de CRM voor patiënten met een lokaal voortgeschreden rectum carcinoom (LARC) beschreven. Alle LARC patiënten die beschreven worden in dit hoofdstuk hebben een bedreigde CRM (kleiner dan 2 mm op “magnetische resonantie” (MR) beeldvorming) en hebben radio(chemo)therapie (RCT) gehad om de lokale controle te vergroten. In dit hoofdstuk wordt onderzocht welke factoren de beste indicatoren zijn voor de voorspelling van overleving of het optreden van een lokaal recidief na dit uitgebreide RCT schema. Histopathologische factoren en vier verschillende tumor regressie systemen worden onderzocht. Uit de analyses blijkt dat de bepaling van de CRM de belangrijkste prognostische factor is na RCT. De prognostische rol van histologische tumor regressie is ondergeschikt aan de CRM, echter een hogere mate van tumor regressie resultert significant vaker in een negatieve CRM. Een goede tumor respons op de radiochemotherapie draagt dus wel degelijk bij aan het verhogen van de lokale controle, mits een vrije CRM verkregen wordt.
Na de pathologische aspecten omtrent de behandeling van rectum tumoren te hebben beschreven in hoofdstuk 1 t/m 3 komen de biologische aspecten aan bod in hoofdstuk 4 t/m 7. **Hoofdstuk 4** beschrijft het “Epitheliaal Cel Adhesie Molecuul” (Ep-CAM) aan het invasieve tumor front van colorectale tumoren. Met behulp van immunohistochemie, waarbij gebruik is gemaakt van drie verschillende anti Ep-CAM antilichamen met verschillende epitopen, hebben we aangetoond dat tumor cellen waarbij het extracellulaire deel van het eiwit vermindert aanwezig is een verhoogde invasieve capaciteit hebben en zich vaker afsplitsen van ander tumor cellen (tumor budding). Analyses van patiënten van de TME trial laten zien dat afname van het extracellulaire Ep-CAM significant vaker gepaard gaat met het optreden van tumor budding en een lokaal recidief. Verder wordt in dit hoofdstuk beschreven dat er kliefing van het Ep-CAM molecuul lijkt plaats te vinden aan het invasieve tumor front onder invloed van β-catenine waardoor het extracellulaire deel van het eiwit afgesplitst wordt en de rest van het eiwit wordt geinternaliseerd.

**Hoofdstuk 5** worden de klinische relevantie en onderliggende biologische mechanismen van de aanwezigheid van zegelringcellen in mucineuze rectum carcinomen beschreven. De aanwezigheid van zegelringcellen in mucineuze tumoren is vrij zeldzaam en daarmee gepaard gaande moleculaire mechanismen zijn nog onbekend. Cellijnen met verschillende fenotype zijn onderzocht met behulp van de Multiplex Ligation-dependent Probe Amplification (MPLA) techniek. Uit de resultaten blijkt dat het Bcl-2 gen is geamplificeerd in de cellijnen met zegelringcellen en mucineuze fenotypen en niet in de cellijnen met het niet-mucineuze fenotype. Veder is met behulp van immunohistochemie aangetoond dat E-cadherine en β-catenine in verminderde mate aanwezig zijn in zegelringcellen en dat deze cellen sterk positief zijn voor ITF en MUC2. Veder blijkt dat de aanwezigheid van zegelringcellen gepaard gaat met een slechtere prognose.

**Hoofdstuk 6** wordt de prognostische waarde van het enzym “cyclooxygenase 2” (Cox-2), dat betrokken is bij het ontstaan van colorectale tumoren en de productie van prostaglandines, voor het rectum carcinoom onderzocht. Hiertoe is patiënten materiaal uit de TME trial onderzocht met behulp van immunohistochemische kleuringen tegen Cox-2. Hieruit blijkt dat Cox-2 vaker verhoogd aanwezig is in bestraalde patiënten dan in onbestraalde patiënten. In tumoren met een verhoogde aanwezigheid van Cox-2 is het niveau van apoptose significant lager dan in tumoren met een lagere Cox-2 expressie. Verder blijkt dat een verhoogde aanwezigheid van het Cox-2 enzym geassocieerd is met een slechtere prognose (overleving en metastase vrije overleving). Dit is echter alleen het geval als patiënten preoperatief bestraald waren. Dit hoofdstuk eindigt met de suggestie dat het toedienen van Cox-2 remmers tijdens radiotherapie zou kunnen bijdragen aan het voorkomen van het optreden van metastasen en het verlengen van de overleving.
In hoofdstuk 7 wordt de predictieve waarde van Thymidylaat Synthase (TS) bij patiënten met een stadium III colon carcinoom onderzocht op DNA en op eiwit niveau. TS is het doelwitzym van 5-Fluorouracil (5-FU), de belangrijkste component van de adjuvante chemotherapie die al de bestudeerde patiënten hebben ontvangen. TS gen amplificatie en de verschillende functionele polymorfismen zijn met behulp van PCR in kaart gebracht en onderzoek naar TS op eiwit niveau werd met behulp van immunohistochemie uitgevoerd. Uit de analyses blijkt dat het variabele aantal tandem repeats (VNTR) polymorfisme in normaal weefsel voorspellend is voor het optreden van metastasen en kankerspecifieke overleving. Patiënten die homozygoet zijn voor twee herhalingen in de VNTR regio (2R/2R) hebben een gunstige prognose. Uit de analyses op eiwit niveau blijkt dat deze niet zijn geassocieerd met overleving of metastase vrije overleving. Analyse van TS op DNA niveau lijkt van grotere predictieve waarde te zijn dan analyse op eiwit niveau.

Hoofdstuk 8 beschrijft de prognostische waarde van apoptose en vijf apoptose gerelateerde eiwitten (p53, Bcl-2, Bax, Cox-2 en maspine) in patiënten met LARC die neoadjuvante radiochemotherapie hebben ontvangen. Onbehandelde biopten en posttherapie resectie preparaten zijn onderzocht met behulp van immunohistochemie. Apoptose is geëvalueerd met behulp van het M30 antilichaam. Uit de resultaten valt af te leiden dat zowel apoptose als de apoptose gerelateerde eiwitten (p53, Bcl-2, Bax en Cox-2) niet gerelateerd zijn met overleving of het optreden van een lokaal recidief in deze populatie. Een sterke cytoplasmatische aankleuring van het maspine eiwit in de biopten was significant gecorreleerd met een hoger risico op het optreden van een lokaal recidief maar niet met overleving. Hieruit kan geconcludeerd worden dat het succes van radiochemotherapie in patiënten met LARC niet bepaald wordt door het intrinsieke niveau van apoptose of van een van de apoptose gerelateerde eiwitten.

CONCLUSIES EN TOEKOMSTIG ONDERZOEK

In dit proefschrift zijn verschillende studies beschreven die betrekking hebben met de behandelings van colorectale tumoren. De studies aangaande de pathologische aspecten zijn uitgevoerd op verschillende patiënten populaties met een rectum carcinoom. Deze studies wijzen uit dat de CRM een grote prognostische waarde heeft. Dit geldt zowel voor patiënten met een mobiel rectum carcinoom als patiënten met een voortgeschreden rectum carcinoom (LARC). Derhalve zou de CRM als behandlingsgerelateerde factor opgenomen moeten worden in de moderne stadiëring van rectum tumoren. De waarde van stadiëring die mede berust op de CRM zal in toekomstige gerandomiseerde trials gevalideerd moeten worden alvorens deze daadwerkelijk opgenomen zal worden in de kliniek.
De biologische aspecten die in dit proefschrift beschreven zijn geven aan dat adhesie moleculen zoals Ep-CAM en E-cadherine een rol spelen bij respectievelijk tumor budding en het ontstaan van zegelringcellen in mucineuze tumoren. De studie naar Ep-CAM (hoofdstuk 4) geeft aanwijzingen voor klieving van het eiwit maar additionele studies met bijvoorbeeld cellijnen zouden dit, en met name het tijdgebonden aspect, verder kunnen verhelderen. De studie naar Cox-2 (hoofdstuk 6) geeft aan dat de toevoeging van Cox-2 remmers tijdens kortdurende radiotherapie een gunstige invloed zou kunnen hebben op de overleving en metastasevrije overleving van patiënten met een rectum carcinoom. Om dit te bestuderen zou een prospectieve studie opgezet moeten worden waarbij gerandomiseerd wordt tussen wel of geen toediening van deze remmers. Echter, het toedienen van Cox-2 remmers is redelijk omstreden geraakt vanwege de vrij ernstige cardiovasculaire bijwerkingen van de selectieve Cox-2 remmer rofecoxib.

Een belangrijk actueel onderzoeksgebied heeft als doel om prognostische biomarkers en voorspellend biomarkers voor de respons op therapie te vinden. Veel studies hebben getracht om deze voorspellende markers in tumor weefsel te vinden maar het aantal markers wat heden ten dagen in de kliniek wordt gebruikt voor colorectale tumoren is teleurstellend laag. Onderzoek naar TS, microsatelliet instabiliteit (MSI), p53, K-ras en deleted in colon cancer (DCC) gaf aan dat er onvoldoende bewijs is voor routinematig gebruik van deze makers als predicatieve en prognostische handvaten. De studies beschreven in dit proefschrift hebben onderzocht in hoeverre TS (hoofdstuk 7) en apopptose (hoofdstuk 8) een predictieve waarde hebben voor respectievelijk patiënten met een stadium III colon carcinoom en patiënten met LARC. De waarde van voorspellende waarde van intrinsieke apopptose bij patiënten met LARC lijkt zeer gering te zijn, de tumor respons op radiochemotherapie is onafhankelijk van deze parameter. Analyses van het VNTR polymorfisme van het TS gen wijzen uit dat dit een interessante marker lijkt te zijn maar omdat deze conclusie is gebaseerd op een kleine groep patiënten is het een aanbeveling om deze analyse in toekomstige trials zeker niet buiten beschouwing te laten.
LIST OF PUBLICATIONS


CURRICULUM VITAE

DANKWOORD

Tien september 2008, zo mijn promotie datum is geprikt. Als je er zo naar kijkt is het eigenlijk best een mooie datum, het is namelijk net aftellen. 10, 9, 8.....7, 6, 5, 4, 3, 2, 1, en dan.....gepromoveerd!

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COLOR FIGURES
Chapter 4, Figure 3
Chapter 4, Figure 4

Double staining of Ep-CAM (Ber-EP4 epitope) and β-catenin of tumor masses with sprouting tumor cells. The Ber-EP4 epitope was visualized with DAB (brown) and β-catenin was displayed with fast blue staining (blue); nuclei were counterstained with Nuclear Fast Red. A, Tumor nodule with distinct budding of tumor cells which show a nuclear staining pattern of β-catenin and decreased staining of the Ber-EP4 epitope, arrows (original magnification 100X). This staining pattern of β-catenin was not observed centrally in the tumor nodule. B, Budding tumor cells spreading into the mesorectal fat. Isolated tumor cells exhibit loss of the Ber-EP4 epitope and nuclear β-catenin, arrow (original magnifications: 100X).

Figure 5: Ep-CAM mRNA in situ hybridization (ISH) and Ber-EP4 immunohistochemistry on serial sections. A, Ep-CAM mRNA ISH. B, Ber-EP4 staining of clusters of tumors cells at the tumor front of the same tumor area. Tumor cells with decreased Ber-EP4 staining do not present to have lower amounts of Ep-CAM mRNA (arrows, original magnifications: 100X).
Chapter 4, Figure 5

Ep-CAM mRNA in situ hybridization (ISH) and Ber-EP4 immunohistochemistry on serial sections. A, Ep-CAM mRNA ISH. B, Ber-EP4 staining of clusters of tumor cells at the tumor front of the same tumor area. Tumor cells with decreased Ber-EP4 staining do not present to have lower amounts of Ep-CAM mRNA (arrows, original magnifications: 100X).
Immunofluorescence double staining of Ep-CAM with both monoclonal Ber-EP4 and polyclonal antibody. A, Normal mucosa of the colon shows a membranous staining pattern with Ber-EP4. B, The polyclonal antibody also presents a membranous staining pattern in normal colon mucosa. C. Merge Ber-EP4 is visualized in green and the polyclonal antibodies in red, nuclei are counterstained with DAPI (blue). D, An isolated tumor cluster (arrow head) next to a tumor gland (arrow), immunohistochemistry was performed with the Ber-EP4 antibody. E, immunohistochemical expression of Ep-CAM evaluated with the polyclonal antibody. F, Merge Ber-EP4 is visualized in green and the polyclonal antibody in red, nuclei are counterstained with DAPI (blue). Loss of Ber-EP4 staining intensity is associated with a cytoplasmic staining pattern with the polyclonal anti Ep-CAM antibody (original magnifications: 400X).
Chapter 5, Figure 1
Illustration of amplifications and deletions of 41 genes (divided into four graphs). Normal range is between 0.8 and 1.2 (dotted line). Some cell lines showed an amplification of more than 2 in certain genes (*). Genes described in results (MYC, CDH1, Bcl2) are marked.
Chapter 5, Figure 2
Illustration of E-cadherin by immunofluorescence staining. The nuclei are represented by the blue colour. Subtype clone 36 (transmembrane) is represented by the green colour, subtype HECD-1 (extracellular) is represented by the red colour. The yellow colour of the normal mucosa (A) indicates that both epitopes are still present. (B) A signet ring cell at high magnification (100x), the transmembranal epitope is still available, while the extracellular epitope was partly not detected.
Chapter 5, Figure 3

**Immunofluorescence illustration of a normal mucosa (A) with goblet cells (arrows) secreting ITF (red, arrowheads) into the lumen; (B) signet ring cell showing a strong membranous and cytoplasmic staining of β-catenin (green) and weak cytoplasmic staining of ITF; and (C) a signet ring cell showing a weak membranous staining of β-catenin and strong cytoplasmic staining of ITF, while β-catenin has moved to the nucleus (D).**
Chapter 5, Figure 4

ITF distribution in clustered signet ring cells (A) and solitary signet ring cells (B). Signet ring cells were positive for ITF mRNA (C, dark purple) and negative using the sense probe (D, negative control), indicating that signet ring cells can produce their own ITF. Expression of MUC2 in normal mucosa (E) and solitary signet ring cells (F).
Chapter 6, Figure 1
Representative stainings of Cox-2 expression in tissue microarray cores from the 1231 rectal cancer specimens evaluated in this study. Figure 1A: Cox-2 negative tumor (score 0). Figure 1B: weak diffuse cytoplasmic staining (score 1). Figure 1C: moderate to strong granular cytoplasmic staining (score 2). Figure 1D: strong intensity of the staining (score 3).
Chapter 7, Figure 1
Different staining intensities observed after TS IHC with monoclonal antibody TS-106 (DakoCytomation) in tumor tissue (B, D, F and H) and normal tissue (A, C, E and G), original magnifications: 200X. Arrows depict intensely stained immature lymphocytes. Staining intensities of 0, 1, 2 and 3 are respectively indicated by panel A and B, C and D, E and F and G and H.
Chapter 8, Figure 1
Examples of immunohisto-chemical staining patterns observed after staining with M30 CytoDEATH (A), p53 (B), Bcl-2 (C), Bax (D), Cox-2 (E) and maspin (F). The arrows in panel A depict examples of apoptotic tumour cells that demonstrate intense staining with the M30 antibody. Original magnifications B-F: 200X, A: 400X.
Color figures