Molecular and biological interactions in colorectal cancer

P. de Heer

ISBN: 978 90 8562 050 1

Ontwerp omslag: Philip Bais
Ontwerp binnenwerk: Beeldvorm, Pijnacker
Druk: Wilco, Amersfoort

Cover art: 2 Cox apples illustrating the COX-2 enzyme and a tribute to the famous Beatles Apple logo designed by Robert Fraser. The back cover is a Cox apple's core illustrating the classic apple core lesion as seen on barium enema radiograph imaging in colon cancer.

Molecular and biological interactions in colorectal cancer

Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 19 september 2007 klokke 16.15 uur

door
Pieter de Heer
Geboren te Hong Kong
in 1976

Promotiecommissie:

Promotor:	Prof. Dr. C.J.H. Van de Velde
Co-promotor:	Dr. P.J.K. Kuppen
Referent:	Prof. Dr. D.J. Richel (Academisch Medisch Centrum)
Overige leden:	Prof. Dr. B. van de Water Prof. Dr. H. Morreau

"The time has come,"

The Walrus said,

"To talk of many things……"

Lewis Carroll (*Through the Looking Glass, 1872)*

Aan Paul en mijn ouders

Voor Kyra

Table of Contents

CHAPTER 1

General introduction and outline of this thesis

General introduction

Cancer of the large bowel (colorectal cancer) includes all cancer originating from the cecum to the anus. Colorectal cancer can be subdivided in colon cancer, which ranges from caecum to the sigmoid (approximately 15 cm above the anal verge), and rectal cancer, that ranges from the recto-sigmoid to the anus. Rectal cancer constitutes approximately 25% of all colorectal cancers. In the year 2000, colorectal cancer was ranked the third most common form of cancer worldwide in terms of incidence. An estimated 300.000 new cases of colorectal cancer are diagnosed each year in Europe, accounting for 8% of all malignant tumors in adults[1]. In the Netherlands, yearly approximately 8600 new cases of colorectal cancer are diagnosed and colorectal cancer accounts for 4500 deaths each year[2]. High incidence rates are found in western world populations, i.e. Western Europe, North America, and Australia. The lowest rates of colorectal cancer are found in the sub-Saharan Africa, South America and Asia, but are increasing in countries adopting western life-style and dietary habits[3].

Aetiology and risk factors

Colorectal cancer most commonly occurs sporadically and is inherited in only 5% of the cases. Studies on migrants suggest that colorectal cancer is determined largely by environmental exposure[4]. Diet is the most important exogenous factor in the aetiology of colorectal cancer. The substantial differences in incidence of colorectal cancer between western world and developing countries can be explained by a high fiber and low fat containing diet in developing countries[5,6] compared with increasing intake of fat and alcohol in western countries[7].

Genetic background of colorectal cancer

The majority of colorectal cancers develop from benign pre-neoplastic lesions: the adenomatous polyps or adenomas. Progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, which is referred to as the adenoma-carcinoma sequence[8].

Two major mechanisms of genomic instability have been identified that give rise to colorectal carcinoma development and progression: chromosomal instability (CIN) and microsatellite instability (MIN). CIN is associated with a series of genetic changes that involve the activation of oncogenes as k-ras and inactivation of tumor suppressor genes as p53, DCC/SMAD4 and APC[9-11] and contributes predominantly to carcinogenesis in the distal segments of the colorectum. Familial Adenomatous Polyposis represents the hereditary syndrome dealing with APC mutation[12,13]. Mutations in DNA mismatch repair (MMR) genes result in a failure to repair errors that occur during DNA replication in repetitive sequences (microsatellites), resulting in an accumulation of frameshift mutations in genes that contain microsatellites. This failure leads to MIN type of tumor and is the hallmark of hereditary non-polyposis colorectal cancer (HNPCC)[14]. MIN is also found in 12-15% of sporadic colorectal cancers. In addition to the genetic disparity of CIN and MIN, MIN tumors are more frequently right-sided and poorly differentiated, and more often display unusual histologic type (mucinous), and marked peritumoral and intratumoral lymphocytic infiltration[15]. Finally, MIN colorectal carcinomas have been associated with a more favorable clinical outcome and do not benefit from adjuvant chemotherapy[16-18].

Treatment of colon and rectal cancer

The overall treatment strategy for colon cancer consists of surgical resection of the primary tumor and regional lymph nodes by a hemi-colectomy, comprising resection of a proportion of the colon and the surrounding mesocolon including the draining veins. Recently the use of laparoscopy in resection of colon cancer was successfully evaluated in a randomized clinical study[19]. Since the late 1980s the role of adjuvant chemotherapy has become increasingly important. A significant improvement in survival has been observed by the addition of 5-Fluoro-Uracil with Leukovorin (5-FU/LV) to surgical resection[20]. Recently the addition of Oxaliplatin to 5FU-containing regimens has become the standard treatment of high risk stage II (patients without lymph node metastases, but with a tumor that invades the serosa of the bowel or adjacent structures) and stage III (lymph node positive) colon cancer patients[21]. The addition of monoclonal antibodies as bevacizumab and cetuximab to adjuvant treatment is currently under investigation[22,23].

The primary treatment of rectal cancer is surgical resection of the primary tumor by total mesorectal excision (TME). TME consists of sharp dissection of the mesorectum between visceral and parietal pelvic fascia, which generally allows tumor-free margins to be achieved and simultaneously allows the reduction of sexual and urinary dysfunction[24]. TME is now generally accepted as standard procedure, at least for lower rectal cancer, and is performed in the majority of European countries. Several reports have been published showing a low local recurrence rate after TME surgery as compared to the blunt dissection for rectal cancer[25-28]. Due to their pelvic location, preoperative radiation therapy is indicated in rectal tumors leading to less local recurrences[29-31]. The evidence that the addition of chemotherapy to preoperative radiotherapy improves local control of tumor growth in locally advanced rectal cancer has recently been shown in two randomized studies[32,33]. Local recurrence was reduced from 17.1% to 8.7%, although in the first results no significant differences in overall 5-year survival were found[33]. Both trials demonstrated an increase in toxicity in the combined modality arm. It is therefore important to select only patients at risk for a positive resection margin for chemoradiotherapy as it is expected that only these patients will benefit.

As stated before, the introduction of TME surgery and the addition of preoperative (chemo-) radiotherapy has led to a drastic reduction in local recurrences in rectal cancer[33-36]. However, despite the improved local control, distant tumor recurrences still cause substantial mortality and overall survival has remained unaffected[29]. Several studies have been undertaken to investigate the role of systemic chemotherapy. Taal et al. randomized patients with stage II or III colorectal cancer between surgery followed by 5-FU/levamisole and surgery alone[37]. No beneficial effect was found for the addition of chemotherapy in patients with rectal cancer. This was probably due to the high percentages of local recurrences (22%) in this study as patients were treated by conventional surgery instead of TME[38]. The addition of systemic chemotherapy to preoperative radiotherapy as treatment of rectal carcinoma is currently being investigated in several clinical trials. The effects of chemotherapy on distant recurrence and patient survival in rectal cancer have to be awaited.

When considering patients with colorectal cancer, oncologists and surgeons typically differentiate colon from rectal tumors on the basis of their location within the peritoneal cavity and the pelvis, respectively. However, the transition of sigmoid colon into rectum, is not clearly

defined. Distinction between colon and rectum is made on the basis of location of the organ to the level of the third sacral vertebra, 10 to 15 cm proximal of the anorectal line. Determination of the location of a tumor is made by coloscopy/MRI/clinical assessment. Due to the flexible and distensible nature of the bowel, determination of the location is notoriously imprecise. As described above, the clinical consequences of allocation of a tumor to the colon or the rectum are substantial as this will determine the type of surgery that is performed and whether or not preoperative (chemo) radiotherapy or adjuvant chemotherapy will be administered.

Biological markers in colorectal cancer

Currently, colorectal cancer is considered a relatively homogeneous disease, which should be treated according to tumor location in the bowel. However, recent advances in molecular biological studies to colorectal cancer may challenge this concept; from a molecular viewpoint, there is increasing evidence that tumors located in the distal colorectum represent a distinct entity, with specific clinical and pathological characteristics[39]. Tumors originating in the distal colorectum have been proposed to arise and progress by pathways distinct from the originating in the proximal colon. The molecular mechanisms giving rise to a tumor are likely to influence its clinical behavior[40]. Rather than focusing on tumor location and tumor staging to allocate patients to treatment, the current thesis investigates and describes molecular markers that influence the clinical behavior of colorectal cancers as local and distant recurrence of tumors and patient survival.

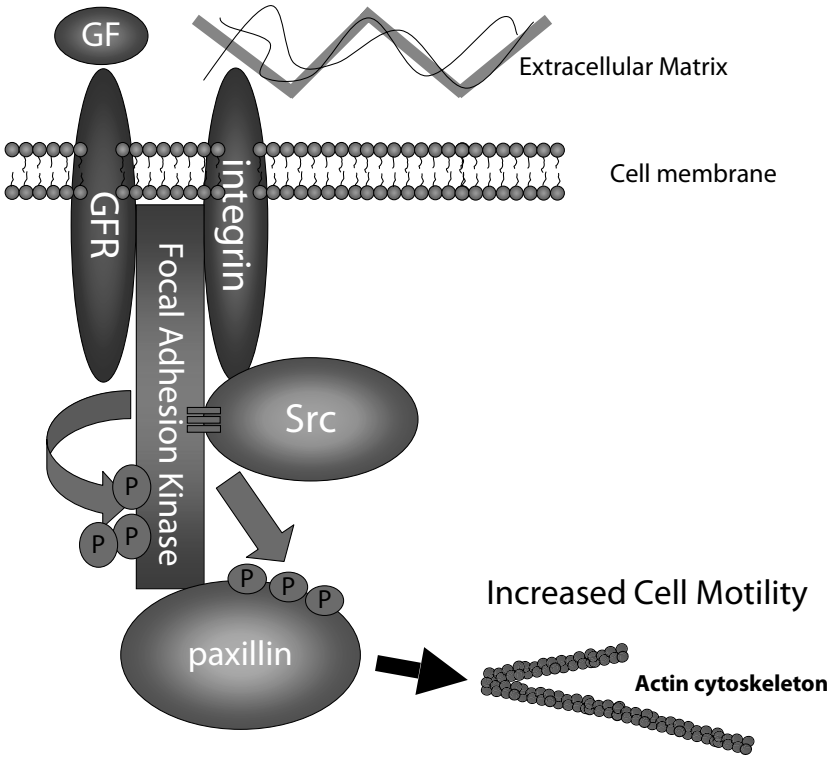
By assessing markers that influence cell motility (chapter 2), that allow tumor cells to escape apoptosis (chapter 3 and 4) and that allow unregulated production of growth-stimulating compounds (chapter 5) we have set out to construct a model in which an accurate prediction of clinical behavior and response to treatment can be made. Ultimately, by combining tumor biological markers with characteristics that can be assessed by preoperative radiological imaging, as tumor location, circumferential margin[41] and lymph node status[42], clinical behavior of the tumor can be predicted enabling patients to receive a tailor-cut treatment suited to optimally treat their disease. Biological markers derived from molecular mechanisms of tumor growth that were studied in this thesis will now be discussed in more detail.

Regulation of cell motility

Invasion of cancer cells into surrounding tissues is an important denominator of clinical behavior of cancers. The loss of adhesion of epithelial cells from its surrounding environment is an important step in promoting tumor cell migration, invasion and metastasis. Regulatory mechanisms of cell motility are critical in this process. Key factors are the non-receptor kinase proteins. The non-receptor protein tyrosine focal adhesion kinase (FAK) is localized at integrin-enriched cell adhesion sites (focal adhesions) and acts as an integrator of several signaling pathways regulating cell motility (illustrated in Figure 1)[43]. These signaling pathways include growth factor stimuli, and signaling through interaction between integrins and extracellular matrix proteins[44]. Autophosphorylation of FAK occurs in response to integrin or growth factor receptor engagement and creates a binding site for the Src family of protein tyrosine kinases[45]. The FAK-Src kinase complex subsequently mediates the phosphorylation of several focal adhesion-associated proteins including the scaffolding molecule paxillin[46,47]. Pax-

illin recruits other signaling molecules to adhesion sites and, thereby, indirectly regulates the dynamic organization of the actin cytoskeleton during the process of cell migration[48]. FAK-, Src- and paxillin-transduced signals control cellular processes such as migration, invasion[49,50] and anchorage independent growth[51]. These processes are vital to cell motility and the ability of tumor cells to metastasize. The association between the molecules FAK, Src and paxillin and the impact of these molecules on the clinical behavior of colorectal cancer will be further dicussed in this thesis.

Figure 1

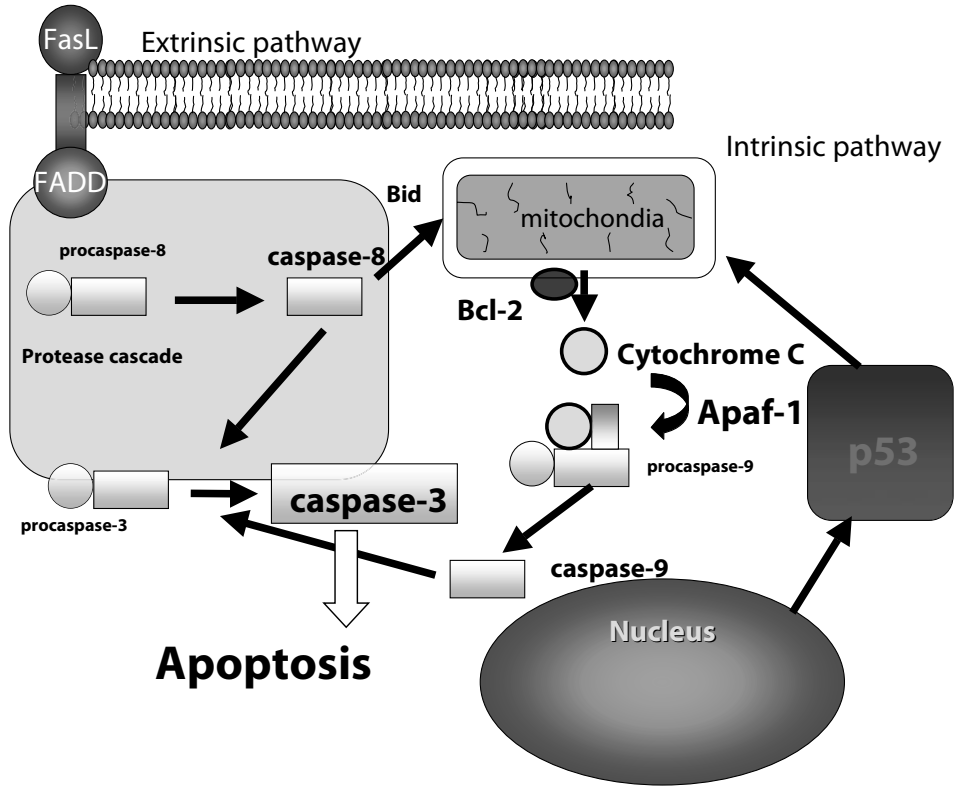


Apoptosis

Apoptosis, or programmed cell death, is important in maintaining homeostasis in tissues during growth and development[52]. Deregulation of apoptosis is considered to be one of the fundamental hallmarks of cancer[53]. Under normal conditions processes as maturation and defects in several processes as DNA repair, DNA-damage checkpoint function, and telomere maintenance, may result in apoptosis. The induction of apoptosis in cells is closely regulated by pro-apoptotic (BAX, BAC) and anti-apoptotic proteins (Bcl-2, Survivin). Higher organisms have developed a second, extrinsic apoptosis pathway that is triggered by activation of the cell surface death receptor Fas by cytotoxic immune cells. Activation of the extrinsic pathway leads to cleavage and activation of initiator caspase 8 and 10, which in turn cleave and activate effector caspases 3, 6, and 7, resulting in apoptosis. In the intrinsic (mitochondrial) pathway, proapoptotic proteins

results in a net increase of free cytosolic cytochrome c. Once released, cytochrome c interacts with apoptosis-activating factor-1 (Apaf-1), adenosine triphosphate, and procaspase 9 to form the apoptosome. The apoptosome activates caspase 9, which leads to caspases 3, 6, and 7 activity, thus stimulating apoptosis[54,55]. Both apoptosis pathways are visualized in figure 2.

Figure 2



Central to the regulation of apoptosis in colorectal cancer are the tumor suppressor proteins APC and p53. It is therefore not surprising that APC and p53 are inactivated in approximately 80%[56] of colorectal cancers, whereas genes involved in the suppression of apoptosis as Bcl-2 and survivin are often highly expressed[57]. Many publications have evaluated the value of apoptosis-relevant genes in predicting clinical behavior and response to treatment in colorectal cancer, but no clear-cut pattern has emerged[58]. In the current thesis the reasons for the lack of a clear relationship between levels of proteins involved in apoptosis and clinical response will be further discussed.

COX-2

Multiple lines of evidence support a protective effect of non-steroidal anti-inflammatory drugs (NSAIDs, e.g., aspirin, indomethacin, ibuprofen, piroxicam, sulindac) against development and growth of colorectal cancer[59,60]. Extensive experimental and clinical research has provided

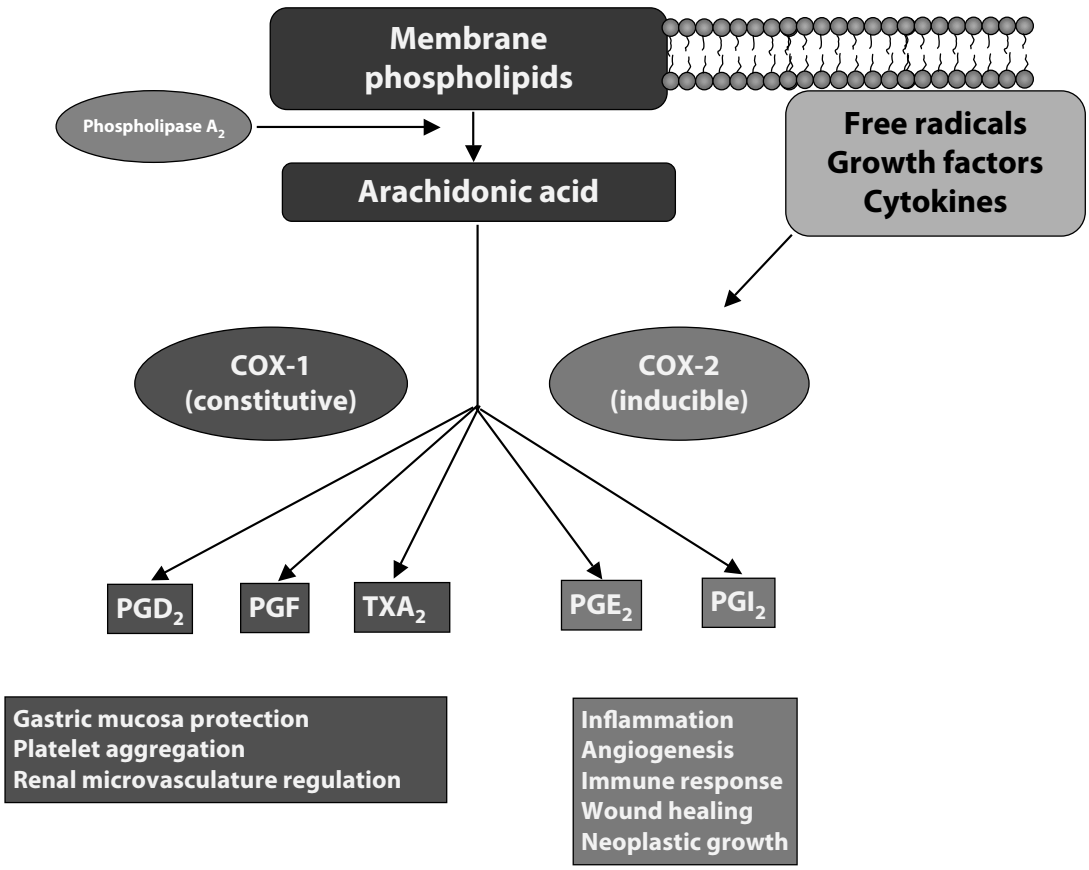
convincing data that NSAIDs and selective cyclooxygenase-2 (COX-2) inhibitors have substantial anti-carcinogenic effects in colorectal cancer[61-65]. Potential mechanisms by which the traditional NSAIDs are chemopreventive include: inhibition of procarcinogen activation and carcinogen formation[66], tumor cell invasion and metastasis[66], and tumor angiogenesis[67], as well as the induction of tumor cell apoptosis[68] and stimulation of immune surveillance[69].
NSAIDs are generally perceived to function by interfering with the cyclooxygenase pathway by temporarily blocking the attachment site for arachidonic acid (AA) on the COX enzyme (figure 3). Several COX isoforms exist. COX-1 is constitutively expressed in different cell types and is considered to be mainly associated with the production of prostaglandins (PGs) under normal physiological conditions. In contrast, COX-2 is induced by cytokines, growth factors and free radicals and is expressed in inflammatory cells. Both COX isoforms are responsible for the production of different types of PGs, tromboxane and leukotriens[70-72]. A large body of evidence suggests that the antineoplastic activities of NSAIDs are independent of COX-1 or COX-2: NSAIDs sulindac and piroxicam decreased proliferation and increased apoptosis in colon cancer cell lines that have no detectable COX activity[73]. Aspirin has well-documented non-COX effects, including inducing of apoptosis by inhibition of nuclear factor κB (NF-κB) activation[74]. More recently, induction of apoptosis by sulindac has shown to be mediated by polyamines as PPARγ, demonstrating direct gene transcriptional effects[75]. The COX-2 independent effects of NSAIDs and their potential value in the treatment of cancer will be further discussed in this thesis.
COX-2 activity is regulated by several mechanisms in human cancer. The COX-2 enzyme is induced in response to growth factors like tumor necrosis factor α (TNFα), endotoxins, cytokines, products of oncogenes (β-catenin and the Wnt-signalling pathway[76]) interleukins (IL-1β) and interferon γ (IFNγ)[60,77,78]. Upon binding of the previously mentioned factors to the promoter region of COX-2 the activation of I-κB kinases (IKKs) and subsequent activation of NF-κB[79] is induced, leading to active transcription of the COX-2 gene and other pro-inflammatory genes[80]. Overexpression of the COX-2 protein occurs in approximately 70% of colorectal cancers[81] and is likely to be mediated through a combination of the previously described mechanisms; however, the clinical consequences of overexpression of COX-2 and treatment of cancer with COX-2 inhibitors remain debated and will be discussed in this thesis.

COX-2 inhibition in the clinical practice

Preclinical data using COX-2 inhibitors for the prevention and treatment of cancer showed encouraging results[60,82-84]. The convincing epidemiological and preclinical data were followed by a chemoprevention trial. The effects of celecoxib 800mg per day were studied in a double blind, placebo-controlled study in patients with familial adenomatous polyposis (FAP). Patients in the celecoxib arm of the study had a significant reduction in the number of colorectal polyps after 6 months of treatment as compared to the placebo arm of the study[85]. This study led to the registration of celecoxib as an oral adjunct to the usual care of FAP. Several studies evaluating the long term beneficial effects of COX-2 inhibitors on the prevention of colorectal adenoma's were started[86,87]. Two randomized, placebo-controlled phase III European trials were started evaluating the effectiveness of the COX-2 inhibitors rofecoxib (the VICTOR study: VIOXX in Colorectal Therapy, definition of Optimal Regime) and celecoxib (the ACTION study: Adju-

vant Celecoxib Treatment in Oncology). The ACTION study was coordinated by the department of Surgery of the Leiden University Medical Center. The trial was designed to randomize patients with a stage II or III colon cancer between celecoxib 800mg/day and placebo for three years to improve overall survival and decrease the number of new primary tumors and polyps in patients. The inclusion criteria were later narrowed to stage III patients after the decision to conduct the study through the EORTC (European Organisation for Research and Treatment of Cancer) datacenter and more specifically, the PETACC organisation (Pan-European Trials in Adjuvant Colon Cancer). The PETACC organisation was at that time conducting a study with stage II patients and did not allow competing trials. The VICTOR trial was conducted in England and randomized patients with a stage II or III colorectal cancer to rofecoxib 25 or 50 mg daily or placebo. Both trials were, however, terminated before patient inclusion was completed. The reasons for termination of the trials will be extensively discussed in the discussion of chapter 9 of this thesis. Though the ACTION trial was prematurely ended, supportive translational research to COX-2 led to a number of articles in the current thesis and will be discussed in the next chapters.

Figure 3



Outline of this thesis

The aim of the current thesis was to evaluate the impact of tumor biological markers on clinical behavior, in order to enable development of tailor-made treatment strategies. The thesis focuses specifically on regulators of cell motility (FAK, Src and paxillin), apoptosis (M30 and caspase-3) and COX-2 pathways. In addition, the effects of the use of selective COX-2 inhibitors are studied in a rat model for colorectal cancer.

Chapter 2 describes the expression of regulators of cell motility in primary colorectal cancers and liver metastases. Several preclinical studies have implicated FAK, Src and paxillin to increase the metastatic potential of colorectal cancer. This chapter provides, for the first time, information on the prognostic value of FAK, Src and paxillin in colorectal cancer.

A large body of evidence suggests that the development of rectal and colon cancers may involve different mechanisms and results in different clinical behavior. **Chapter 3** and **Chapter 4** describe the impact of tumor cell apoptosis on clinical tumor behavior in colorectal (**Chapter 3**) and rectal cancer (**Chapter 4**) and show that patients can be selected by preoperative determination of the level of apoptosis in tumors in which preoperative radiotherapy may be redundant.

In **Chapter 5** cyclooxygenase-2 (COX-2) expression in tumors of rectal cancer patients from the Dutch TME trial was evaluated. Results from this study indicated that upregulation of COX-2 expression can occur after radiotherapy and this suggests that the addition of COX-2 inhibitors to the treatment of rectal cancer could improve patient prognosis. **Chapter 6** describes the results from an experimental study in which the effect of COX-2 inhibitors on a rat model for colorectal liver metastases was evaluated. **Chapter 7** describes side effects of COX-2 inhibition when combined with tumor treatment by radio frequency ablation in the same rat model. An overall summary and discussion of the data presented in this thesis are provided in **Chapter 8**.

References

1. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
2. Siesling, S., van Dijck, J. A., Visser, O., and Coebergh, J. W. Trends in incidence of and mortality from cancer in The Netherlands in the period 1989-1998. Eur.J.Cancer, *39*: 2521-2530, 2003.
3. Vainio, H. and Miller, A. B. Primary and secondary prevention in colorectal cancer. Acta Oncol., *42*: 809-815, 2003.
4. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
5. Burkitt, D. P. Epidemiology of cancer of the colon and rectum. Cancer, *28*: 3-13, 1971.
6. Jacobs, E. T., Lanza, E., Alberts, D. S., Hsu, C. H., Jiang, R., Schatzkin, A., Thompson, P. A., and Martinez, M. E. Fiber, sex, and colorectal adenoma: results of a pooled analysis. Am.J.Clin.Nutr., *83*: 343-349, 2006.
7. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
8. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. N.Engl.J.Med., *319*: 525-532, 1988.
9. Conlin, A., Smith, G., Carey, F. A., Wolf, C. R., and Steele, R. J. The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. Gut, *54*: 1283-1286, 2005.
10. Esteller, M., Gonzalez, S., Risques, R. A., Marcuello, E., Mangues, R., Germa, J. R., Herman, J. G., Capella, G., and Peinado, M. A. K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. J.Clin.Oncol., *19*: 299-304, 2001.
11. Hsieh, J. S., Lin, S. R., Chang, M. Y., Chen, F. M., Lu, C. Y., Huang, T. J., Huang, Y. S., Huang, C. J., and Wang, J. Y. APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am.Surg., *71*: 336-343, 2005.
12. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. N.Engl.J.Med., *319*: 525-532, 1988.
13. Fearon, E. R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61*: 759-767, 1990.
14. Boland, C. R., Thibodeau, S. N., Hamilton, S. R., Sidransky, D., Eshleman, J. R., Burt, R. W., Meltzer, S. J., Rodriguez-Bigas, M. A., Fodde, R., Ranzani, G. N., and Srivastava, S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res., *58*: 5248-5257, 1998.
15. Dolcetti, R., Viel, A., Doglioni, C., Russo, A., Guidoboni, M., Capozzi, E., Vecchiato, N., Macri, E., Fornasarig, M., and Boiocchi, M. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. Am.J.Pathol., *154*: 1805-1813, 1999.
16. Benatti, P., Gafa, R., Barana, D., Marino, M., Scarselli, A., Pedroni, M., Maestri, I., Guerzoni, L., Roncucci, L., Menigatti, M., Roncari, B., Maffei, S., Rossi, G., Ponti, G., Santini, A., Losi, L., Di Gregorio, C., Oliani, C., Ponz, d. L., and Lanza, G. Microsatellite instability and colorectal cancer prognosis. Clin.Cancer Res., *11*: 8332-8340, 2005.
17. Ribic, C. M., Sargent, D. J., Moore, M. J., Thibodeau, S. N., French, A. J., Goldberg, R. M., Hamilton, S. R., Laurent-Puig, P., Gryfe, R., Shepherd, L. E., Tu, D., Redston, M., and Gallinger, S. Tumor

microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N.Engl.J.Med., *349*: 247-257, 2003.
18. Carethers, J. M., Smith, E. J., Behling, C. A., Nguyen, L., Tajima, A., Doctolero, R. T., Cabrera, B. L., Goel, A., Arnold, C. A., Miyai, K., and Boland, C. R. Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. Gastroenterology, *126*: 394-401, 2004.
19. A comparison of laparoscopically assisted and open colectomy for colon cancer. N.Engl.J.Med., *350*: 2050-2059, 2004.
20. Moertel, C. G., Fleming, T. R., Macdonald, J. S., Haller, D. G., Laurie, J. A., Goodman, P. J., Ungerleider, J. S., Emerson, W. A., Tormey, D. C., Glick, J. H., and . Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N.Engl.J.Med., *322*: 352-358, 1990.
21. Andre, T., Boni, C., Mounedji-Boudiaf, L., Navarro, M., Tabernero, J., Hickish, T., Topham, C., Zaninelli, M., Clingan, P., Bridgewater, J., Tabah-Fisch, I., and de Gramont, A. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N.Engl.J.Med., *350*: 2343-2351, 2004.
22. Sun, W. and Haller, D. G. Adjuvant therapy of colon cancer. Semin.Oncol., *32*: 95-102, 2005.
23. Iqbal, S. and Lenz, H. J. Integration of novel agents in the treatment of colorectal cancer. Cancer Chemother.Pharmacol., *54 Suppl 1*: S32-S39, 2004.
24. Quirke, P., Durdey, P., Dixon, M. F., and Williams, N. S. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histopathological study of lateral tumour spread and surgical excision. Lancet, *2*: 996-999, 1986.
25. Heald, R. J. and Ryall, R. D. Recurrence and survival after total mesorectal excision for rectal cancer. Lancet, *1*: 1479-1482, 1986.
26. Heald, R. J. Total mesorectal excision is optimal surgery for rectal cancer: a Scandinavian consensus. Br.J.Surg., *82*: 1297-1299, 1995.
27. MacFarlane, J. K., Ryall, R. D., and Heald, R. J. Mesorectal excision for rectal cancer. Lancet, *341*: 457-460, 1993.
28. Bulow, S., Christensen, I. J., Harling, H., Kronborg, O., Fenger, C., and Nielsen, H. J. Recurrence and survival after mesorectal excision for rectal cancer. Br.J.Surg., *90*: 974-980, 2003.
29. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
30. Adjuvant radiotherapy for rectal cancer: a systematic overview of 8,507 patients from 22 randomized trials. Lancet, *358*: 1291-1304, 2001.
31. Camma, C., Giunta, M., Fiorica, F., Pagliaro, L., Craxi, A., and Cottone, M. Preoperative radiotherapy for resectable rectal cancer: A meta-analysis. JAMA, *284*: 1008-1015, 2000.
32. Gerard, J. P., Bonnetain, F., Conroy, T., Chapet, O., Bouche, O., Closon-Dejardin, M. T., Untereiner, M., Leduc, B., Francois, E., and Bedenne, L. Preoperative (preop) radiotherapy (RT) {+/-} 5 FU/ folinic acid (FA) in T3-4 rectal cancers: results of the FFCD 9203 randomized trial. J Clin Oncol (Meeting Abstracts), *23*: 3504, 2005.
33. Bosset, J. F., Calais, G., Mineur, L., Maingon, P., Radosevic-Jelic, L., Daban, A., Bardet, E., Beny, A., Briffaux, A., and Collette, L. Enhanced tumorocidal effect of chemotherapy with preoperative radiotherapy for rectal cancer: preliminary results--EORTC 22921. J.Clin.Oncol., *23*: 5620-5627, 2005.
34. Sauer, R., Becker, H., Hohenberger, W., Rodel, C., Wittekind, C., Fietkau, R., Martus, P., Tschmelitsch, J., Hager, E., Hess, C. F., Karstens, J. H., Liersch, T., Schmidberger, H., and Raab, R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N.Engl.J.Med., *351*: 1731-1740, 2004.

35. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
36. Heald, R. J. and Ryall, R. D. Recurrence and survival after total mesorectal excision for rectal cancer. Lancet, *1*: 1479-1482, 1986.
37. Taal, B. G., Van Tinteren, H., and Zoetmulder, F. A. Adjuvant 5FU plus levamisole in colonic or rectal cancer: improved survival in stage II and III. Br.J.Cancer, *85*: 1437-1443, 2001.
38. Taal, B. G., Van Tinteren, H., and Zoetmulder, F. A. Adjuvant 5FU plus levamisole in colonic or rectal cancer: improved survival in stage II and III. Br.J.Cancer, *85*: 1437-1443, 2001.
39. Iacopetta, B. Are there two sides to colorectal cancer? Int.J.Cancer, *101*: 403-408, 2002.
40. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100*: 57-70, 2000.
41. Beets-Tan, R. G., Lettinga, T., and Beets, G. L. Pre-operative imaging of rectal cancer and its impact on surgical performance and treatment outcome. Eur.J.Surg.Oncol., *31*: 681-688, 2005.
42. Koh, D. M., Brown, G., Temple, L., Raja, A., Toomey, P., Bett, N., Norman, A. R., and Husband, J. E. Rectal cancer: mesorectal lymph nodes at MR imaging with USPIO versus histopathologic findings--initial observations. Radiology, *231*: 91-99, 2004.
43. Webb, D. J., Parsons, J. T., and Horwitz, A. F. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. Nat.Cell Biol., *4*: E97-100, 2002.
44. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. Nat.Rev.Mol.Cell Biol., *6*: 56-68, 2005.
45. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der, G. P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature, *372*: 786-791, 1994.
46. Tachibana, K., Urano, T., Fujita, H., Ohashi, Y., Kamiguchi, K., Iwata, S., Hirai, H., and Morimoto, C. Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase. A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates. J.Biol.Chem., *272*: 29083-29090, 1997.
47. Thomas, J. W., Cooley, M. A., Broome, J. M., Salgia, R., Griffin, J. D., Lombardo, C. R., and Schaller, M. D. The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. J.Biol.Chem., *274*: 36684-36692, 1999.
48. Bellis, S. L., Miller, J. T., and Turner, C. E. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. J.Biol.Chem., *270*: 17437-17441, 1995.
49. Hsia, D. A., Mitra, S. K., Hauck, C. R., Streblow, D. N., Nelson, J. A., Ilic, D., Huang, S., Li, E., Nemerow, G. R., Leng, J., Spencer, K. S., Cheresh, D. A., and Schlaepfer, D. D. Differential regulation of cell motility and invasion by FAK. J.Cell Biol., *160*: 753-767, 2003.
50. van Nimwegen, M. J., Verkoeijen, S., van Buren, L., Burg, D., van de Water, B. Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. Cancer Res., *65*: 4698-4706, 2005.
51. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J.Cell Biol., *134*: 793-799, 1996.
52. Brown, J. M. and Attardi, L. D. The role of apoptosis in cancer development and treatment response. Nat.Rev.Cancer, *5*: 231-237, 2005.
53. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100*: 57-70, 2000.
54. Brown, J. M. and Wilson, G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? Cancer Biol.Ther., *2*: 477-490, 2003.
55. Watson, A. J. Apoptosis and colorectal cancer. Gut, *53*: 1701-1709, 2004.

56. Brown, J. M. and Attardi, L. D. The role of apoptosis in cancer development and treatment response. Nat.Rev.Cancer, *5*: 231-237, 2005.
57. Brown, J. M. and Wilson, G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? Cancer Biol.Ther., *2*: 477-490, 2003.
58. Brown, J. M. and Wilson, G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? Cancer Biol.Ther., *2*: 477-490, 2003.
59. DuBois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. Cyclooxygenase in biology and disease. FASEB J., *12*: 1063-1073, 1998.
60. Tuynman, J. B., Peppelenbosch, M. P., and Richel, D. J. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev.Oncol.Hematol., *52*: 81-101, 2004.
61. Labayle, D., Fischer, D., Vielh, P., Drouhin, F., Pariente, A., Bories, C., Duhamel, O., Trousset, M., and Attali, P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. Gastroenterology, *101*: 635-639, 1991.
62. Akre, K., Ekstrom, A. M., Signorello, L. B., Hansson, L. E., and Nyren, O. Aspirin and risk for gastric cancer: a population-based case-control study in Sweden. Br.J.Cancer, *84*: 965-968, 2001.
63. Coogan, P. F., Rosenberg, L., Palmer, J. R., Strom, B. L., Zauber, A. G., Stolley, P. D., and Shapiro, S. Nonsteroidal anti-inflammatory drugs and risk of digestive cancers at sites other than the large bowel. Cancer Epidemiol.Biomarkers Prev., *9*: 119-123, 2000.
64. Funkhouser, E. M. and Sharp, G. B. Aspirin and reduced risk of esophageal carcinoma. Cancer, *76*: 1116-1119, 1995.
65. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. Aspirin use and reduced risk of fatal colon cancer. N.Engl.J.Med., *325*: 1593-1596, 1991.
66. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc.Natl.Acad.Sci.U.S.A, *94*: 3336-3340, 1997.
67. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93*: 705-716, 1998.
68. Tsujii, M. and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83*: 493-501, 1995.
69. Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., Lee, J. C., and Dubinett, S. M. Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J.Immunol, *164*: 361-370, 2000.
70. Bos, C. L., Richel, D. J., Ritsema, T., Peppelenbosch, M. P., and Versteeg, H. H. Prostanoids and prostanoid receptors in signal transduction. Int.J.Biochem.Cell Biol., *36*: 1187-1205, 2004.
71. Williams, C. S. and DuBois, R. N. Prostaglandin endoperoxide synthase: why two isoforms? Am.J.Physiol, *270*: G393-G400, 1996.
72. Smith, W. L., Marnett, L. J., and DeWitt, D. L. Prostaglandin and thromboxane biosynthesis. Pharmacol.Ther., *49*: 153-179, 1991.
73. Hanif, R., Pittas, A., Feng, Y., Koutsos, M. I., Qiao, L., Staiano-Coico, L., Shiff, S. I., and Rigas, B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem.Pharmacol., *52*: 237-245, 1996.
74. Din, F. V., Dunlop, M. G., and Stark, L. A. Evidence for colorectal cancer cell specificity of aspirin effects on NF kappa B signalling and apoptosis. Br.J.Cancer, *91*: 381-388, 2004.
75. Babbar, N., Ignatenko, N. A., Casero, R. A., Jr., and Gerner, E. W. Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. J.Biol. Chem., *278*: 47762-47775, 2003.

76. Andre, T. and de Gramont, A. An overview of adjuvant systemic chemotherapy for colon cancer. Clin.Colorectal Cancer, *4 Suppl 1*: S22-S28, 2004.
77. Tuynman, J. B., Peppelenbosch, M. P., and Richel, D. J. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev.Oncol.Hematol., *52*: 81-101, 2004.
78. Brown, J. R. and DuBois, R. N. COX-2: a molecular target for colorectal cancer prevention. J.Clin. Oncol., *23*: 2840-2855, 2005.
79. Dempke, W., Rie, C., Grothey, A., and Schmoll, H. J. Cyclooxygenase-2: a novel target for cancer chemotherapy? J.Cancer Res.Clin.Oncol., *127*: 411-417, 2001.
80. Baldwin, A. S., Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu.Rev. Immunol., *14*: 649-683, 1996.
81. Kojima, M., Morisaki, T., Izuhara, K., Uchiyama, A., Matsunari, Y., Katano, M., and Tanaka, M. Lipopolysaccharide increases cyclo-oxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kappa B activation. Oncogene, *19*: 1225-1231, 2000.
82. Yamauchi, T., Watanabe, M., Kubota, T., Hasegawa, H., Ishii, Y., Endo, T., Kabeshima, Y., Yorozuya, K., Yamamoto, K., Mukai, M., and Kitajima, M. Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. Dis.Colon Rectum, *45*: 98-103, 2002.
83. Tuynman, J. B., Peppelenbosch, M. P., and Richel, D. J. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev.Oncol.Hematol., *52*: 81-101, 2004.
84. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res., *58*: 409-412, 1998.
85. Reddy, B. S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K., and Rao, C. V. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res., *60*: 293-297, 2000.
86. Rao, C. V., Rivenson, A., Simi, B., Zang, E., Kelloff, G., Steele, V., and Reddy, B. S. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. Cancer Res., *55*: 1464-1472, 1995.
87. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N.Engl.J.Med., *342*: 1946-1952, 2000.
88. Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanas, A., Konstam, M. A., and Baron, J. A. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. N.Engl.J.Med., *352*: 1092-1102, 2005.
89. Solomon, S. D., McMurray, J. J., Pfeffer, M. A., Wittes, J., Fowler, R., Finn, P., Anderson, W. F., Zauber, A., Hawk, E., and Bertagnolli, M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N.Engl.J.Med., *352*: 1071-1080, 2005.

CHAPTER 2

Combined expression of the non-receptor protein tyrosine kinases FAK and Src in primary colorectal cancer is associated with tumor recurrence and metastasis formation

P. de Heer, M.M. Koudijs, C.J.H. van de Velde, R.I.J.M. Aalbers, R.A.E.M. Tollenaar, H. Putter, H. Morreau, B. van de Water, P.J.K. Kuppen

Submitted for publication

Abstract

The protein tyrosine kinase Focal Adhesion Kinase (FAK) and Src in association with phosphorylation of the adapter protein paxillin are essential in tumor metastasis formation. Elevated levels of FAK, Src and paxillin may increase the metastatic potential of primary colorectal tumor cells. The aim of the current study was to examine the expression of FAK, Src, and paxillin using immunohistochemistry in the context of disease progression and to evaluate its clinical significance as a prognostic factor.

The impact of FAK, Src and paxillin levels on colorectal cancer progression was evaluated by immunohistochemistry in 104 primary colorectal cancer specimens with clinical follow up. In addition, FAK, Src and paxillin expression levels were quantified in 68 primary colorectal tumors and corresponding liver metastases.

FAK and paxillin expression individually did not significantly impact time to recurrence (p=0.09, and p=0.89 respectively). Src expression was associated with tumor recurrence p=0.03. However, tumors that expressed both high FAK and Src levels had a significant shorter time to recurrence (p=0.004, hazard ratio: 2.98, 95% CI 1.14-6.31). FAK, Src and paxillin showed equivalent levels in corresponding liver metastases compared to the primary tumors (p=0.67, p=0.28 and p=0.34 respectively).

These findings show that high levels of FAK and Src combined were predictive for recurrence of colorectal cancer. In addition, expression of FAK, Src and paxillin in primary colorectal cancer were maintained in corresponding distant metastases.

Introduction

Tumor cell metastasis involves several steps including detachment, migration and invasion. These processes are tightly regulated by protein tyrosine kinase activity downstream of integrin-mediated cell adhesion. The non-receptor protein tyrosine focal adhesion kinase (FAK) is localized at integrin-enriched cell adhesion sites (focal adhesions) and acts as an integrator of several signaling pathways regulating cell motility[1]. These signaling pathways include growth factor signaling, mechanical stimuli and biochemical signaling through interaction between integrins and extracellular matrix proteins[2]. Autophosphorylation of FAK occurs in response to integrin engagement and creates a binding site for the Src family of protein tyrosine kinases[3]. The FAK-Src kinase complex subsequently mediates the phosphorylation of several focal adhesion-associated proteins including the scaffolding molecules paxillin[4]. Paxillin recruits other signaling molecules to adhesion sites and, thereby, indirectly regulates the dynamic organization of the actin cytoskeleton during the process of cell migration[5]. FAK-, Src- and paxillin-transduced signals control cellular processes such as migration, invasion[6,7] and anchorage independent growth[8], processes vital for cell motility and the ability of tumor cells to metastasize. Previous studies in laboratory animals indicate a direct role for both FAK and Src in tumor development as well as disease progression, *i.e.* metastasis formation[6].
Increased expression of FAK, Src and paxillin has been reported in several malignancies including breast[9,10], head and neck[11], colorectal cancer[12,13], and in colorectal liver metastases[14]. Elevated levels of FAK expression in metastases as compared to the primary tumor have been described[13,15], but these observations are not consistent[16,17]. The prognostic value of FAK levels has been established in several malignancies[18-21], but remains debated in colorectal cancer[22]. Increased Src expression is associated with malignant disease and poor patient prognosis in colorectal cancer[23]. Given the direct functional relationship between FAK, Src and downstream substrates such as paxillin, in the biological process of cell migration, the aim of the current study was to examine the clinical impact of FAK, Src and paxillin expression in colorectal cancer using immunohistochemistry.

Material and Methods

Patients and tumors

Randomly selected, formalin-fixed, paraffin-embedded archival tissue tumor samples were obtained from the tissue archives of the Leiden University Medical Center. Analyses were performed in two separate groups of patients. The study population consisted of 2 panels of pathological material: For survival analyses with FAK, Src and paxillin a randomly selected group of 104 stage II and III patients with colorectal cancer was used. These patients underwent curative resection of their tumor with available clinical follow up. For analyses of FAK, Src and paxillin expression in matched tissue samples of primary colorectal tumors and corresponding synchronous and metachronous liver metastases a group of 68 selected patients was used. All patients underwent surgery at Leiden University Medical Center between 1980 and 1992. Patient follow-up was derived from hospital files. Clinical follow-up included physical examination, chest X-ray, hematology, blood chemistry and screening for tumor marker CEA. Abdominal

and pelvic computed tomographic scans and colonoscopies were performed when indicated. The study was performed according to the Dutch medical ethical regulations for good clinical practice. Patients did not receive pre- or post operative chemo- or radiotherapy for their primary tumor or metastases.

Figure 1

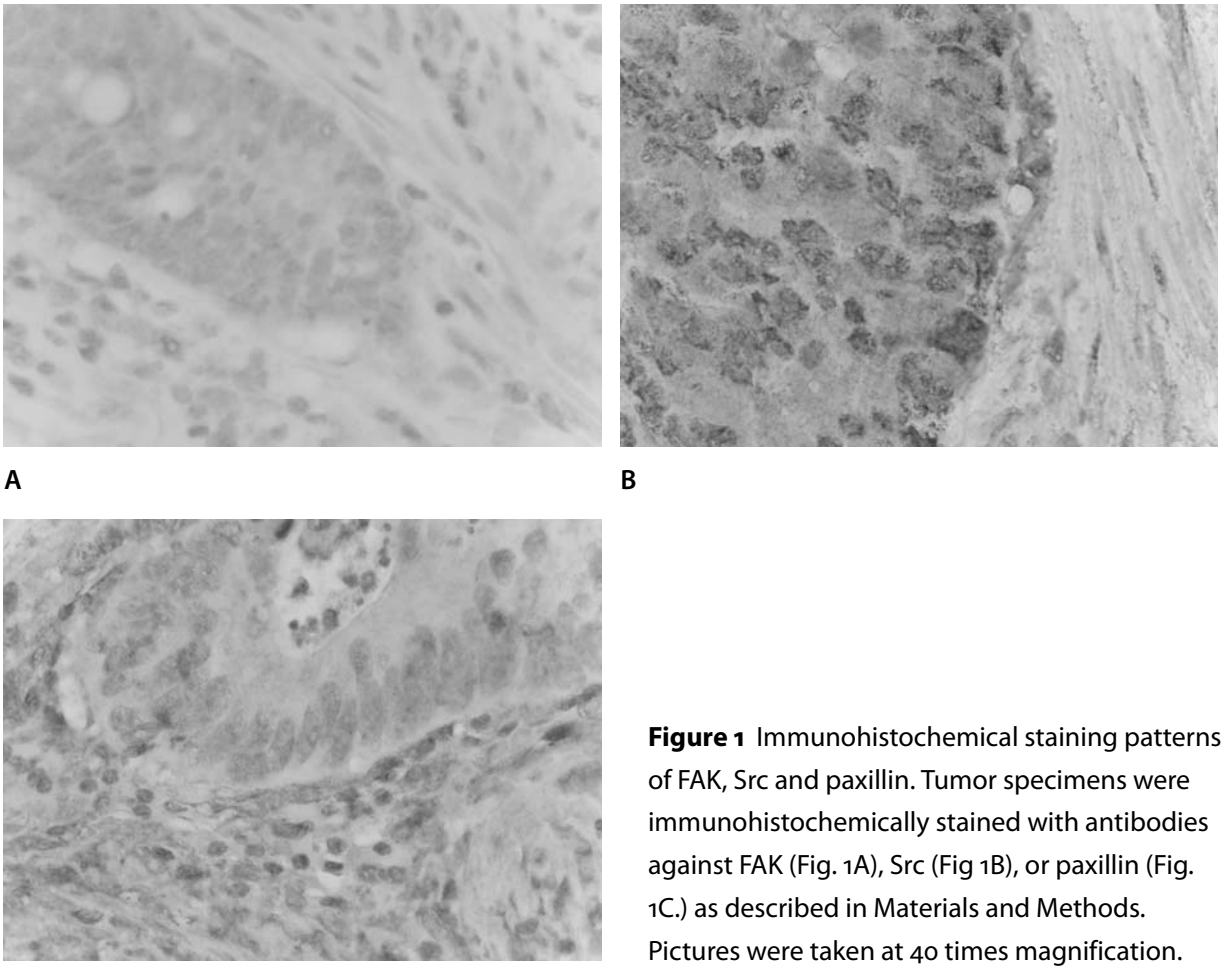


Figure 1 Immunohistochemical staining patterns of FAK, Src and paxillin. Tumor specimens were immunohistochemically stained with antibodies against FAK (Fig. 1A), Src (Fig 1B), or paxillin (Fig. 1C.) as described in Materials and Methods. Pictures were taken at 40 times magnification.

Immunohistochemistry

Paraffin-embedded archival tissue sections of 4 μm were prepared on aminopropylethoxysilane (APES) coated slides, dried overnight at 37°C, deparaffinized in xylol and subsequently rinsed in ethanol. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol for 20 min. After immersion in alcohol the sections were rehydrated. Antigen retrieval was performed for FAK and Src IHC stainings by boiling the sections in 10 mM EDTA solution (pH 6.0) for 10 min, cooling them for 2 hr, and washing them in demiwater and PBS (pH 7.4). For paxillin staining antigen retrieval was performed by incubation for 30 min. in freshly prepared, preheated trypsin solution (0,5 gram 0.1% Trypsin (Sigma T-7409) with 0,5 gram $CaCl_2$ (anhydrous) (pH: 7,4), demineralised water was added to a total volume of 500ml) at 37°C in water

bath, after which slides were thoroughly washed in demineralised water and transferred to PBS. Sections were incubated overnight at room temperature with monoclonal antibodies against human FAK (Mouse anti-FAK clone 77: 1:100, BD Biosciences, Alphen a/d Rijn, The Netherlands), Src (Mouse anti-Src clone GD11: 1:35, Upstate, Waltham, MA, USA) or paxillin (Mouse anti-paxillin clone 349: 1:25, BD Biosciences, Alphen a/d Rijn, The Netherlands) in PBS with 1% BSA (PBS/BSA). After three washing steps in PBS, sections were incubated for 30 min with Envision (DAKO, Denmark). The sections were then washed in PBS, rinsed in 0.05M Tris/HCl-buffer (ph 7.6) and developed in 3.3 diaminobenzidine tetrahydrochloride (DAB) with 0.002% hydrogen-peroxide for 10 min, which results in a brown signal. Sections were counterstained with haematoxylin, dehydrated with ethanol, cleared in xylene and mounted with pertex. To avoid inter-assay variability, all slides were stained in one batch.

Analysis of staining patterns

FAK, Src and paxillin staining was scored under light microscopy, blinded for tumor number and clinical outcome using a scoring system by Lark et al.[17] that measured relative intensity of antibody expression: 0, none; 1, borderline/weak; 2, moderate; 3, strong expression. In addition, cellular localisation (cytoplasm and nucleus) and the percentage of positive cells was evaluated. All slides were double-blindly scored by an independent observer (M.K.) and the results were subsequently validated by an independent pathologist (H.M.). The degree of FAK, Src and paxillin expression in tumor specimens was determined by multiplying intensity of staining (0, 1, 2, or 3) with percentage of positive cells/100 resulting in a continuous score from 0 to 3. In order to assess the impact of FAK, Src and paxillin expression level on patient survival and tumor recurrence and for correlations with clinical parameters, the score of the degree of expression was empirically dichotomized at the median, comparing the survival of patients whose levels were above the median (high expression levels) to those below the median (low expression levels).
Of the 104 primary tumor specimens stained for FAK, Src and paxillin expression respectively 10, 3, and 9 tumors were not assessable. Of the 68 primary liver tumors and corresponding liver metastases stained for FAK, Src and paxillin expression respectively 3, 2 and 2 tumors were not assessable, due to technical failures, unavailability of tumor material or excessive tumor necrosis. One patient was lost to follow-up; due to relocation to another hospital where patient files were deleted after 10 years.

Statistical analyses

Statistical analysis between groups was performed using the Pearson's χ^2 test and McNemar-Bowker test. Correlations between continuous variables were evaluated using Spearman rank correlation test, one way anova, or student's T-test. For survival analysis grouping with FAK, Src or paxillin expression, Kaplan-Meier analysis was used and differences between the survival curves were analysed using the log-rank test. Events for time to recurrence, disease-free and overall survival were defined as follows: time from surgical resection to disease relapse, time from surgical resection to disease relapse or death, and time from surgical resection to death respectively.
To correct the univariate results for correlations with established risk factors in colorectal carcinoma, a multivariate analysis was performed for the time to recurrence of all 104 patients (Cox

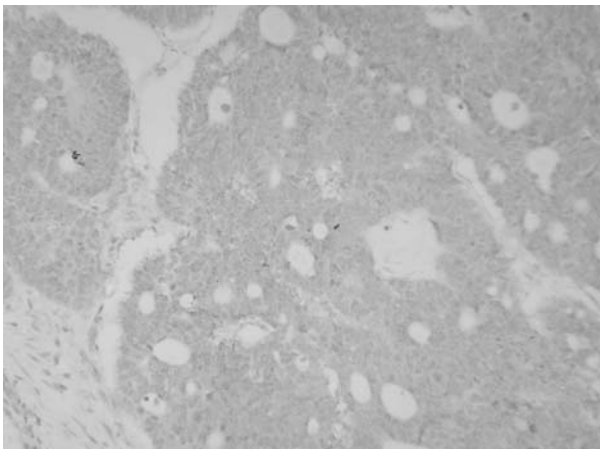
proportional hazard model). All variables with a p-value<0.10 in the univariate analysis were selected for a forward selection procedure. The clinicopathological factors were entered into the model; molecular characteristics were then entered in a forward selection procedure.
The statistical package SPSS version 12.0 (SPSS Inc, Chicago, IL) was used to conduct statistical analyses. A p-value < 0.05 (two-tailed) was considered significant. P-values < 0.10 were considered near significant and included in the multivariate analysis.

Table 1 Clinicopathological characteristics of 104 randomly selected primary colorectal cancer specimens and their association with FAK, Src and paxillin expression levels and tumor recurrence

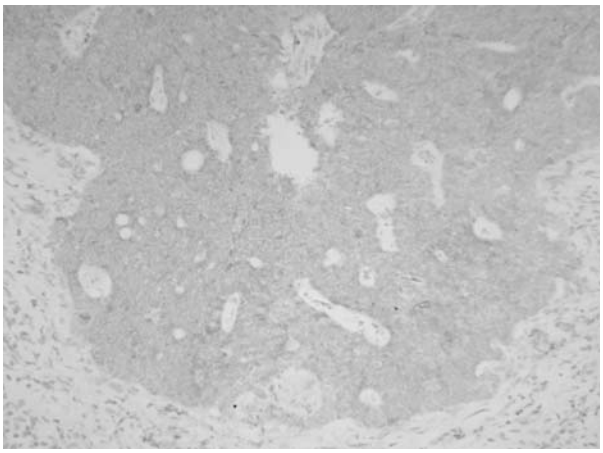
Clinicopathological Characteristics	**n(%)**	**FAK expression level****	**Src expression level****	**paxillin expression level****	**Association with tumor recurrence**
Gender: Female Male	 43 (41) 61 (59)	p=0.20 2.0 1.9	 p=0.15 2.2 2.0	 p=0.69 2.3 2.4	p=0.31
Pathological staging: II III	 51 (49) 53 (51)	 p=0.60 2.0 1.9	 p=0.60 2.0 2.2	 p=0.14 2.3 2.5	p=**0.001**
Tumor Location: left of the L.F.* right of the L.F.*	 45 (43) 59 (57)	 p=**0.05** 1.78 2.0	 p=**0.01** 1.9 2.2	 p=0.45 2.3 2.4	p=0.95
Age: 0-50 >50	 13 (12) 91 (88)	 p=0.60 2.0 1.9	 p=0.82 2.1 2.1	 p=0.18 2.6 2.3	p=0.09
Grade of differentiation: -Undifferentiated -Poorly -Moderately -Well -Not assessable	 2 (2) 57 (55) 24 (23) 17 (16) 4 (4)	p=0.71 1.8 2.0 1.9 1.9 1.8	 p=0.22 1.1 2.1 2.2 2.1 1.6	 p=0.66 2.8 2.4 2.3 2.4 2.1	p=0.39

*: Lienate Flexure
**: Expression score levels in Arbitrary Units (AU)

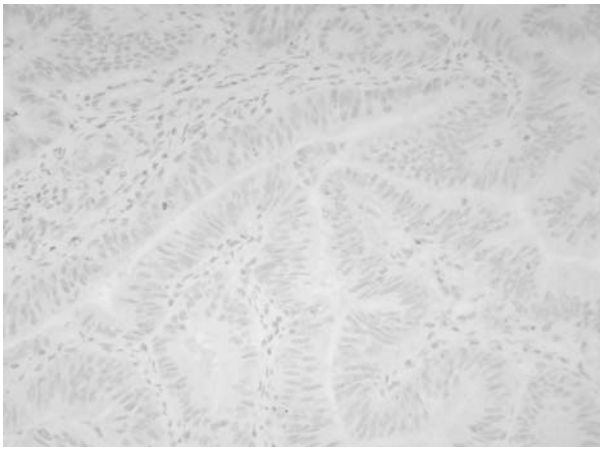
Figure 2



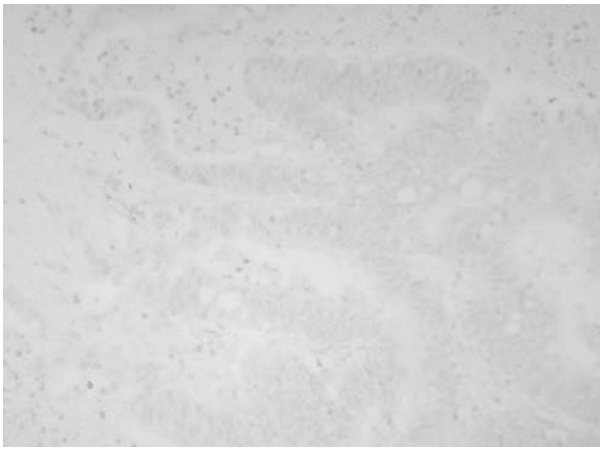
A1



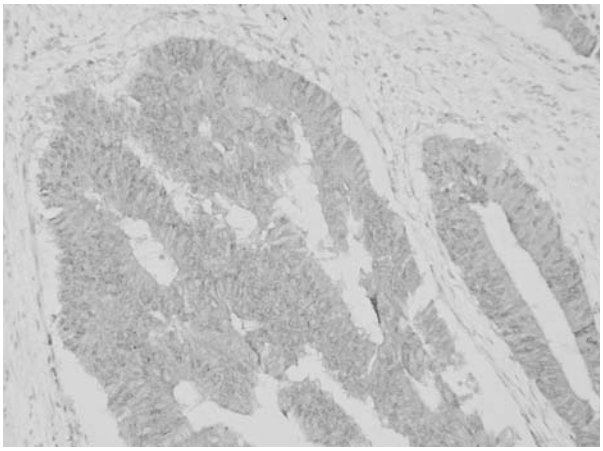
A2



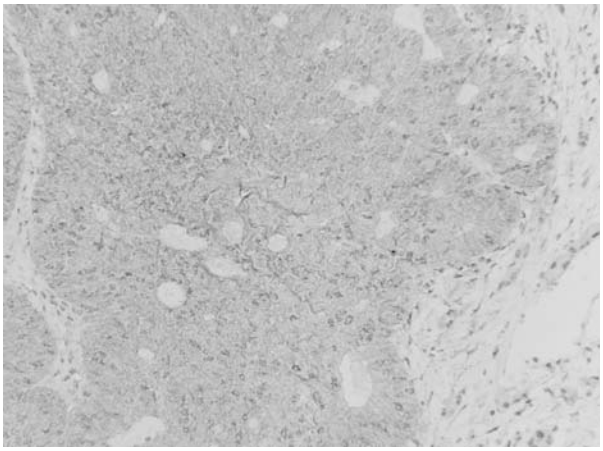
A3



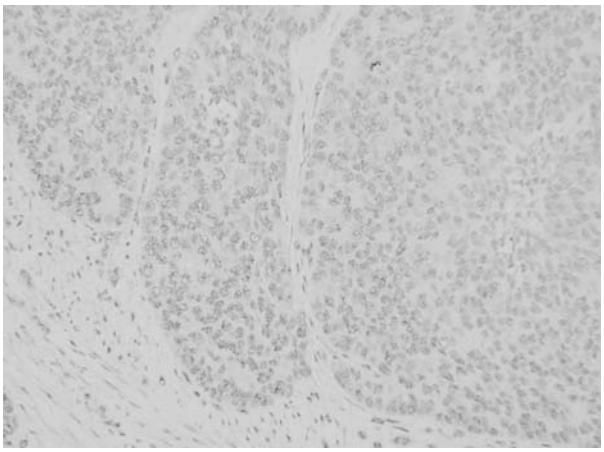
A4



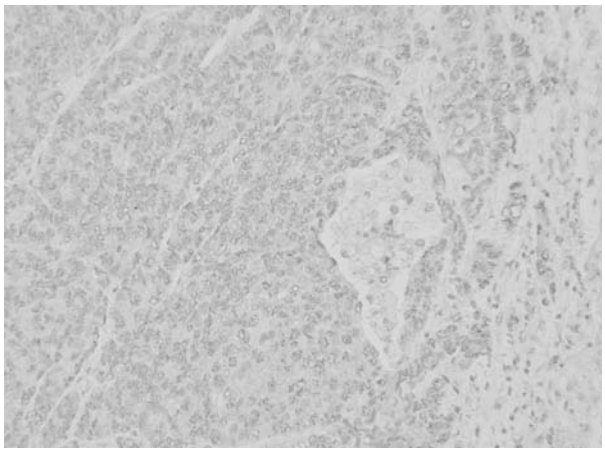
B1



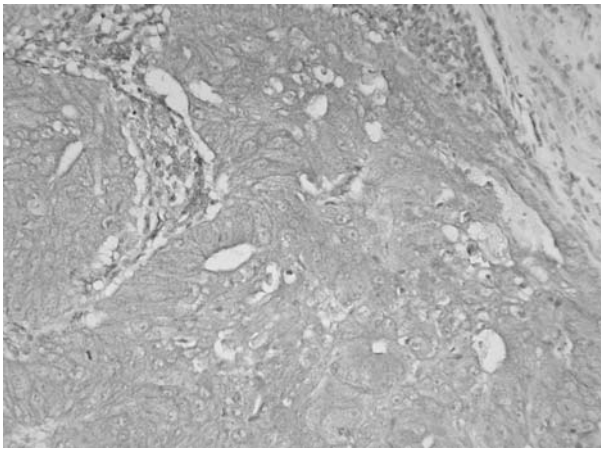
B2



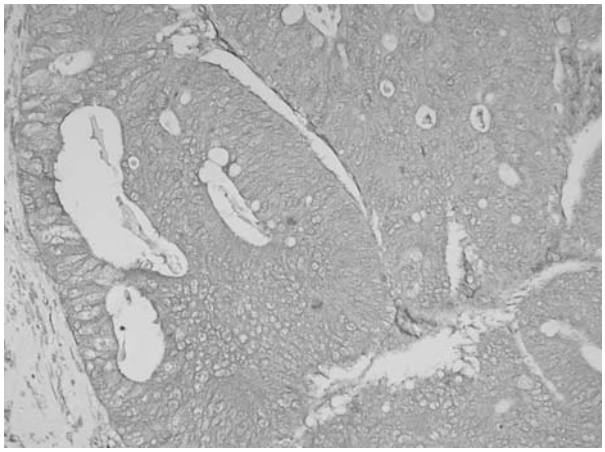
B3



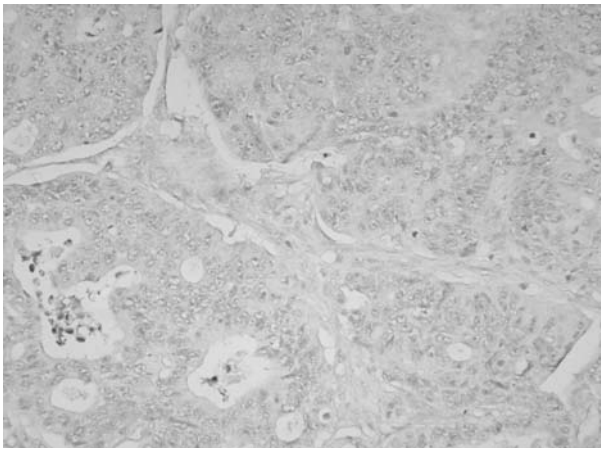
B4



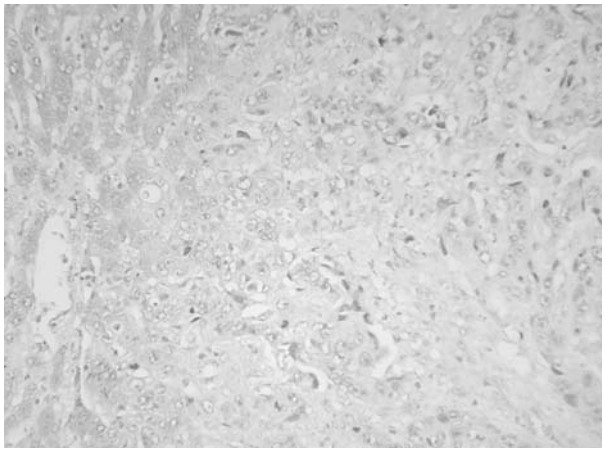
C1



C2



C3



C4

Figure 2 Immunohistochemical staining patterns of FAK, Src and paxillin expression in 68 primary colorectal cancers and corresponding liver metastases. Paired samples were stained with antibodies against FAK (Fig 2A1-A4), Src (Fig 2B1-B4) or paxillin (Fig 2C1-C4). Pictures were taken at 20 times magnification. Figures 2ABC: strong FAK (A), Src, (B) or paxillin (C) expression in primary tumor (1) and corresponding liver metastasis (2) and weak FAK (A), Src, (B) and paxillin (C) expression in primary tumor (3) and corresponding liver metastasis (4).

Results

Analysis of FAK, Src and paxillin expression in primary colorectal cancer specimens

Expression of FAK, Src and paxillin was immunohistochemically detected in a panel of colorectal tumors (Fig. 1A-C). Expression levels differed not only among tumors, but were also heterogeneously distributed throughout the whole tumor area. Therefore, the intensity of immunohistochemical staining as well as the percentage of positive cells was determined in each tumor specimen. Variations in intensity of the immunohistochemical staining can be seen in figure 2.

Next, we assessed the impact of various clinicopathological parameters on FAK, Src and paxillin expression in 104 randomly selected, curatively resected colorectal carcinoma patients with clinical follow up. Patients were retrospectively followed up for a median of 5.9 years (range, 0.1-18.6 years; SD, 5.2 years). An overview of tumor characteristics for all 104 patients is provided in table 1. None of the evaluated patient characteristics was associated with FAK, Src and paxillin expression, except that tumors with high FAK and high Src expression were significantly more often localized left of the lienate flexure ($p=0.05$ and $p=0.01$ respectively).

FAK and Src form a protein tyrosine complex that is involved in the regulation of focal adhesion turnover and cell motility. One of the protein targets of the FAK/Src complex is paxillin, and phosphorylation of the latter is important for cell migration processes. Because of this interrelationship we anticipated that the expression levels of the individual proteins would be correlated in individual tumor specimens. Indeed, tumor expression levels of FAK significantly correlated with Src and paxillin expression levels ($p=0.005$ and $p=0.006$ respectively). In addition, Src expression levels were significantly correlated with paxillin expression ($p=0.04$) (Spearman rank correlation test). These results suggest a possible overlapping mechanism of regulation of FAK, Src and paxillin expression in colorectal carcinoma cells.

FAK and Src but not paxillin are prognostic markers for colorectal cancer progression

We determined whether expression level of FAK and Src had an impact on the clinical behavior of colorectal cancer specimens. Moreover, we evaluated the prognostic impact of the FAK/Src downstream effector paxillin. High tumor expression levels of FAK were near significantly associated with a shorter TTR ($p=0.09$) (Fig. 3A). Patients with high expression of SRC showed a shorter TTR ($p=0.03$) (Fig. 3B), Paxillin expression was not associated with tumor recurrence ($p=0.89$) (Fig 3C). Because FAK and Src functionally act as protein kinase complex, we reasoned that the combined expression may predict colorectal cancer progression. Indeed, tumors with high levels of FAK as well as high levels of Src were highly significant associated with a shorter TTR ($p=0.005$, Fig 3D) as compared to all other tumors. This did not translate in a survival benefit DFS ($p=0.34$) and OS ($p=0.50$) for tumors with a high FAK and Src expression. Pathological stage III was significantly correlated with a shorter time to recurrence ($p=0.001$), DFS ($p=0.05$) and OS ($p=0.06$). Patient age, grade of differentiation, tumor location and gender were not associated with patient survival or tumor recurrence.

Next, all variables with a p-value<0.10 in the univariate analysis were selected for a forward selection procedure (Cox proportional hazard model). First, the clinicopathological factors were entered into the model. Second, the molecular characteristics were entered in a forward selec-

tion procedure. In the multivariate forward selection procedure high FAK and Src combined proved to be an independent prognostic factor for TTR (p=0.004, hazard ratio: 2.98, 95% CI 1.14-6.31), when corrected for age (p=0.043, HR=0.38, 95% CI 0.15-0.97) and stage (p=0.004, HR=3.47, 95% CI 1.47-8.17).

Figure 3 Predictive value of FAK, Src and paxillin expression for time to recurrence.

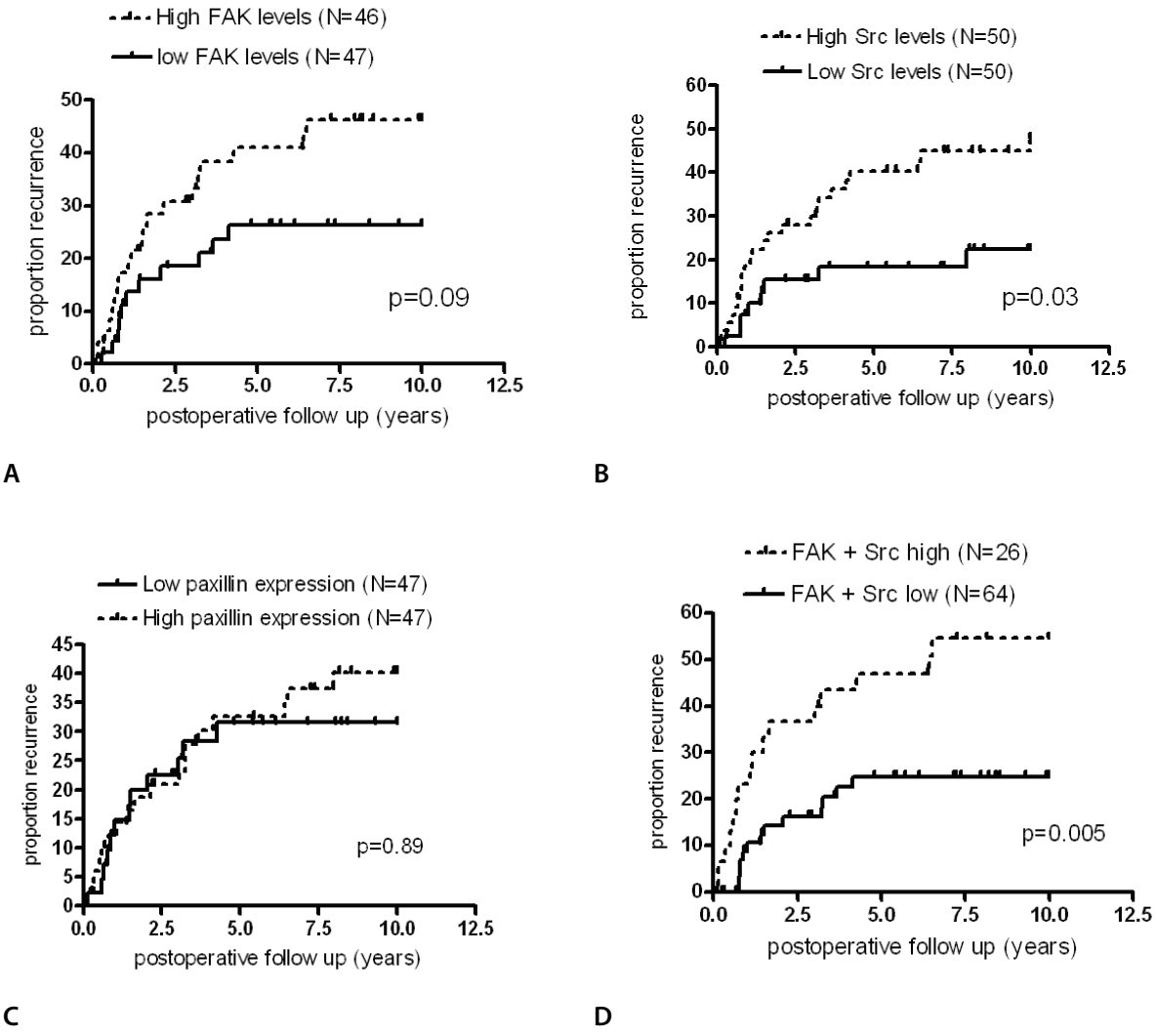


Figure 3A-D Time to local or distant tumor recurrence in patients according to FAK, Src and paxillin expression levels. Patients with high (above median expression) versus patients with low (below median expression) level of FAK (3A), Src (3B) and paxillin (3C). Time to tumor recurrence of patients with high level of both FAK and Src expression is shown in figure 3D. P-values for significance of statistical difference between both groups in each figure are shown.

Table 2 Clinicopathological characteristics of 68 patients with primary colorectal cancers and corresponding liver metastases and their correlation with FAK, Src and paxillin expression levels

Clinicopathological Characteristics	**n(%)**	**FAK expression level****	**Src expression level****	**paxillin expression level****
Gender: Male Female	 22 (32) 46 (68)	p=0.69 1.3 1.6	p=0.50 2.2 2.0	p=0.30 2.2 2.3
Pathological staging: III IV	 41 (60) 27 (40)	p=0.36 1.5 1.7	p=0.88 2.1 2.1	p=0.84 2.1 2.2
Tumor Location: left of the L.F.* right of the L.F.*	 55 (81) 13 (19)	p=0.72 1.7 1.6	p=0.59 2.2 2.1	p=0.48 2.3 2.4
Age: 0-50 >50	 13 (12) 91 (88)	p=0.34 2.0 1.7	p=0.81 2.2 2.1	p=0.71 2.2 2.0
Grade of differentiation: -Poorly -Moderately -Well	 4 (8) 42 (84) 4 (8)	p=0.49 1.5 1.7 2.0	p=0.11 1.9 2.1 2.7	p=0.20 1.7 2.2 2.5

*: Lienate Flexure

**: Expression score levels in Arbitrary Units (AU)

Analysis of FAK, Src and paxillin expression in colorectal carcinomas and corresponding liver metastases

FAK, Src and paxillin levels were quantified in a panel of 68 primary tumors and corresponding liver metastases. Patient characteristics are shown in table 2. IHC evaluation indicated that FAK, Src and paxillin showed equivalent levels in corresponding liver metastases compared to the primary tumors (Fig. 4). No significant differences in levels of expression of FAK, Src and paxillin between primary tumors and paired liver metastases were observed (p=0.67, p=0.28 and p=0.34, respectively; paired t-test) (Fig 4).

Figure 4

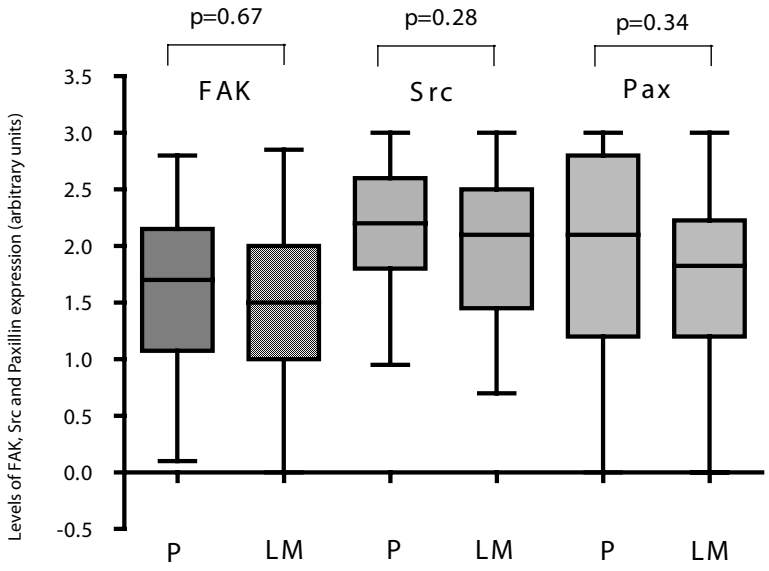


Figure 4 Immunohistochemical analysis of levels of expression of FAK, Src and paxillin in 68 primary colorectal tumors (P) and corresponding liver metastases (LM). Box plots denote Mean, 25th and 75th percentiles and range of expression levels. Expression levels of FAK, Src and paxillin do not significantly differ between primary cancers and corresponding liver metastases (paired samples t-test), as p-values shown indicate.

Discussion

The major observation from the current study is that tumors expressing high FAK and Src levels showed higher recurrence rates. This finding suggests close interdependency of FAK and Src in determining clinical behavior. FAK interacts with Src and the latter kinase mediates the trans-phosphorylation of FAK within the kinase domain activation loop, hereby promoting maximal FAK catalytic activation[24]. Combined targeting of FAK and Src may thus be beneficial for the outcome of colorectal cancer and may provide an opportunity for therapeutic intervention. This is supported by recent *in vitro* studies in which selective small molecular Src inhibitors and dominant negative deletion mutants of FAK act synergistically to promote colon tumor cell apoptosis[6]. Protein tyrosine kinases and related focal adhesion molecules have been associated with invasion and metastasis; however, the clinical relevance of elevated levels has been reported in only a small number of colorectal cancer cohort studies. Increased expression of FAK did not have an impact on patient survival when analysed in a recent study with 80 colorectal patients using IHC[22]. Src, however, was found to be an independent prognostic factor for DFS and OS in 45 patients in a study using an immune complex kinase assay[6]. The FAK/Src tyrosine kinase complex phosphorylates downstream signaling proteins, including paxillin[6]. In our studies an elevated level of paxillin was not associated with increased ability

to metastasize, indicating that integration of signaling pathways by paxillin is not a significant factor in determining the clinical outcome of colorectal cancer. However, since the function of paxillin is regulated by tyrosine phosphorylation on tyrosine residues 31 and 118 by Src and FAK, it is well possible that instead of expression level, paxillin phosphorylation is prognostic for colorectal cancer progression.

The current study used IHC as quantification method, as we were keen to focus on post transcriptional and thus stable protein expression. In addition, IHC is a technique easily applicable and widely used in the clinical setting. However, there can be limitations in relation to i.e. staining variability, epitope stability, fixation and sample area for counting. We avoided most limitations by staining all tissue sections one batch, evaluating slides by an independent observer, and validating the scoring results by an independent pathologist.

The equal expression levels of FAK, Src and paxillin in colorectal carcinomas compared to corresponding liver metastases in this study indicate that colorectal liver metastases recapitulate the organization of their primary tumors. The analysis provided the advantage of directly comparing levels of FAK, Src and paxillin levels in colorectal cancer cells in primary tumors and metastases of the same patient. To our knowledge this is the first time that Src expression was evaluated in primary colorectal cancer and corresponding liver metastases. The results with paxillin in our study were similar to those in a study by Ayaki et al[17]. However, our data on FAK contradict several studies in which FAK was found to be up-regulated[13-16] or down-regulated[17] in the liver metastases, as compared to the primary tumor. As the aforementioned studies use different techniques for evaluation of FAK expression level, direct comparisons between results should be made with caution. The tumors in the current study were evaluated by IHC as IHC provides information about FAK, Src and paxillin expression on a cellular level and is sensitive to slight changes in expression levels, yielding an accurate evaluation. A recent study found FAK expression to be up-regulated in RT-PCR analysis in 17 patients, but no significant differences in FAK levels were detected when evaluated with IHC[16]. Ayaki et al.[17] report down regulation of FAK expression level in liver metastases as compared to the primary tumor. However, both studies have as limitation that they include small sample sizes of respectively 17 and 10 patients. The present study provided robust evidence by utilizing the largest sample size up to date.

Recently a model for cancer metastasis was proposed by Brabletz et al.[25], that integrates both genetic alteration and the tumor environment as combined driving forces of malignant progression. This model proposes the existence of mobile cancer stem cells that transiently develop from stationary cancer cells by induction of epithelial to mesenchymal transition. The possibility exists that the comparable expression of FAK, Src and paxillin is indeed related to a mobile cancer stem cell population that is responsible for both the primary tumor growth as well as the formation of liver metastases. In summary, the present study showed that high levels of the non-receptor protein tyrosine kinases FAK and Src in the primary tumor, but not paxillin, predict tumor recurrence in colorectal cancer patients. In addition, the levels of expression of FAK, Src as well as paxillin in colorectal cancer were maintained in corresponding distant metastases.

References

1. Webb, D. J., Parsons, J. T., and Horwitz, A. F. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. Nat.Cell Biol., *4*: E97-100, 2002.
2. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. Nat.Rev.Mol.Cell Biol., *6*: 56-68, 2005.
3. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der, G. P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature, *372*: 786-791, 1994.
4. Thomas, J. W., Cooley, M. A., Broome, J. M., Salgia, R., Griffin, J. D., Lombardo, C. R., and Schaller, M. D. The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. J.Biol.Chem., *274*: 36684-36692, 1999.
5. Bellis, S. L., Miller, J. T., and Turner, C. E. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. J.Biol.Chem., *270*: 17437-17441, 1995.
6. van Nimwegen, M. J., Verkoeijen, S., van Buren, L., Burg, D., van de Water, B. Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. Cancer Res., *65*: 4698-4706, 2005.
7. Hsia, D. A., Mitra, S. K., Hauck, C. R., Streblow, D. N., Nelson, J. A., Ilic, D., Huang, S., Li, E., Nemerow, G. R., Leng, J., Spencer, K. S., Cheresh, D. A., and Schlaepfer, D. D. Differential regulation of cell motility and invasion by FAK. J.Cell Biol., *160*: 753-767, 2003.
8. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J.Cell Biol., *134*: 793-799, 1996.
9. Oktay, M. H., Oktay, K., Hamele-Bena, D., Buyuk, A., and Koss, L. G. Focal adhesion kinase as a marker of malignant phenotype in breast and cervical carcinomas. Hum.Pathol., *34*: 240-245, 2003.
10. Madan, R., Smolkin, M. B., Cocker, R., Fayyad, R., and Oktay, M. H. Focal adhesion proteins as markers of malignant transformation and prognostic indicators in breast carcinoma. Hum. Pathol., *37*: 9-15, 2006.
11. Kornberg, L. J. Focal adhesion kinase and its potential involvement in tumor invasion and metastasis. Head Neck, *20*: 745-752, 1998.
12. Cance, W. G., Harris, J. E., Iacocca, M. V., Roche, E., Yang, X., Chang, J., Simkins, S., and Xu, L. Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. Clin. Cancer Res., *6*: 2417-2423, 2000.
13. Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., and Cance, W. G. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res., *55*: 2752-2755, 1995.
14. Han, N. M., Fleming, R. Y., Curley, S. A., and Gallick, G. E. Overexpression of focal adhesion kinase (p125FAK) in human colorectal carcinoma liver metastases: independence from c-src or c-yes activation. Ann.Surg.Oncol., *4*: 264-268, 1997.
15. Weiner, T. M., Liu, E. T., Craven, R. J., and Cance, W. G. Expression of focal adhesion kinase gene and invasive cancer. Lancet, *342*: 1024-1025, 1993.
16. Lark, A. L., Livasy, C. A., Calvo, B., Caskey, L., Moore, D. T., Yang, X., and Cance, W. G. Overexpression of focal adhesion kinase in primary colorectal carcinomas and colorectal liver metastases: immunohistochemistry and real-time PCR analyses. Clin.Cancer Res., *9*: 215-222, 2003.
17. Ayaki, M., Komatsu, K., Mukai, M., Murata, K., Kameyama, M., Ishiguro, S., Miyoshi, J., Tatsuta, M., and Nakamura, H. Reduced expression of focal adhesion kinase in liver metastases compared with matched primary human colorectal adenocarcinomas. Clin.Cancer Res., *7*: 3106-3112, 2001.

18. Sood, A. K., Coffin, J. E., Schneider, G. B., Fletcher, M. S., DeYoung, B. R., Gruman, L. M., Gershenson, D. M., Schaller, M. D., and Hendrix, M. J. Biological significance of focal adhesion kinase in ovarian cancer: role in migration and invasion. Am.J.Pathol., *165*: 1087-1095, 2004.
19. Fujii, T., Koshikawa, K., Nomoto, S., Okochi, O., Kaneko, T., Inoue, S., Yatabe, Y., Takeda, S., and Nakao, A. Focal adhesion kinase is overexpressed in hepatocellular carcinoma and can be served as an independent prognostic factor. J.Hepatol., *41*: 104-111, 2004.
20. Recher, C., Ysebaert, L., Beyne-Rauzy, O., Mansat-De Mas, V., Ruidavets, J. B., Cariven, P., Demur, C., Payrastre, B., Laurent, G., and Racaud-Sultan, C. Expression of focal adhesion kinase in acute myeloid leukemia is associated with enhanced blast migration, increased cellularity, and poor prognosis. Cancer Res., *64*: 3191-3197, 2004.
21. Itoh, S., Maeda, T., Shimada, M., Aishima, S., Shirabe, K., Tanaka, S., and Maehara, Y. Role of expression of focal adhesion kinase in progression of hepatocellular carcinoma. Clin.Cancer Res., *10*: 2812-2817, 2004.
22. Theocharis, S. E., Kouraklis, G. P., Kakisis, J. D., Kanelli, H. G., Apostolakou, F. E., Karatzas, G. M., and Koutselinis, A. S. Focal adhesion kinase expression is not a prognostic predictor in colon adenocarcinoma patients. Eur.J.Surg.Oncol., *29*: 571-574, 2003.
23. Aligayer, H., Boyd, D. D., Heiss, M. M., Abdalla, E. K., Curley, S. A., and Gallick, G. E. Activation of Src kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. Cancer, *94*: 344-351, 2002.
24. Hanks, S. K., Ryzhova, L., Shin, N. Y., and Brabek, J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. Front Biosci., *8*: d982-d996, 2003.
25. Brabletz, T., Jung, A., Spaderna, S., Hlubek, F., and Kirchner, T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat.Rev.Cancer, *5*: 744-749, 2005.

CHAPTER 3

Caspase-3 activity predicts local recurrence in rectal cancer

P. de Heer, E.C. de Bruin, E. Klein-Kranenbarg, R.I.J.M. Aalbers, C.A.M. Marijnen, H. Putter, H.J. de Bont, J.F. Nagelkerke, J.H.J.M. van Krieken, H.W. Verspaget, P.J.K. Kuppen, C.J.H. van de Velde

Clinical Cancer Research, in press

Abstract

Radiotherapy followed by total mesorectal excision (TME) surgery has been shown to significantly reduce local recurrence rates in rectal cancer patients. Radiotherapy, however, is associated with considerable morbidity. The present study evaluated the use of biochemical detection of enzymatic caspase-3 activity as preoperative marker for apoptosis to preselect patients that are unlikely to develop a local recurrence to spare these patients from overtreatment and the negative side effects of radiotherapy.

Non-irradiated freshly-frozen tissue samples from 117 stage III rectal cancer patients were collected from a randomized clinical trial that evaluated preoperative radiotherapy in TME surgery. Additional frozen archival tissues from 47 preoperative biopsies and corresponding resected colorectal tumors were collected. Level of apoptosis was determined by measuring the enzymatic activity of caspase-3 in a biochemical assay.

Results: In tumor tissue, caspase-3 activity lower than the median were predictive of 5-year local recurrence (HR=7.4, 95%CI: 1.7-32.8; p=0.008), which was unaffected by adjustment for type of resection, tumor location and T status (adjusted HR=7.5, 95%CI 1.7-34.1; p=0.009). Caspase-3 activity in preoperative biopsies was significantly correlated with caspase-3 activity in corresponding resected tumors (r: 0.56, p<0.0001).

Detection of tumor apoptosis levels by measuring caspase-3 activity, for which a pre-operative biopsy can be used, accurately predicted local recurrence in rectal cancer patients. These findings indicate that caspase-3 activity is an important denominator of local recurrence and should be evaluated prospectively to be added to the criteria to select rectal cancer patients in which radiotherapy is redundant.

Introduction

Local recurrence is a major problem after rectal cancer surgery as it is the cause of severely disabling symptoms and is difficult to treat[1,2]. Local recurrence rates historically vary between 15% and 45%[2,6,7]. The introduction of total mesorectal excision (TME) as treatment for patients with rectal cancer has led to an improved local control and survival when compared to historical controls[2,6,7]. In addition to improved surgery, the administration of preoperative radiation therapy has further decreased local recurrence rates in several randomized clinical trials[8-12]. Radiation therapy, however, is associated with considerable morbidity. Several studies have evaluated the short- and long term morbidity of radiation therapy; Preoperative radiation therapy is associated with faecal incontinence, urgency and anal blood loss[13]. In addition to the general bowel dysfunction, an increase in venous thromboembolisms, pelvic fractures and sexual dysfunction have been reported[14,15]. These negative side-effects of radiation therapy emphasize the need for finding predictive factors for local recurrence to exclude patients with a very high probability for cure with surgery alone.

Among the predictive factors for survival and local tumor recurrence are lymph node metastasis, stage of the disease and the presence of a tumor-positive circumferential margin[12]. Most criteria, however, can only be determined postoperatively. Several recent studies in rectal cancer have showed that tumors with high levels of apoptosis show low local recurrence rates and favourable prognosis[16-19]. These results indicate that only patients with low levels of apoptosis may benefit from radiation therapy with respect to the development of local recurrences. Therefore, the level of apoptosis in tumors may provide a criterion to select patients for radiation therapy. The present study evaluated the use of biochemical detection of caspase-3 activity as a simple and quantitative technique to measure apoptosis in tissue samples and preoperative biopsies of rectal cancer to predict local recurrences in rectal cancer. The proapoptotic enzyme caspase-3 is activated at a point of convergence for the intrinsic and extrinsic apoptosis-induction pathways[20], so its activity should give a reliable measure of on-going levels of apoptosis in tumor samples. The study was performed in stage III rectal cancer patients as these are the patients that are most likely to benefit from preoperative radiation therapy[11,12].

Materials & Methods

Patients

The study population consisted of 2 sources of pathological material.

The first consisted of stage III rectal cancer patients who participated in the Dutch TME trial. These were collected and analysed for caspase-3 activity in order to evaluate the prognostic value of caspase-3 activity. In the TME study patients were randomized to receive radiation therapy before undergoing surgery according to a standardized TME protocol[12]. Patients were selected from the trial-arm that did not receive preoperative radiation therapy. All stage III patients who complied with the eligibility criteria of the TME trial[12] and of whom frozen tumor material was available were selected for this study, resulting in a study cohort of 117 patients. Frozen tissue samples of adjacent normal rectum tissue were available from 29 of the patients. A pathology review committee reviewed all tumors[12]. Any specimen that had tumor (i.e., pri-

mary tumor or lymph node metastasis) ≤1 mm from the circumferential margin was recorded as having a positive resection margin[21].
The second source consisted of 47 frozen biopsies and corresponding frozen rectal and rectosigmoideal, non-irradiated tumor tissues. These were collected and analysed for caspase-3 activity in order to evaluate the feasibility of caspase-3 detection in biopsies and to assess whether caspase-3 activity in preoperative biopsies is representative for that of the primary tumor. As preoperative biopsies were not collected in the TME trial, biopsies and corresponding non-irradiated tumor specimens were collected from the tissue archives of the Leiden University Medical Center. As these patients did not receive TME surgery and therefore have poor local control, they were not included in survival analyses.
The study was conducted following the regulations according to Dutch law for human material for research. Ethics board approval was obtained for gathering all study material and patient data in the current study.

Measurement of Caspase-3 Activity

The enzymatic activity of caspase-3 in tissue samples was measured as previously described[22]. Briefly, five 10-μM crysostat sections of tumor or normal tissue were suspended in a lysis buffer consisting of 10 mM HEPES, pH 7.0, 40mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl 2, and 5 mM EGTA. After 10 min on ice, the cells were disrupted by 10 seconds of sonification followed by four cycles of freezing and thawing and stored at -80°C. Protein concentration was determined using the method described by Bradford[23]. For measurement of caspase-3 enzymatic activity, samples containing 15 μg protein were incubated with 2.5 nmol of the enzyme substrates DEVD-AMC (Bachem, Heidelberg, Germany) in a 100-mM HEPES buffer, pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, and 10 mM dithiothreitol. During incubation at 37°C, fluorescent AMC was cleaved off by active caspases, corresponding with the level of caspase activity in the sample. The fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a Fluostar Optima plate reader (BMG Labtech gmbh, Offenburg, Germany). Calibration curves were constructed using free AMC. Caspase-3 activity was indicated in pmolAMC/min/mg protein.
Tumor sections and preoperative biopsies may contain various proportions of tumor epithelium and tumor stroma. To assess whether caspase-3 activity depended on the ratio of tumor epithelium and tumor stroma in sections of the tumor tissues used for analysis, the percentage of tumor epithelium was assessed by two independent observers (R.I.J.M.A. and N.G.E.) in adjacent Hematoxylin-Eosin stained slides.

Statistical analysis

Analysis was performed with SPSS statistical software (version 11.0 for Windows, SPSS Inc, Chicago, IL). Mann-Whitney U, Kruskal-Wallis, Wilcoxon Signed Rank and Spearmans' Rho tests were used to compare continuous variables. The entry date for the recurrence analyses was the time of surgery of the primary tumor. To guarantee sufficient number of patients in both groups, the patients were dichotomized at the median level of apoptosis by caspase-3 activity. Kaplan-Meier analyses and log rank tests were performed to compare recurrence rates in patients from the high and low apoptotic groups. Cox' regression analyses were used to calculate

Hazard Ratios (HR) with 95% confidence intervals (CI) for categorical variables. Variables with a p-value of ≤0.10 in the univariate analyses were subjected to a multivariate analysis. In order to enable comparisons of the outcome of caspase-3 data with recurrence rate of stage III rectal cancer patients treated with radiation therapy, Kaplan-Meier analysis was performed for these patients in the follow up data of the Dutch TME trial[12].

Results

Level of apoptosis in rectal cancer

We determined caspase-3 activity in rectal cancer specimens and adjacent normal tissue in a biochemical cleavage assay. Caspase-3 activity in the rectal tumor specimens was significantly higher than in the 29 normal tissue samples: median 15.2, interquartile range (IQR: 10.6-26.9) compared with 4.9 pmolAMC/min/mg protein (IQR: 3.4-10.5) ($p<0.0001$, Wilcoxon Signed Rank). There was no significant correlation between caspase-3 activity in tumor tissue and adjacent normal tissue ($p=0.85$; correlation coefficient 0.04, Spearmans' Rho test).

Correlation between clinical parameters and apoptosis

The characteristics of the stage III rectal cancer patients included in this study are summarized in table 1. Patient characteristics and several markers that have an impact on disease recurrence in rectal cancer, or that can be used as diagnostic tool[24-26], were evaluated for their association with level of apoptosis caspase-3 activity.

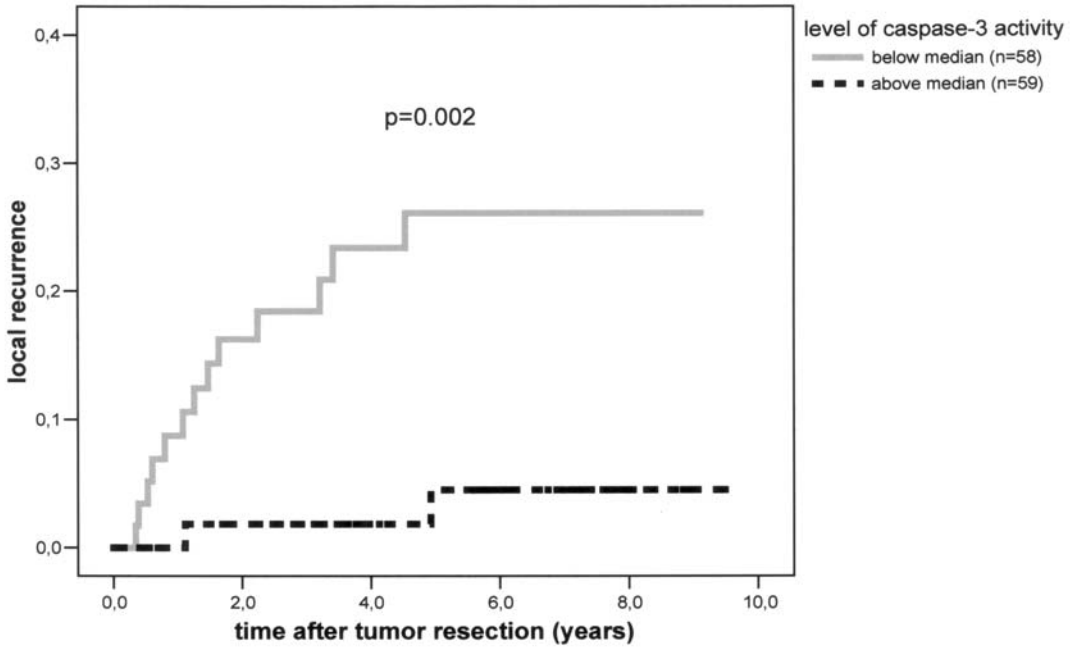
Mucinous type of carcinoma and tumors with tumor-positive circumferential margins were associated with lower caspase-3 activity ($p=0.04$ and $p=0.04$ respectively); all other variables were not significant (table 1).

Predictive value of caspase-3 activity for local recurrence

In the current study, caspase-3 activity with the median as cut-off point was accurately predictive for local tumor recurrence ($p=0.002$, log-rank test) (figure 1). After 5 years of follow-up the local recurrence rates were 28.1% in the tumors with caspase-3 activity below the median and 5.9% in tumors with caspase-3 activity above the median. Caspase-3 activity below the median was also associated with high distant recurrence rates (69.0% vs. 42.2% in the group with high caspase-3 activity after 5 years, $p=0.05$, log-rank test), but did not have an impact on patient survival ($p=0.10$, log-rank test).

In order to evaluate the benefit of short-term radiotherapy for the above described recurrence rates of low and high levels of apoptosis, we calculated the 5-years local recurrence rate of all stage III rectal cancer patients treated with preoperative radiation therapy included in the Dutch TME trial (N=243). Stage III patients receiving preoperative radiation therapy had a 5-years local recurrence rate of 11.4%. This percentage demonstrates that patients with high levels of apoptosis have sufficiently low recurrence rates (5.9%) to make preoperative radiation therapy redundant.

Figure 1 Impact of level of caspase-3 activity on local recurrence



No. at risk:

Caspase-3 Activity					
≤ median:	58	41	29	20	9
> median:	59	50	38	29	8

Recurrence rates since TME surgery for low (grey) and high (black, dotted) levels of apoptosis in tumors of 117 rectal cancer patients. Levels of caspase-3 activity above the median was associated with significantly lower local recurrence rates (5-years risks: 5.9% vs. 28.1%; p=0.002) (log rank analysis).

Univariate & Multivariate analysis

In order to assess the independent predictive value of caspase-3 activity on local recurrences, variables with a significant impact on local recurrence, shown in Table 2, were analysed in a multivariate analysis. Proximal location of the tumor from the anal verge (p=0.007), abdominoperineal resection (p=0.06), T status (p=0.10) and high caspase-3 activity (p=0.008) proved to be associated with low local recurrence rates in stage III rectal cancer and were subjected to a multivariate analysis. In the multivariate Cox' regression analysis (Table 3), caspase-3 levels below the median proved to be an independent predictor of a high risk of local recurrence in stage III rectal cancer (p=0.009, HR: 7.5, C.I.: 1.7-34.1), whereas type of operation, T status and distance of tumor from anal verge had no independent prognostic value with regard to this endpoint (table 3).

If the circumferential margin (p=0.13 in the univariate analysis) was included in the multivariate analysis, caspase-3 levels below the median remained an independent predictor of a low risk of local recurrence in stage III rectal cancer (p=0.007, HR: 8.0, C.I.: 1.8-36.5)

Table 1 Patient characteristics of the 117 included rectal cancer patients.

Patient Characteristic	**No. of patients** (%) N=117 (100%)	**Caspase-3 activity** (pmolAMC/min/mg protein) Median (IQR)	P-value
Gender			0.32
Male	81(69)	14.5 (9.8-26.6)	
Female	36(31)	16.1 (13.1-27.2)	
Age (median: 65, range: 26-85)			0..94
< median	61 (52)	15.5 (5.8-26.5)	
> median	56 (48)	15.1 (5.2-27.2)	
Preoperative CEA levels (median: 3.0, range: 2-41)			0.20
< median	47 (40)	17.8 (12.2-30.4)	
> median	70 (60)	14.5 (9.5-23.6)	
Maximum tumor diameter (median: 4.5, range: 0-11)			0.09
< median	51 (44)	13.8 (9.3-22.7)	
> median	64 (56)*	16.2 (12.7-31.5)	
Distance of tumor from anal verge			0.73
10.1-15 cm	38(33)	15.3 (11.6-26.4)	
5.1-10 cm	49(42)	15.0 (9.7-24.8)	
≤ 5 cm	30(25)	16.3 (9.9-31.2)	
Grade of differentiation			0.12
well	4(3)	13.7 (12.4-31.1)	
moderate	79(68)	16.0 (12.0-29.8)	
poor	34(29)	13.3 (5.8-20.2)	
WHO classification			**0.04**
adenocarcinoma	105(90)	16.2 (10.4-28.6)	
mucinous carcinoma	12(10)	12.8 (11.2-14.0)	
Number of positive lymph nodes			0.64
1-3	71 (61)	16.0 (10.1-29.8)	
4 or more	46 (39)	14.2 (11.0-22.8)	
T status			0.62
T1-T2	22 (19)	14.7 (8.5-27.9)	
T3	89 (76)	16.2 (11.0-27.1)	
T4	6 (5)	12.7 (9.9-22.7)	
Circumferential margin			**0.04**
negative	81(69)	16.4 (11.8-30.1)	
positive	36(31)	13.4 (9.5-17.6)	
Type of resection			0.18
Abdominoperineal	40 (34)	17.4 (12.2-26.8)	
Low anterior	77 (66)	14.5 (9.4-27.1)	
Adjuvant therapy			0.37
None	83	16.2 (12.0-29.8)	
Chemotherapy	6	16.2 (10.4-22.1)	
Radiation therapy	24	13.1 (19.4-17.6)	
Chemoradiation therapy	4	11.2 (6.1-55.6)	

* tumor diameter of 2 tumors was not determined

Table 1 Association of clinical and pathological parameters with caspase-3 activity as determined in the material and methods section. Significant associations are stated in bold.

Correction for percentage of tumor epithelium

Because the cleavage assay used in the current study did not discriminate between caspase-3 activity in tumor epithelium and tumor stroma, we corrected the caspase-3 activity for the percentage tumor epithelium in order to evaluate the influence variety in the percentages of epithelium and stroma. Caspase-3 levels were compared to levels corrected for percentage tumor epithelium. Median caspase-3 levels before and after adjustment were 15.2 (IQR: 10.6-26.9) and 17.3 (IQR: 10.4-38.3) pmolAMC/min/mg protein respectively.

All statistical analyses in the current study were repeated using caspase-3 levels adjusted for percentage tumor epithelium in the frozen tissue sections. Results were unaffected whether the original or adjusted levels were used (data not shown), indicating that caspase-3 activity can be assessed in tumor tissue specimens without previous knowledge of the tumor epithelium/stroma ratio in the tissue specimen.

Evaluation of possible selection bias

A total of 271 stage III patients were included in the TME trial. To investigate whether the patients in the current study were subject to a selection bias, patient and tumor characteristics of the 117 included patients were compared to all remaining eligible non-irradiated stage III patients included in the TME trial (N=154). No significant differences in patient age (p=0.80) and gender (p=0.10) were found. No differences in tumor characteristics as T-stage (p=0.49), N-stage (p=0.30), grade of differentiation (p=0.22), localisation (p=0.72), WHO classification (p=0.65), CEA levels (p=0.07) or circumferential margins (p=0.43) were found. The maximum tumor diameters in the current study were significantly larger (5.0 cm vs 4.5 cm, p=0.02) than in non-included tumors. This can be explained by the fact that tumors with sufficient material for study tended to be large specimens. As tumor size was not of prognostic value in the current study we concluded that patients were not subject to a selection bias.

Caspase-3 activity in preoperative biopsies and resected rectal tumors

Finally, we determined the feasibility of measuring caspase-3 activity in preoperative biopsies in archive samples of 47 fresh frozen preoperative biopsies and corresponding resected rectal tumors. Caspase-3 activity levels in preoperative biopsies and in tumor samples were highly correlated (correlation coefficient: 0.56, $p<0.0001$ Spearmans' Rho test). Levels were significantly higher in the tumor specimens (median [IQR] 31.4 pmolAMC/min/mg protein [15.1-109.9] than in the preoperative biopsies (23.3 pmolAMC/min/mg protein [8.7-48.5]); p=0.002 (Wilcoxon signed rank).

These results indicate that determination of levels of apoptosis by caspase-3 activity in preoperative biopsies can be used in predicting level of apoptosis in rectal tumors.

Table 2 Univariate Cox regression analysis of the impact of clinical and pathological parameters on local recurrence rates in stage III rectal cancer

	Association with local recurrence		
Variable	Hazard Ratio	95% CI	p-value
Age (years)	1.0	0.98-1.03	0.50
Preoperative CEA levels (ng/ml)	1.0	0.98-1.03	0.58
Maximum tumor diameter (cm)	0.91	0.77-1.10	0.26
Gender			0.24
Male	1		
Female	0.73	0.42-1.25	
Distance of tumor from anal verge			0.007
10.1-15 cm	1		
5.1-10 cm	3.7	1.6-8.7	
≤ 5 cm	2.3	1.0-5.3	
Grade of differentiation			0.30
well	1		
moderate	0.49	0.2-1.2	
poor	0.63	0.2-1.7	
WHO classification:			0.82
adenocarcinoma	1		
mucinous carcinoma	1.1	0.7-1.7	
Number of positive lymph nodes			0.68
1-3	1		
4 or more	1.1	0.6-1.9	
T status			0.10
T1-T2	1		
T3	2.6	1.0-6.6	
T4	3.4	0.9-12.5	
Circumferential margin:			0.13
negative	1		
positive	1.6	0.9-2.7	
Caspase-3 activity			**0.008**
> median	1		
≤ median	7.4	1.7-32.8	
Type of resection			**0.06**
Abdominoperineal	1		
Low anterior	1.7	1.0-3.0	
Adjuvant therapy			0.24
No	1		
Yes	1.9	0.7-5.2	

Table 3 Results of multivariate Cox regression analysis of local recurrence among 117 non-irradiated stage III rectal cancer patients.

	Association with local recurrence (Cox regression analysis)		
Variable	Hazard ratio	95% CI	p-value
Caspase-3 activity			**0.009**
> median	1		
≤ median	7.5	1.7-34.1	
Type of resection			0.87
Abdominoperineal	1		
Low anterior	1.12	0.3-4.6	
Distance of tumor from anal verge			0.36
10.1-15 cm	1		
5.1-10 cm	2.3	0.3-16.3	
≤ 5 cm	3.2	0.7-15.3	
T status			0.11
T1-T2	1		
T3	4.4	0.2-72.1	
T4	5.7	0.7-39.7	

Discussion

Preoperative radiation therapy has shown to be of benefit for the prevention local recurrence rates in rectal cancer patients[11,12]. Long-course preoperative chemoradiotherapy has been shown to be of benefit in stage T3/T4 rectal cancer patients and long-course preoperative chemoradiation is the standard of care in the United States[27]. However, considering the extensive morbidity of preoperative radiation therapy[13-15], it is of great importance to identify patients with a low risk of local recurrence in which radiation therapy is redundant. With this intention, the current study was performed in patients with stage III rectal cancer, as these patients are at the highest risk for local recurrence[12]. Our results demonstrate that biochemical detection of caspase-3 levels can be used as a marker to identify patients with a very high probability for local cure with surgery alone.

In order to select patients who can be refrained from preoperative radiation therapy, a marker should provide accurate prediction of clinical behaviour and it must be applicable in a pretreatment biopsy of the tumor. Several markers for prediction of radiation therapy efficacy have been suggested in previous studies including analysis of Ki-67, BAX, COX-2, survivin and M30 staining amongst others[17-19,28-30]. As the benefit of radiation therapy is, at least in part, mediated by the induction of tumor cell apoptosis and other forms of cell death[20,31,32] it is not surprising that the majority of these markers involve pro-, or anti-apoptotic proteins. A recent study evaluated the predictive value of tumor cell apoptosis as quantified by immunohistochemical staining of paraffin-embedded tumor tissue arrays with the M30 antibody in rectal cancer specimens from the Dutch TME trial[19]. In this study the number of M30-positive cells showed to be a significant predictor of local recurrence, however, with at much lower levels of

statistical significance than we found measuring caspase-3 activity. This may be due to limited accuracy of quantifying cells by immunohistochemical staining. Preoperative biopsies may yield a limited number of tumor cells[16], thus further limiting the use of immunohistochemical markers like M30 to detect the number of apoptotic tumor cells to select patients for radiation therapy. By measuring enzymatic caspase-3 activity, apoptosis can be determined even before the phenotypic changes of these cells are clearly detectable. In addition, our study showed that only 10 μg of tumor-derived protein was necessary for a single assay, making biochemical detection of caspase-3 activity a feasible assay to determine apoptotic levels in preoperative biopsies of rectal tumors.

Caspase-3 activity in the current study was evaluated in non-irradiated tumors as several studies have convincingly demonstrated that radiation therapy-induced apoptosis is not of prognostic value[17,19]. Evaluation of enzymatic caspase-3 activity as an indicator of apoptotic cell death appeared in this study an accurate parameter for prediction of local recurrences. Preoperative biopsies are routinely taken for diagnosis in diseases of the large bowel. For biochemical quantification of caspase-3 activity, an additional biopsy can be taken and either processed immediately or freshly frozen for analysis later.

A highly significant association between caspase-3 activity in the biopsies and corresponding tumor suggests that determining levels of caspase-3 activity in pre-treatment biopsies can be used in predicting level of apoptosis in tumors. It must be taken into account that caspase-3 levels were significantly lower in adjacent normal tissue and a risk of misinterpretation of caspase-3 levels can be apparent if a biopsy contains normal tissue. Caspase-3 activity in the archival biopsies that we studied was significantly lower than in the corresponding resected tumor specimens, suggesting an effect of tumor resection on apoptosis.

One of the factors predictive for local recurrences in rectal cancer is a positive circumferential resection margin[33] and short-term preoperative radiation therapy is of limited effect in patients with a circumferential margin of ≤1mm[21]. In the non-irradiated stage III rectal cancer patients evaluated in this study, the presence of positive resection margins was not a prognostic factor. Apparently, other factors are of more importance in this subset of stage III patients. A possible explanation could be lymphatic spread beyond the surgical resection. In this study, a positive resection margin was associated with low caspase-3 activity in the residual tumor specimens, suggesting that tumors with low levels of apoptosis do not have a clear invasive front and, therefore, are difficult to resect completely. Several clinical and pathological factors as lymph node metastases and tumors located within 10 cm from anal verge are known to be predictors of local recurrence[12,24,25,33]. It has already been demonstrated that magnetic resonance imaging can improve the selection of patients who may have a positive circumferential margin[34]. Preoperative detection of positive lymph nodes by magnetic resonance imaging in combination with ultra small particles of iron oxide (USPIO) is currently being reviewed and tested in clinical studies[34,35]. This is likely to result in a better pre-operative staging of patients and this will enable accurate identification of stage III patients, which are candidates for preoperative radiation therapy. In order to establish a caspase-3 activity level that can be generalized to a broader population of rectal cancer patients we are currently prospectively collecting preoperative rectal cancer biopsies for analyses of caspase-3 activity. Selection of patients who will not benefit from

preoperative radiation therapy by determination of caspase-3 activity will drastically further decrease the number of patients who receive unnecessary preoperative radiation therapy.
In conclusion, the present study demonstrates that caspase-3 activity is an important denominator of local recurrence in rectal cancer and suggests that identification of patients with a low risk of recurrence can be achieved by caspase-3 measurement in preoperative biopsies. If an independent study can confirm our results, determination of caspase-3 levels should be added to other selection criteria to select rectal cancer patients in whom radiation therapy is redundant.

References

1. Kapiteijn, E., Marijnen, C. A., Colenbrander, A. C., Klein, K. E., Steup, W. H., van Krieken, J. H., van Houwelingen, J. C., Leer, J. W., and van de Velde, C. J. Local recurrence in patients with rectal cancer diagnosed between 1988 and 1992: a population-based study in the west Netherlands. Eur.J.Surg.Oncol., *24*: 528-535, 1998.
2. Kapiteijn, E., Putter, H., and van de Velde, C. J. Impact of the introduction and training of total mesorectal excision on recurrence and survival in rectal cancer in The Netherlands. Br.J.Surg., *89*: 1142-1149, 2002.
3. Phillips, R. K., Hittinger, R., Blesovsky, L., Fry, J. S., and Fielding, L. P. Local recurrence following 'curative' surgery for large bowel cancer: II. The rectum and rectosigmoid. Br.J.Surg., *71*: 17-20, 1984.
4. Porter, G. A., Soskolne, C. L., Yakimets, W. W., and Newman, S. C. Surgeon-related factors and outcome in rectal cancer. Ann.Surg., *227*: 157-167, 1998.
5. MacFarlane, J. K., Ryall, R. D., and Heald, R. J. Mesorectal excision for rectal cancer. Lancet, *341*: 457-460, 1993.
6. Enker, W. E. Total mesorectal excision--the new golden standard of surgery for rectal cancer. Ann. Med., *29*: 127-133, 1997.
7. Aitken, R. J. Mesorectal excision for rectal cancer. Br.J.Surg., *83*: 214-216, 1996.
8. Gerard, A., Buyse, M., Nordlinger, B., Loygue, J., Pene, F., Kempf, P., Bosset, J. F., Gignoux, M., Arnaud, J. P., Desaive, C., and . Preoperative radiotherapy as adjuvant treatment in rectal cancer. Final results of a randomized study of the European Organization for Research and Treatment of Cancer (EORTC). Ann.Surg., *208*: 606-614, 1988.
9. Goldberg, P. A., Nicholls, R. J., Porter, N. H., Love, S., and Grimsey, J. E. Long-term results of a randomized trial of short-course low-dose adjuvant pre-operative radiotherapy for rectal cancer: reduction in local treatment failure. Eur.J.Cancer, *30A*: 1602-1606, 1994.
10. Cedermark, B., Johansson, H., Rutqvist, L. E., and Wilking, N. The Stockholm I trial of preoperative short term radiotherapy in operable rectal carcinoma. A prospective randomized trial. Stockholm Colorectal Cancer Study Group. Cancer, *75*: 2269-2275, 1995.
11. Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. N.Engl.J.Med., *336*: 980-987, 1997.
12. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
13. Peeters, K. C., van de Velde, C. J., Leer, J. W., Martijn, H., Junggeburt, J. M., Kranenbarg, E. K., Steup, W. H., Wiggers, T., Rutten, H. J., and Marijnen, C. A. Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients--a Dutch colorectal cancer group study. J.Clin.Oncol., *23*: 6199-6206, 2005.
14. Holm, T., Singnomklao, T., Rutqvist, L. E., and Cedermark, B. Adjuvant preoperative radiotherapy in patients with rectal carcinoma. Adverse effects during long term follow-up of two randomized trials. Cancer, *78*: 968-976, 1996.
15. Marijnen, C. A., Kapiteijn, E., van de Velde, C. J., Martijn, H., Steup, W. H., Wiggers, T., Kranenbarg, E. K., and Leer, J. W. Acute side effects and complications after short-term preoperative radiotherapy combined with total mesorectal excision in primary rectal cancer: report of a multicenter randomized trial. J.Clin.Oncol., *20*: 817-825, 2002.

16. Adell, G. C., Zhang, H., Evertsson, S., Sun, X. F., Stal, O. H., and Nordenskjold, B. A. Apoptosis in rectal carcinoma: prognosis and recurrence after preoperative radiotherapy. Cancer, *91*: 1870-1875, 2001.
17. Marijnen, C. A., Nagtegaal, I. D., Mulder-Stapel, A. A., Schrier, P. I., van de Velde, C. J., van Krieken, J. H., and Peltenburg, L. T. High intrinsic apoptosis, but not radiation-induced apoptosis, predicts better survival in rectal carcinoma patients. Int.J.Radiat.Oncol.Biol.Phys., *57*: 434-443, 2003.
18. Rodel, F., Hoffmann, J., Distel, L., Herrmann, M., Noisternig, T., Papadopoulos, T., Sauer, R., and Rodel, C. Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. Cancer Res., *65*: 4881-4887, 2005.
19. E.C.de Bruin, C. J. H. van de Velde S. van de Pas I. D. Nagtegaal J. H. J. M. van Krieken M. J. E. M. Gosens L. T. C. Peltenburg J. P. Medema C. A. M. Marijnen. Intrinsic and radiotherapy-induced apoptosis in rectal cancer patients of the Dutch TME trial: no need for radiotherapy in intrinsically high apoptotic tumours. Clin.Cancer Res. 2006 Nov 1;12(21):6432-6.
20. Brown, J. M. and Attardi, L. D. The role of apoptosis in cancer development and treatment response. Nat.Rev.Cancer, *5*: 231-237, 2005.
21. Marijnen, C. A., Nagtegaal, I. D., Kapiteijn, E., Kranenbarg, E. K., Noordijk, E. M., van Krieken, J. H., van de Velde, C. J., and Leer, J. W. Radiotherapy does not compensate for positive resection margins in rectal cancer patients: report of a multicenter randomized trial. Int.J.Radiat.Oncol.Biol.Phys., *55*: 1311-1320, 2003.
22. Jonges, L. E., Nagelkerke, J. F., Ensink, N. G., van der Velde, E. A., Tollenaar, R. A., Fleuren, G. J., van de Velde, C. J., Morreau, H., and Kuppen, P. J. Caspase-3 activity as a prognostic factor in colorectal carcinoma. Lab Invest, *81*: 681-688, 2001.
23. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem., *72*: 248-254, 1976.
24. Wibe, A., Syse, A., Andersen, E., Tretli, S., Myrvold, H. E., and Soreide, O. Oncological outcomes after total mesorectal excision for cure for cancer of the lower rectum: anterior vs. abdominoperineal resection. Dis.Colon Rectum, *47*: 48-58, 2004.
25. Gunderson, L. L., Sargent, D. J., Tepper, J. E., Wolmark, N., O'Connell, M. J., Begovic, M., Allmer, C., Colangelo, L., Smalley, S. R., Haller, D. G., Martenson, J. A., Mayer, R. J., Rich, T. A., Ajani, J. A., MacDonald, J. S., Willett, C. G., and Goldberg, R. M. Impact of T and N stage and treatment on survival and relapse in adjuvant rectal cancer: a pooled analysis. J.Clin.Oncol., *22*: 1785-1796, 2004.
26. Nagtegaal, I. D., Marijnen, C. A., Kranenbarg, E. K., Mulder-Stapel, A., Hermans, J., van de Velde, C. J., and van Krieken, J. H. Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. BMC.Cancer, *1*: 7, 2001.
27. Sauer, R., Becker, H., Hohenberger, W., Rodel, C., Wittekind, C., Fietkau, R., Martus, P., Tschmelitsch, J., Hager, E., Hess, C. F., Karstens, J. H., Liersch, T., Schmidberger, H., and Raab, R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N.Engl.J Med., *351*: 1731-1740, 2004.
28. Pachkoria, K., Zhang, H., Adell, G., Jarlsfelt, I., and Sun, X. F. Significance of Cox-2 expression in rectal cancers with or without preoperative radiotherapy. Int.J.Radiat.Oncol.Biol.Phys., *63*: 739-744, 2005.
29. Nehls, O., Okech, T., Hsieh, C. J., Sarbia, M., Borchard, F., Gruenagel, H. H., Gaco, V., Porschen, R., Gregor, M., and Klump, B. Low BAX protein expression correlates with disease recurrence in preoperatively irradiated rectal carcinoma. Int.J.Radiat.Oncol.Biol.Phys., *61*: 85-91, 2005.
30. Rodel, C., Grabenbauer, G. G., Papadopoulos, T., Bigalke, M., Gunther, K., Schick, C., Peters, A., Sauer, R., and Rodel, F. Apoptosis as a cellular predictor for histopathologic response to

neoadjuvant radiochemotherapy in patients with rectal cancer. Int.J.Radiat.Oncol.Biol.Phys., *52*: 294-303, 2002.

31. Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science, *300*: 1155-1159, 2003.
32. Paris, F., Fuks, Z., Kang, A., Capodieci, P., Juan, G., Ehleiter, D., Haimovitz-Friedman, A., Cordon-Cardo, C., and Kolesnick, R. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science, *293*: 293-297, 2001.
33. Nagtegaal, I. D., Marijnen, C. A., Kranenbarg, E. K., van de Velde, C. J., and van Krieken, J. H. Circumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit. Am.J.Surg.Pathol., *26*: 350-357, 2002.
34. Beets-Tan, R. G., Lettinga, T., and Beets, G. L. Pre-operative imaging of rectal cancer and its impact on surgical performance and treatment outcome. Eur.J.Surg.Oncol., *31*: 681-688, 2005.
35. Koh, D. M., Brown, G., Temple, L., Raja, A., Toomey, P., Bett, N., Norman, A. R., and Husband, J. E. Rectal cancer: mesorectal lymph nodes at MR imaging with USPIO versus histopathologic findings--initial observations. Radiology, *231*: 91-99, 2004.

CHAPTER 4

Apoptosis is a poor prognostic factor in colorectal cancer

P. de Heer, F.M. Speetjens, N.G. Ensink, R.I.J.M. Aalbers, C.P.E. Asselbergs, H. Putter, R.A.E.M. Tollenaar, H. Morreau, C.J.H. van de Velde, P.J.K. Kuppen

Submitted for publication

Abstract

To determine parameters that predict prognosis of colorectal cancer patients, this study evaluated the correlation and prognostic value of apoptosis and microsatellite instability and colorectal cancer.

One hundred and four patients with sporadic colorectal cancer with a median clinical follow-up of 5.9 years were evaluated immunohistochemically for presence of apoptotic tumor cells using a monoclonal antibody, M30 that recognizes a cleavage fragment of cytokeratin 18 (M30 neoantigen) specific for apoptotic epithelial cells. Eight patients were excluded from evaluation as they had received preoperative radiotherapy. The number of apoptotic tumor cells was associated with clinicopathological parameters including tumor microsatellite stability. The latter was immunohistochemically determined by evaluation of nuclear PMS2 and MHL-1 expression. A negative nuclear staining for either PMS2 or MLH-1 was used to indicate microsatellite instability (MSI), a positive staining to indicate microsatellite stable (MSS).

A relatively high number of apoptotic tumor cells was significantly associated with unfavorable prognosis (p=0.005). Of the patients evaluated, 14 tumors showed MSI, the other 82 MSS. Although MSI was not associated with an increase in number of apoptotic tumor cells (p=0.44), these patients showed better prognosis than MSS patients (p=0.05). Apoptosis proved to be an independent prognostic factor for disease free survival and tumor recurrence in the multivariate analysis (p=0.003, HR 2.2 CI: 1.32-3.94), and p=0.05, HR: 2.1 CI: 0.98-4.78 respectively).

Our data indicate that apoptosis and microsatellite instability are independent mechanisms that determine patient outcome in colorectal cancer.

Introduction

Apoptosis, or programmed cell death, is a subject of extensive research as it has been implicated in the development and progression of cancer. Abnormalities in apoptotic function contribute to both the pathogenesis of colorectal cancer, as to its resistance to chemotherapeutic drugs and radiotherapy[1]. Apoptosis results from activation of several closely regulated pathways that ultimately lead to chromatin condensation, membrane blebbing, and DNA and protein degradation by downstream effector caspases[2]. The clinical significance of apoptosis on patient survival in colorectal cancer has extensively been studied. However, its role remains contradictory as high levels of apoptosis of tumor cells has been associated with improved[3,4] as well as impaired prognosis[5-8].

Tumors of the colorectum originate from two main genetic pathways[9]. A subgroup of colorectal cancers are characterized by high levels of DNA microsatellite instability (MSI) and are associated with an improved prognosis and favorable prognostic characteristics as increased immune cell infiltration[10]. In general, the mutational burden MSI tumors is suspected to be detrimental to tumor growth and development, however an association with apoptosis remains unclear.

One of the principal problems of determining the impact of apoptosis on patient prognosis in colorectal cancer is that large inconsistencies exist in evaluation methods. This complicates direct comparisons between studies and restricts analysis of the independent prognostic value of apoptosis. To ensure adequate characterization of tumor cell apoptosis in the current study we evaluated apoptosis by immunohistochemical staining with the monoclonal antibody M30 that recognizes a cleavage fragment of Cytokeratin 18 (M30 neoantigen) and is specific for apoptotic epithelial cells[3].

Material and methods

Tumor specimens

The study comprised a random series of 104 consecutive sporadic colorectal carcinomas obtained from the department of Pathology of the Leiden University Medical Center. Tumor samples were derived from patients who underwent curative surgery between 1980 and 1987 and from whom follow up information was available. Patients presenting with colon cancer received a hemicolectomy. Patients with a rectal cancer were operated by conventional blunt dissection of the cancer. Patient follow-up was completed until January 2003, with a median follow-up of 5.4 years (range, 0.1-18.6 years; SD, 5.2 years). Patients with stage II and III colorectal cancer (as defined by American Joint committee on Cancer and Union Internationale Contre le Cancer-criteria) were selected for this study. Patient data were collected retrospectively from hospital records. Patients with another primary malignancy, or patients who deceased within 30 days after surgery were not eligible for this study. Colon tumors were defined as those originating 15 cm above the anal verge; rectal tumors were defined as those distal to this site. Right-sided tumors were defined as those originating proximal to the splenic flexure and left sided as those arising distal to splenic flexure.

Immunohistochemistry

Tissue sections were immunostained using antibodies against a caspase-cleaved product of cytokeratin 18, (clone M30, Roche diagnostics, Germany, 1:800)[11]. Staining of mismatch repair proteins was performed using anti-PMS2 (clone A16-4; 1:50; BD Biosciences) and MLH-1 antibodies (1:50, Zymed laboratories, San Francisco, USA).

Paraffin sections (4 μm) were prepared on aminopropylethoxysilane (APES) coated slides, and dried overnight at 37°C. Tissue sections were deparaffinized in xylol and subsequently rinsed in ethanol. Endogenous peroxidase was blocked by 0.3% hydrogen peroxidase-methanol for 20 min. After being immersed in alcohol the sections were rehydrated. Antigen retrieval was performed by boiling the sections in 10mM citrate buffer (pH 6.0) for 10 min, after which the sections were allowed to cool down in this buffer for 2 hours at room temperature. Subsequently the sections were washed in demi water and phosphate buffered saline (PBS). Sections were incubated overnight at room temperature with the previously mentioned antibodies. All antibodies were diluted in PBS with 1% Bovine Serum Albumin (PBS/BSA). After three washing steps of 5 minutes in PBS, sections were incubated for 30 minutes with SWaR/biotin or RaM/biotin (1:400; DAKO, Glustrup, Denmark). The sections were washed in PBS, followed by 30-minute incubation with Streptavidin-Biotin-Complex (SABC) (1:100; DAKO, Denmark). The sections were then washed in, rinsed in 0.05M Tris/HCl-buffer (ph 7.6) and developed in a 3.3 diaminobenzidine tetrahydrochloride (DAB) with 0.002% hydrogen-peroxide for 10 minutes. Sections were counterstained with haematoxylin, dehydrated with ethanol, cleared in xylene and mounted with pertex. A negative control was included for each tumor sample by using PBS/BSA instead of the before mentioned antibodies in the overnight incubations.

Quantification of Immunohistochemistry

PMS2 and MLH-1 immunohistochemical staining can be used to distinguish between MSI and MSS[12]. Nuclear PMS2 and MLH-1 analysis was done by a pathologist (H. M.). Tumor cells were scored as positive or negative for PMS2 MLH-1 on condition that the internal stromal control was positive.

M30 immunoreativity on all slides was evaluated by two independent observers (N.G.E. and R.I.J.M.A.) as previously described[3,8]. In brief, the apoptotic index was documented as the number of M30-positive cells per square millimeter of tumor cells and was counted in randomly chosen high-power fields (25 per tumor) at a 200x magnification. For survival analyses the median apoptotic index was used as a cut-off point for low and high number of apoptotic cells. All observers were blinded to clinical outcome.

Two tumors were not assessable after immunohistochemical staining, due to technical failures, unavailability of tumor material or excessive tumor necrosis.

Statistics

Of the 104 primary tumor specimens 8 patients received preoperative radiotherapy and were excluded from this study. Kruskal-Wallis, Mann-Whitney's U-test and Spearman's rho analyses were used to analyse correlation between number of apoptotic cells, clinicopathological features, immunological parameters and mutational pathway. For survival analysis Kaplan-Meier analysis was used and differences between the survival curves were analysed using the log-rank

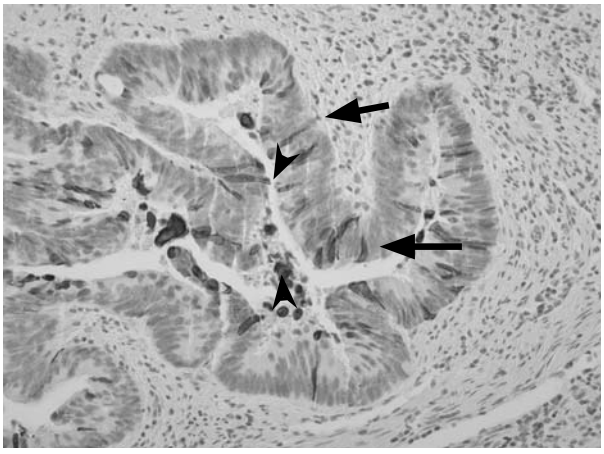
test. Events for time to recurrence, disease-free and overall survival were defined as follows: time from surgical resection to disease relapse, time from surgical resection to disease relapse or death, and time from surgical resection to death respectively.
Cox's stepwise proportional hazard regression models were used for multivariate analyses of apoptosis, MSI and clinicopathological features. The statistical package SPSS version 12.0 (SPSS Inc, Chicago, IL) was used to conduct statistical analyses. A p-value < 0.05 (two-tailed) was considered as indicating a statistical significant result.

Results

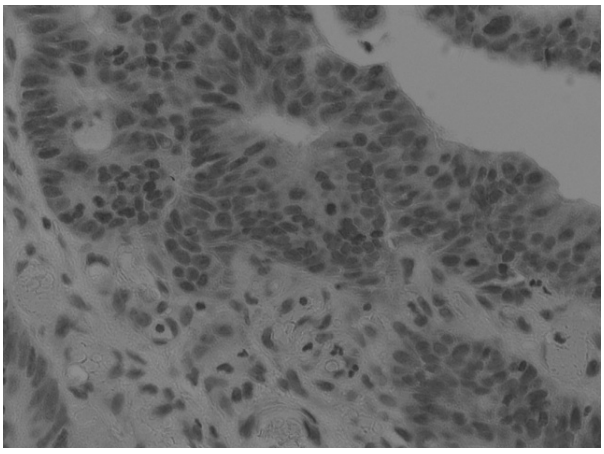
Patient and tumor characteristics

The patient and tumor characteristics are summarized in Table 1. Patients had a median follow-up of 5.9 years (range, 0.1-18.6 years; SD, 5.2 years). Tumors in this study were obtained from a randomly selected group of 96 non-irradiated patients presenting with stage II and III colorectal carcinoma. The average age of the patients in this study was 66.9 years (range, 26.0-85.0 years). In this study 14 of 96 were negative for PMS2 or MLH-1 and thus considered microsatellite instable (MSI) the remaining 90 were considered to be microsatellite stable (MSS).

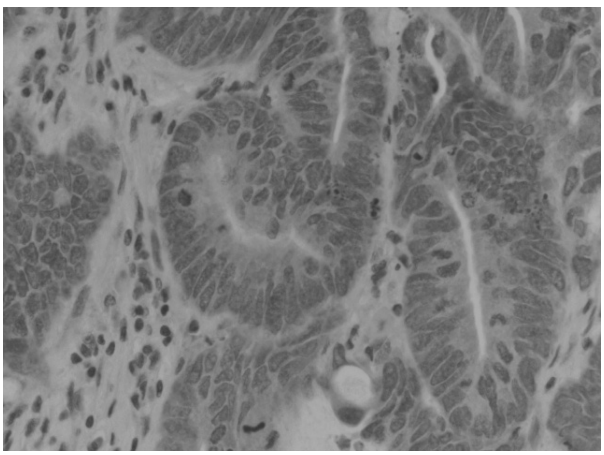
Figure 1



A



B



C

Representative immunohistochemical staining of apoptotic tumor cells, and nuclear PMS2 expression in colorectal cancer specimens. **Figure 1A** Apoptotic tumor cells were detected with M30 monoclonal antibody. Arrows indicate examples of apoptotic epithelial cells. Arrowheads indicate apoptotic bodies, these were not counted. **B** MSI colon adenocarcinoma with loss of PMS2 expression. Nuclear staining of non-epthelial cells serves as internal positive control. **C** adenocarcinoma with normal nuclear PMS2 expression.

Immunohistochemical analysis of apoptosis in colorectal cancer specimens and its relation to clinicopathological variables and survival

Figure 1A shows a representative tumor specimen after immunohsitochemical staining with the M30 monoclonal antibody. Cells from epithelial origin, positive for staining with the M30 monoclonal antibody were clearly recognizable in the tumor tissue. These positive cells represent cells in the first part of the degradation phase of apoptosis (Fig 1.). The median number of apoptotic cells per square mm tumor epithelial cells was 4.2 cells/mm^2.

To evaluate whether apoptosis and clinicopathological features were associated, the number of apoptotic cells was correlated to each feature. Number of apoptotic tumor cells was not associated with any of the clinicopathological variables (table 1).

Table 1 Patient characteristics and association with levels of apoptosis (94 patients)

	No. of patients (%)	Number of apoptotic cells (cells/mm^2) Median (IQR)	p-value
Sex		4.8 (1.9-8.7)	0.81*
Male	56(60)	3.9 (1.3-8.6)	
Female	38(40)		
Age (median [range])	(67.5[26-85])		0.20*
≤ median	48 (50)	3.0 (1.6-8.8)	
> median	48 (50)	5.2 (2.6-8.6)	
Tumor location			0.80*
colon	61(66)	3.7 (1.6-9.6)	
rectum**	33(34)	5.5 (2.4-7.9)	
Tumor stage			0.36*
II	55(59)	4.7 (2.0-9.6)	
III	39(41)	4.6 (1.7-8.5)	
Grade of differentiation			0.81†
poor	54(57)	4.2 (1.7-8.6)	
moderate	21(23)	5.4 (1.3-8.4)	
well	19(16)	5.1 (1.7-21.7)	
MSI			0.53*
MSI	13(15)	4.2 (2.7-8.4)	
MSS	81(85)	4.6 (1.6-8.7)	

*Mann-Whitney, †Kruscal-Wallis

Table 1 Characteristics of the patients 96 investigated in the present study. Apoptosis was defined as number of apoptotic cells/mm2, 2 patients were not evaluable after immunohistochemistry and excluded from further analyses. Apoptosis levels were evenly distributed amongst patient characteristics.

Prognostic significance of tumor cell apoptosis and MSI status

We analysed the number of apoptotic tumor cells in relation with patient survival data. As is shown in figure 2, tumor specimens with a relatively high number of apoptotic cells were bor-

derline significantly associated with high recurrence rates (p=0.09 figure 2A) and significantly associated with a poor disease free survival (p=0.005, figure 2B). MSI tumors were associated with low recurrence rates (p=0.05 figure 2C); this did however not translate into a survival benefit (p=0.59 figure 2D).
When separately analysed in the colon (n=63) and rectal (n=33) tumors, high number of apoptotic tumor cells were associated with a poor DFS in both colon (p=0.04) as well as in rectal (p=0.05) tumors (data not shown).

Figure 2A Apoptosis, TTR

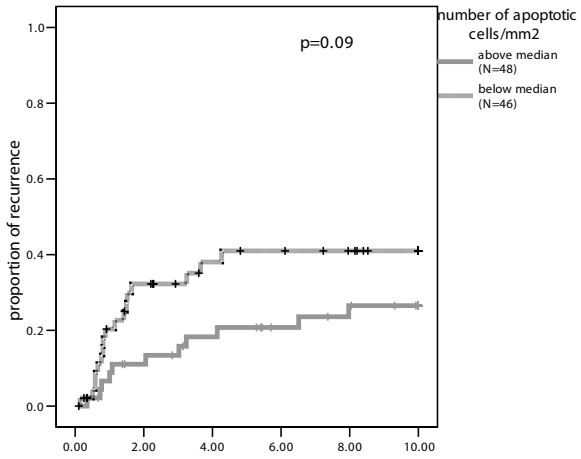


Figure 2B Apoptosis, DFS

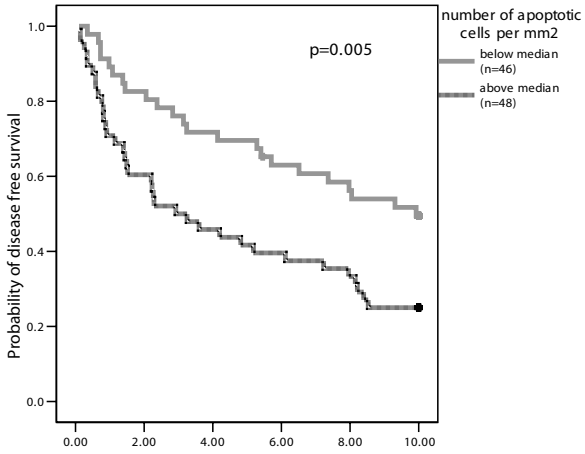


Figure 2C MSI, TTR

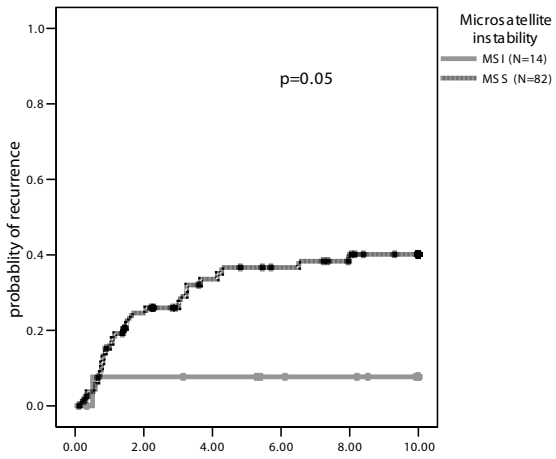
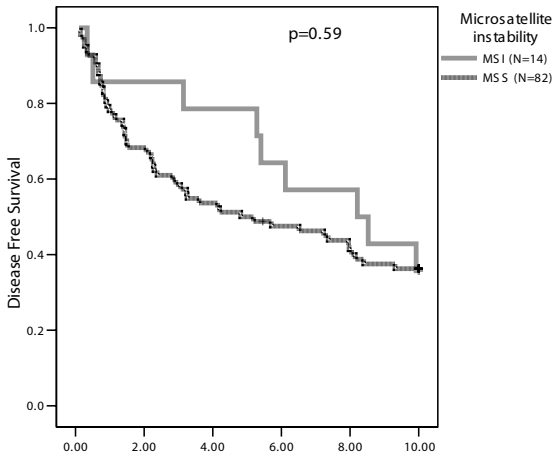


Figure 2D MSI, DFS



Prognostic value of apoptosis and microsatellite instability on time to recurrence (TTR) and DFS in 96 colorectal cancer patients**.** The continuous line represents a relative low number of apoptotic cells per square millimeter (≤ median), the dotted line a relative high number (>median). 2A: High levels of apoptosis was associated with increased recurrence rates in colorectal cancer (p=0.09). 2B: High levels of apoptosis was associated with a poor DFS in colorectal cancer specimens (p=0.005). 2C Microsatellite instability was associated with low recurrence rates in colorectal cancer (0.05). 2D Microsatellite instability did not have a significant impact of DFS in colorectal cancer (p=0.59)

All parameters with a p-value ≤0.10 in the univariate analysis were subjected to a multivariate analysis using a Cox proportional hazards model, to evaluate whether apoptosis showed prognostic significance for tumor recurrence, independent of other factors. Tumor stage, tumor cells apoptosis and microsatellite stability status were included in the analysis. A relatively high number of apoptotic cells (p=0.05, HR: 2.1 CI: 0.98-4.78) and advanced tumor stage (p=0.006, HR 3.0 CI: 1.37-6.63) retained their strength as independent prognostic variables for tumor recurrence (table 2). In addition apoptosis was an independent prognostic factor for disease free survival (p=0.003, HR 2.2 CI: 1.32-3.94). Therefore, these results indicate that the number of apoptotic cells in a tumor resection specimen is highly prognostic in colorectal cancer.

Discussion

We demonstrate in the current study that colorectal tumors with a high number of apoptotic tumor cells show a poor prognosis. This negative impact of high number of apoptotic cells on patient survival is confirmed by several other studies[5-7]. A large number of studies have focused on the prognostic significance of the number of apoptotic cells in colorectal cancer, however, the results of these studies are often contradictory[13]. The inconsistencies in observed impact of apoptosis on clinical outcome can partly be explained by the different methods for detecting apoptosis and partly by the differences between the evaluated patient populations. Several techniques are employed to detect apoptosis, including TUNEL assay[14-16], flow cytometry[6], pro- and anti-apoptotic protein activity[5,17] or the measurement of caspase-3 activity in tumor lysates[7]. The limitation of most of these methods is that they reflect apoptosis of both stromal cells as well as tumor cells. The use of the monoclonal antibody M30 overcomes these limitations as the antibody is directed against the apoptosis-induced cleavage product of cytokeratin 18. This protein is only present in epithelial cells and allows a highly reproducible discrimination of apoptotic tumor cells from other cell types[3,8]. The evaluation of apoptosis with immunohistochemical M30 expression provided information about apoptosis on a cellular level and is sensitive to slight changes in expression levels, thus yielding an accurate evaluation[3,8].

Table 2 Cox proportional hazards model (first model) for Time to Recurrence (TTR)

			DFS	
Variables	Categories	HR	95% CI	P-values
Microsatellite stability	MSI/MSS	4.55	0.99-4.78	0.14
Tumor stage	stage II/ stage III	3.0	1.37-6.63	p=0.006
Apoptosis	≤median/ >median	2.1	0.98-4.78	p=0.05

The type of treatment, including preoperative therapy and type of surgical resection, may a point that should also be taken into account when discussing the impact of the number of apoptotic tumor cells in a resection specimen. Considering the different studies, the prognos-

tic value of apoptosis has been analysed in varying cohorts of patients, making it difficult to compare results. Much of the published literature has evaluated apoptosis in resected colorectal tumor specimens, regardless of adjuvant treatment or type of surgery. However, the introduction of new surgical techniques as the Total Mesorectal Excision[17] and neoadjuvant treatment[18] has greatly improved the clinical outcome of colorectal cancer, but certainly will influence apoptosis in resected tumor material. Not only preoperative radiotherapy[3], but also surgical resection has been shown to induce apoptosis as levels of apoptosis were significantly higher in resected tumors than in preoperative biopsies[14]. A recent study evaluated apoptosis in tumors of patients included in the Dutch TME trial and showed by M30 immunostaining that low numbers of apoptotic tumor cells in rectal cancer were associated with high local recurrence rates. Our study showed different clinical behavior for patients with high numbers of apoptotic tumor cells when operated with conventional surgery for rectal cancer as was used before TME surgery was introduced. In addition, the number of apoptotic cells after TME surgery was much higher than the number we found in the current study[3,8]. Together, these results indicate that type of surgery highly influences tumor cell apoptosis. Therefore, the clinical impact of tumor cells apoptosis should only be considered in the context of patient's treatment.

The mechanism behind the negative impact of high number of apoptotic cells on DFS in the present study could be explained by disturbance in the structural cohesion of the tumor by the presence of apoptotic tumor cells. Disturbance in the structural cohesion may result in an increased dissemination of tumor cells, and thus more tumor cells in blood and lymphatic vessels and thus a higher change of developing distant metastases[7].

The favorable clinical outcome of MSI tumors as seen in the current study has been reported previously[20,21] and has been attributed to increased immune cell infiltration in such cancers [22]. MSI in the current study was not associated with increased tumor cell apoptosis. This lack of association was also apparent in a study evaluating apoptosis and tumor infiltrating lymphocytes in cancer specimens with defective MMR genes[22]. Although MSI tumors in several studies were associated with increased numbers of tumor infiltrating lymphocytes[21], they also did not find increased numbers of apoptotic cells. These studies concluded that the level of apoptosis in MSI cancers is spontaneous in nature. Our study is in line with these data and shows that apoptosis reflects clinical tumor behavior.

In conclusion, the current study demonstrates that high numbers of apoptotic tumor cells in resection specimens is a poor prognostic factor for patient survival in colorectal cancer and also that apoptosis and MSI are independent mechanisms that determine patient outcome. Furthermore, tumor cell apoptosis should be considered in the context of the preoperative and surgical treatment of the patient.

References

1. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. Cell, *108*: 153-164, 2002.
2. Watson, A. J. Apoptosis and colorectal cancer. Gut, *53*: 1701-1709, 2004.
3. Marijnen, C. A., Nagtegaal, I. D., Mulder-Stapel, A. A., Schrier, P. I., van de Velde, C. J., van Krieken, J. H., and Peltenburg, L. T. High intrinsic apoptosis, but not radiation-induced apoptosis, predicts better survival in rectal carcinoma patients. Int.J.Radiat.Oncol.Biol.Phys., *57*: 434-443, 2003.
4. Langlois, N. E., Lamb, J., Eremin, O., and Heys, S. D. Apoptosis in colorectal carcinoma occurring in patients aged 45 years and under: relationship to prognosis, mitosis, and immunohistochemical demonstration of p53, c-myc and bcl-2 protein products. J.Pathol., *182*: 392-397, 1997.
5. Allal, A. S., Waelchli, L., and Brundler, M. A. Prognostic value of apoptosis-regulating protein expression in anal squamous cell carcinoma. Clin.Cancer Res., *9*: 6489-6496, 2003.
6. Rupa, J. D., de Bruine, A. P., Gerbers, A. J., Leers, M. P., Nap, M., Kessels, A. G., Schutte, B., and Arends, J. W. Simultaneous detection of apoptosis and proliferation in colorectal carcinoma by multiparameter flow cytometry allows separation of high and low-turnover tumors with distinct clinical outcome. Cancer, *97*: 2404-2411, 2003.
7. Jonges, L. E., Nagelkerke, J. F., Ensink, N. G., van der Velde, E. A., Tollenaar, R. A., Fleuren, G. J., van de Velde, C. J., Morreau, H., and Kuppen, P. J. Caspase-3 activity as a prognostic factor in colorectal carcinoma. Lab Invest, *81*: 681-688, 2001.
8. Fearon, E. R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61*: 759-767, 1990.
9. Guidoboni, M., Gafa, R., Viel, A., Doglioni, C., Russo, A., Santini, A., Del Tin, L., Macri, E., Lanza, G., Boiocchi, M., and Dolcetti, R. Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. Am.J.Pathol., *159*: 297-304, 2001.
10. Grassi, A., Susca, M., Ferri, S., Gabusi, E., D'Errico, A., Farina, G., Maccariello, S., Zauli, D., Bianchi, F. B., and Ballardini, G. Detection of the M30 neoepitope as a new tool to quantify liver apoptosis: timing and patterns of positivity on frozen and paraffin-embedded sections. Am.J.Clin.Pathol., *121*: 211-219, 2004.
11. de Jong, A. E., van Puijenbroek, M., Hendriks, Y., Tops, C., Wijnen, J., Ausems, M. G., Meijers-Heijboer, H., Wagner, A., van Os, T. A., Brocker-Vriends, A. H., Vasen, H. F., and Morreau, H. Microsatellite instability, immunohistochemistry, and additional PMS2 staining in suspected hereditary nonpolyposis colorectal cancer. Clin.Cancer Res., *10*: 972-980, 2004.
12. Brown, J. M. and Wilson, G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? Cancer Biol.Ther., *2*: 477-490, 2003.
13. Adell, G. C., Zhang, H., Evertsson, S., Sun, X. F., Stal, O. H., and Nordenskjold, B. A. Apoptosis in rectal carcinoma: prognosis and recurrence after preoperative radiotherapy. Cancer, *91*: 1870-1875, 2001.
14. Sugao, Y., Koji, T., Yao, T., Ueki, T., and Tsuneyoshi, M. The Incidence of Apoptosis During Colorectal Tumorigenesis. Int.J.Surg.Pathol., *8*: 123-132, 2000.
15. Schwandner, O., Schiedeck, T. H., Bruch, H. P., Duchrow, M., Windhoevel, U., and Broll, R. Apoptosis in rectal cancer: prognostic significance in comparison with clinical histopathologic, and immunohistochemical variables. Dis.Colon Rectum, *43*: 1227-1236, 2000.
16. Krajewska, M., Kim, H., Kim, C., Kang, H., Welsh, K., Matsuzawa, S., Tsukamoto, M., Thomas, R. G., Assa-Munt, N., Piao, Z., Suzuki, K., Perucho, M., Krajewski, S., and Reed, J. C. Analysis of apoptosis

protein expression in early-stage colorectal cancer suggests opportunities for new prognostic biomarkers. Clin.Cancer Res., *11*: 5451-5461, 2005.

17. Heald, R. J. and Ryall, R. D. Recurrence and survival after total mesorectal excision for rectal cancer. Lancet, *1*: 1479-1482, 1986.
18. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
19. Lanza, G., Gafa, R., Santini, A., Maestri, I., Guerzoni, L., and Cavazzini, L. Immunohistochemical test for MLH1 and MSH2 expression predicts clinical outcome in stage II and III colorectal cancer patients. J.Clin.Oncol., *24*: 2359-2367, 2006.
20. Popat, S., Hubner, R., and Houlston, R. S. Systematic review of microsatellite instability and colorectal cancer prognosis. J.Clin.Oncol., *23*: 609-618, 2005.
21. Dolcetti, R., Viel, A., Doglioni, C., Russo, A., Guidoboni, M., Capozzi, E., Vecchiato, N., Macri, E., Fornasarig, M., and Boiocchi, M. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. Am.J.Pathol., *154*: 1805-1813, 1999.
22. Michael-Robinson, J. M., Biemer-Huttmann, A., Purdie, D. M., Walsh, M. D., Simms, L. A., Biden, K. G., Young, J. P., Leggett, B. A., Jass, J. R., and Radford-Smith, G. L. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut, *48*: 360-366, 2001.

CHAPTER 5

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

P. de Heer, M.J.E.M. Gosens, E.C. de Bruin, N.G. Dekker-Ensink, H. Putter, C.M. Marijnen, A.J.C. van den Brule, J. H.J.M. van Krieken, H.J. Rutten, P.J.K. Kuppen, C.J.H. van de Velde

Clinical Cancer Research 2007 May 15;13(10):2955-60.

Abstract

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

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 pre-operative short-term (5 times 5Gy) radiotherapy or no pre-operative 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.

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: 1.5, C.I.: 1.1-2.0), independent of patient age, tumor stage, tumor location or the presence of tumor cells in the circumferential resection margin.

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 (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, metastatis, and abrogation of the antitumor immune response[1-4]. COX-2 prevents apoptosis by generation of antiapoptotic PGE_2[5] and PGI_2[6] and by removal of the proapoptotic substrate arachidonic acid[7]. PGE 2 induces transformations that result in increased Bcl-2 expression and prolong the cell cycle G1 phase with increased cyclin D1 expression[8]. Numerous epidemiologic studies have indicated that the use of nonsteroidal anti-inflammatory drugs, 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 effect of COX-2 expression has been evaluated in a large number of studies in colorectal cancer and results have not been consistent[12,14,15]. Considering the distinct differences in tumor biology, treatment, recurrence rates, and metastatic behavior[16], 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].

Materials and Methods

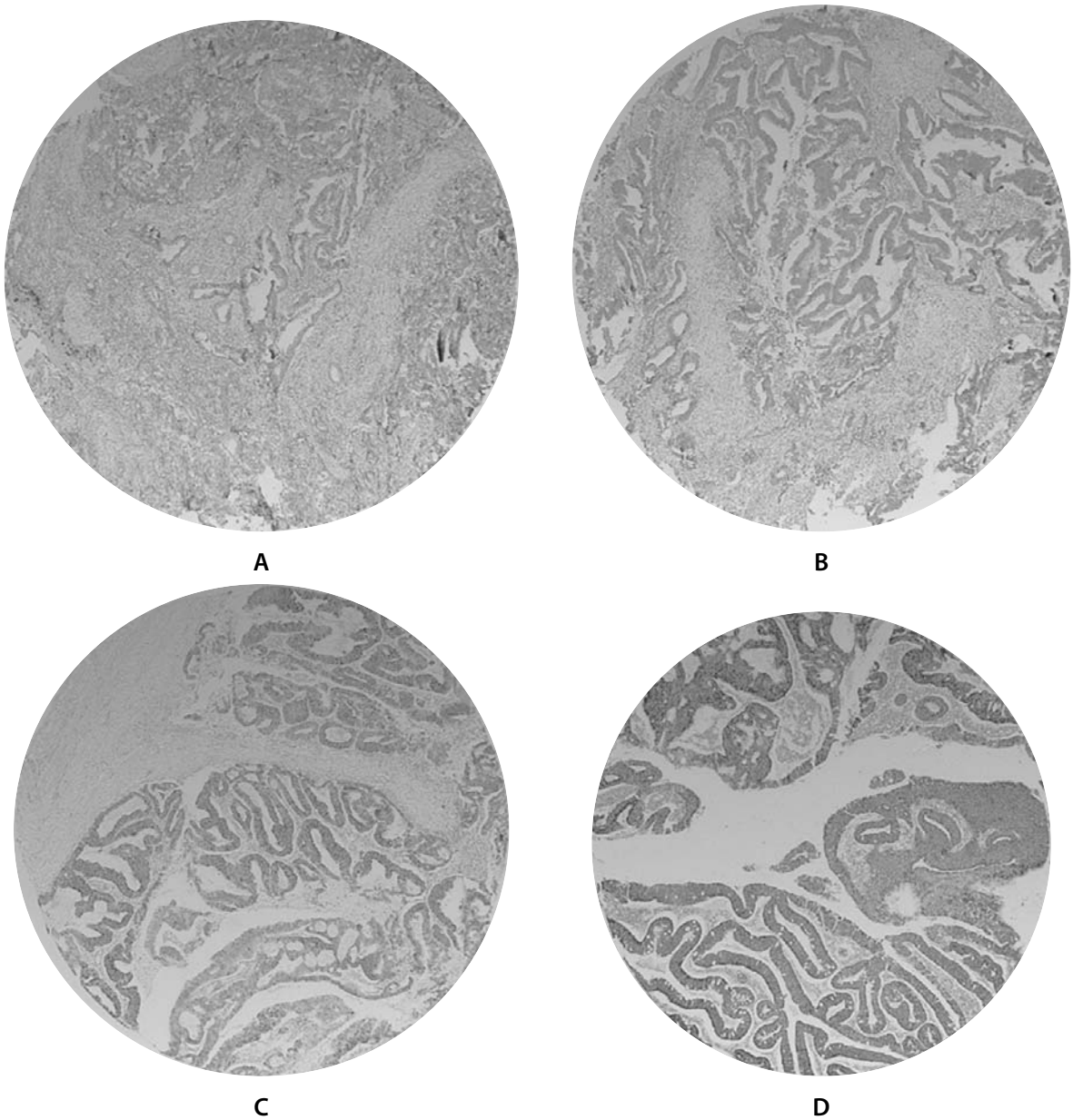
Study population.

Patients were obtained from the Dutch TME trial, a large multicenter trial in which 1,861 patients were included from January 1996 until December 1999. Patients with a respectable carcinoma of the rectum were included in this international multicenter clinical trial and were subsequently randomized for radiotherapy (5x5Gy) followed by TME surgery or for TME surgery alone without preoperative radiotherapy[17]. Radiotherapeutic, surgical, and pathologic procedures were standardized and quality controlled[17-19]. Patients who complied with the eligibility criteria of the TME trial[17] with sufficient paraffin-embedded tumor material were selected for this study. Archival tumor material was collected from the 1,530 Dutch patients who were included in the trial. Tumor material was available from 1,231 patients. For the evaluation of COX-2 expression, patients were only included if at least two of the three included punches on the tissue microarray could be evaluated, leaving 1,038 eligible stages I to III rectal cancer patients for analyses of clinical effect of COX-2 expression.

Tissue microarray preparation.

Tissue microarrays from formalin-fixed, paraffin-embedded tumors included in the Dutch TME trial were constructed with a custom-built precision tissue arrayer (Beecher Instruments) using a 2-mm-diameter punch as described previously[20].

Figure 1 Representative staining of COX-2 expression in tissue micro array cores from the 1231 rectal cancer specimens evaluated in this study.



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 cytoplasmatic staining (score 1). Figure 1C: moderate to strong granular cytoplasmatic staining (score 2). Figure 1D: strong intensity of the staining (score 3).

Immunohistochemistry.

For the quantification of COX-2 expression, 4-Am sections of the tissue microarrays were stained with COX-2– specific mouse anti-human monoclonal antibodies (clone CX229, Cayman Chemical Co.). The immunohistochemical procedures were described in detail elsewhere[21]. Antigen retrieval was done by boiling the sections in 10 mmol/L 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 preabsorption of the primary antibody with human COX-2 antibody–blocking peptides (10 AL/mL; Cayman Chemical) for 1 h at room temperature before the staining procedure. All tumor specimens were stained simultaneously to avoid interassay 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 tissue microarrays, the scoring criteria proposed by Buskes et al.[21] was used: score 0, no staining; score 1, weak diffuse cytoplasmatic staining (may contain stronger intensity in <10% of the cancer cells); score 2, moderate to strong granular cytoplasmatic staining in 10–90% of the tumor cells; and score 3, >90% of the tumor cells stained with strong intensity. The three stained tissue microarray 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, and 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[20]. Data on COX-2 expression and apoptosis was available in 1,024 patients.

Statistical analyses.

All analyses were done with SPSS statistical software (version 12.0 for Windows, SPSS Inc.). Paired samples t test, Mann-Whitney U, Kruskall-Wallis, and Spearman's q tests were used to compare continuous variables. The $\chi 2$ 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 from the time of surgery to the time of local disease relapse, time of 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 (95% CI). Variables with a P value of V0.10 in the univariate analyses were subjected to a multivariate analysis. Interobserver variability was calculated by κ statistic as described by Landis and Koch: κ-values of 0.2 to 0.4 indicate fair; 0.4 to 0.6, moderate; and of > 0.6, excellent results[22].

Results

COX-2 protein expression in rectal cancer tissue microarrays.

The immunohistochemical COX-2 staining pattern exhibited a brown diffuse granular cytoplasmatic staining (Fig.1). No staining of COX-2 was observed in five tumors 0.5%; Fig. 1A). A weak diffuse, moderate, or strong staining was observed in respectively 114 (11.0%), 602 (58.0%),

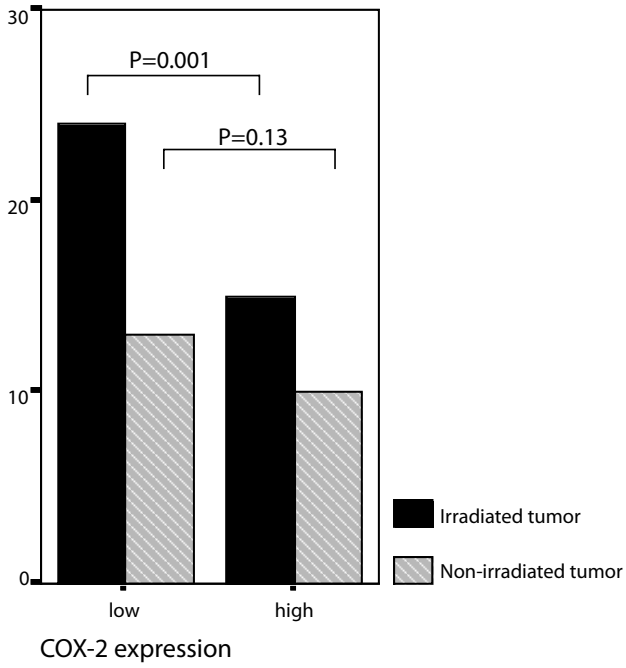
and 317 (30.5%) of the tumors (Fig.1B-D). The interobserver κ -value score for evaluation of COX-2 expression was 0.62, indicating minimal interobserver variation. Ten randomly selected rectal cancer specimens were stained with COX-2 antibodies with or without blocking peptide. All tumor cell signals were blocked by this control procedure in all specimens.

Table 1 Distribution of COX-2 expression in irradiated and non-irradiated rectal cancer specimens

		Treatment group		
		TME	**RT+TME**	**Total**
COX-2	0	3 (0.4%)	2 (0.6%)	5 (0.5%)
	1	51(12.4%)	63 (9.7%)	114 (11.0%)
	2	300 (59.2%)	302 (56.8%)	602 (58.0%)
	3	174 (28.0%)	143 (33.0%)	317 (30.5%)
	Total	528 (100%)	510 (100%)	1038 (100%)

Chi-square test: p=0.24

Figure 2 COX-2 expression and apoptosis in irradiated and non-irradiated rectal cancer specimens



Dichotomized COX-2 expression is associated with decreased levels of apoptosis in irradiated tumor specimens with high levels of COX-2 expression. Black columns represent patients receiving preoperative radiotherapy (RT+) followed by TME surgery. Gray columns represent patients receiving TME surgery alone (RT-).

COX-2 expression and clinicopathologic variables.

Clinical data and conventional prognosis factors (tumor-node-metastasis stage, age, histology, localization) of the patients in the current study have been published previously[17,20]. 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 pathologic variables, such as age, gender, tumor size, depth of invasion, lymph node involvement, tumor-node-metastasis stage, type of surgery, circumferential margin and distance from anal verge. All P values were not significant (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 (compared with tumors of the mucinous type) in irradiated and non-irradiated tumors (P = 0.05/0.04).

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 (20) in irradiated tumors (P = 0.001, Mann-Whitney test). As can be seen in Fig.2, the analysis remained significant when COX-2 scores were dichotomized as scores 0 to 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 (interquartile 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 analysed the effect of COX-2 expression on tumor recurrence and patient survival. Figure 3A-C shows the effect 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 effect on local recurrence (P = 0.44; Fig. 3A), distant recurrences (P = 0.77; Fig. 4), overall survival (P = 0.61; Fig. 3B), or disease-free survival (P = 0.57; Fig.3C) in non-irradiated rectal cancer specimens. As can be seen in Fig.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 disease-free survival (P = 0.004) and overall survival (P = 0.006) but not with local recurrence rates (P = 0.92). Univariate and multivariate analyses in irradiated patients. Univariate COX regression analyses were done to identify prognostic factors for overall survival in irradiated patients. Advanced patient age (HR, 1.03, 95% CI, 1.01-1.05; P <0.0001), advanced pathologic stage (HR, 1.75; 95% CI, 1.47-2.03; P < 0.0001), tumor-positive circumferential resection margins (HR, 2.46; 95% CI, 1.82-3.33; P <0.0001), distal location of the tumor (HR, 1.46; 95% CI, 1.01-2.06; P = 0.05) and high COX-2 expression (HR, 1.48; 95% CI, 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 pathologic stage, tumor-positive circumferential resection margins, and high COX-2 expression (HR, 1.46; 95% CI, 1.10-1.94; P = 0.009) retained their strength as independ-

ent prognostic factors for overall survival (Table 2). In addition, COX-2 proved to be an independent prognostic factor for high distant recurrence rates (P = 0.003; HR, 1.7; 95% CI, 1.2-2.5) and disease-free survival (P = 0.002; HR, 1.8; 95% CI, 1.2-2.5).

Figure 3 Kaplan-Meijer survival estimates by COX-2 tumor epithelial staining in non-irradiated (RT-) rectal tumors

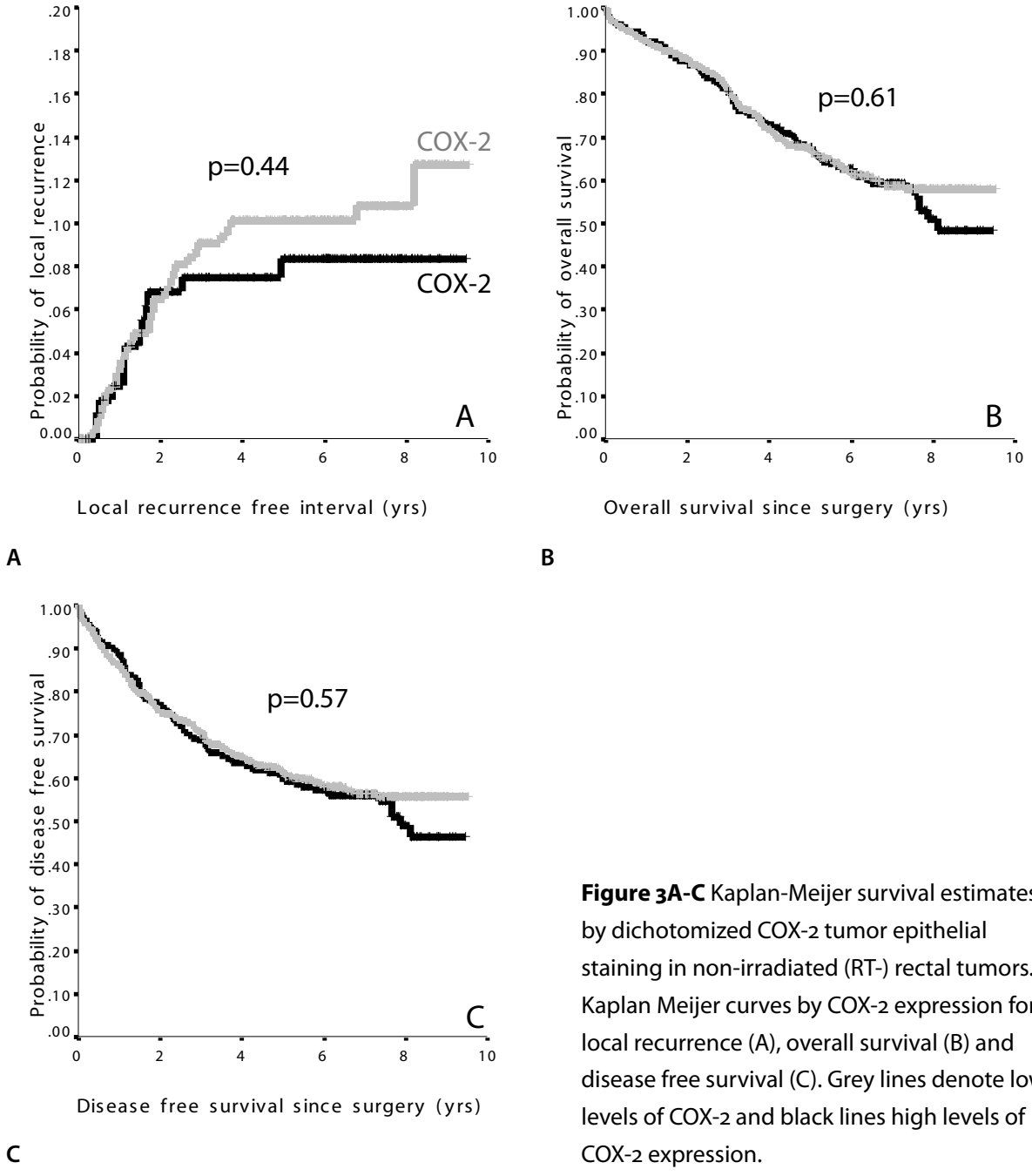
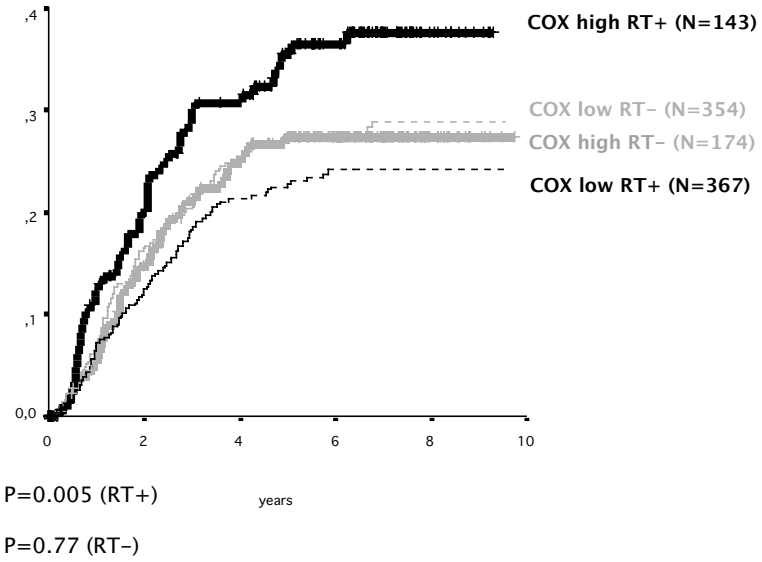


Figure 3A-C Kaplan-Meijer survival estimates by dichotomized COX-2 tumor epithelial staining in non-irradiated (RT-) rectal tumors. Kaplan Meijer curves by COX-2 expression for local recurrence (A), overall survival (B) and disease free survival (C). Grey lines denote low levels of COX-2 and black lines high levels of COX-2 expression.

Figure 4 Kaplan-Meijer survival estimates by high and low COX-2 tumor epithelial staining in irradiated and non-irradiated rectal tumors.



Kaplan-Meijer 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).

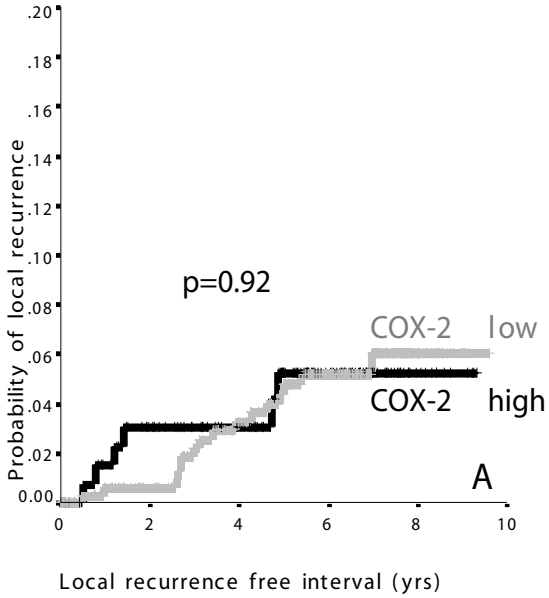
Discussion

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[23,24]. In the current study, high COX-2 expressions after radiotherapy were associated with apoptosis resistance and can therefore lower levels of radiotherapy-induced apoptosis. Antiapoptotic proteins of the Bcl-2 family are able to suppress radiation-induced cell death[24]. COX-2 is known to induce Bcl-2 expression[2] and is associated with apoptosis resistance[8]. De Bruin et al.[20] showed, 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[25]. Because the current study found a prognostic effect of COX-2 in irradiated patients only, whereas apoptotic rates were only prognostic in non-irradiated cases, our findings cannot 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

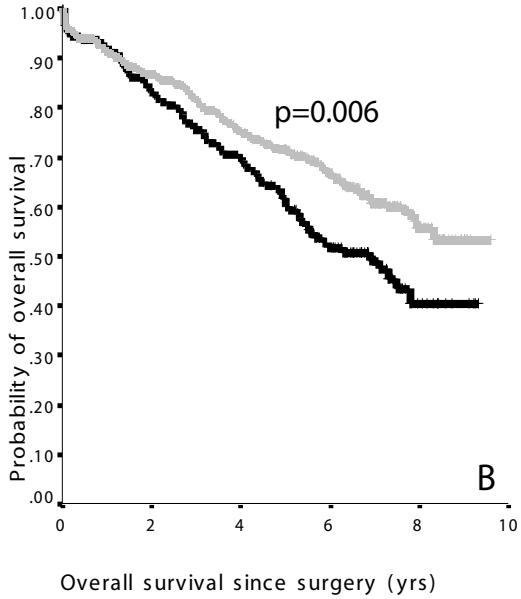
response gene[26]. The interval between the short-term radiotherapy and surgery could be sufficient for a change in COX-2 activity and subsequent prostaglandin production to influence the clinical behavior of the tumor[8,24]. Elevated COX-2 expression has shown to lead to alterations in the invasive and metastatic potential of cancer cells[4]. COX-2 expression and prostaglandin production induce cell-surface glysosyltransferases and type 1 sialyl Lewis antigens, leading to enhanced tumor cell adhesion to endothelial cells[27,28]. And animal studies reported that COX-2 inhibition prevented the formation of distant metastases[29]. Moreover, the immunosuppressive effect of increased prostaglandin production[1] may allow circulating tumor cells to escape the host antitumor 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 given safely[30-33]. 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 effect on local recurrence rates or prognosis in non-irradiated tumors. We have not studied pretreatment biopsies, but our results regarding non-irradiated tumors indicate that evaluation of COX-2 expression in non-irradiated rectal cancer specimens or preradiation 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 (refs.[14,15]; reviewed in ref.[12]), 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%) compared with the 10% to 30% negative tumors reported in studies evaluating COX-2 expression in colorectal cancer specimens[34] 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; refs.[35,36]) in right-sided tumors [37,38] 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 done 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 preoperative radiotherapy may help to reduce distant recurrences and improve patient survival.

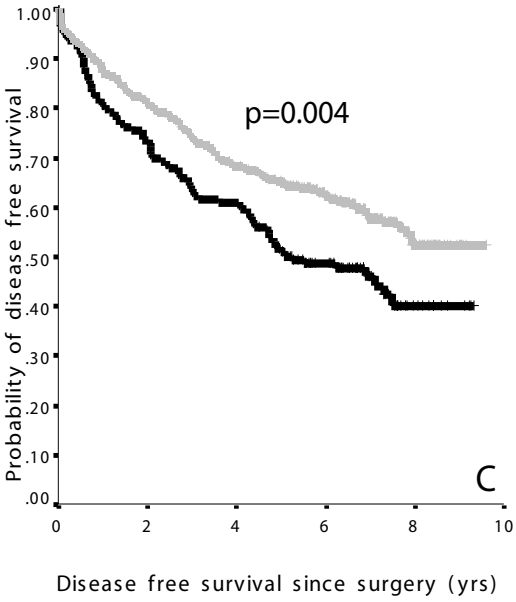
Figure 5 Kaplan-Meijer survival estimates by high and low COX-2 tumor epithelial staining in irradiated (RT+) rectal tumors



A



B



C

Kaplan-Meijer survival estimates by dichotomized COX-2 tumor epithelial staining in irradiated (RT+) rectal tumors. Kaplan Meijer curves by COX-2 expression 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).

Table 2

variable	Hazard ratio	95% CI	p-value
Patient age			<0.0001
Below median	1		
Above median	1.03	1.01-1.05	
TNM stage			<0.0001
I	1		
II	1.83	1.22-2.74	
III	2.88	1.96-4.22	
Circumferential margin			<0.0001
Negative	1		
Positive	1.94	1.41-2.67	
Distance of tumor from the anal verge			0.07
10.1-15 cm	1		
5.1-10 cm	1.48	1.03-2.13	
≤ 5 cm	1.44	1.01-2.03	
COX-2 expression			0.009
Low	1		
High	1.46	1.10-1.94	

Results of multivariate Cox regression analysis of overall survival among 510 irradiated rectal cancer patients. A variable was included in the multivariate analysis if the p-value in the univariate analysis was less than 0.05. Patients with missing data were excluded from the analysis. CI denotes confidence interval.

References

1. Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., Lee, J. C., and Dubinett, S. M. Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J.Immunol., *164*: 361-370, 2000.
2. Tsujii, M. and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83*: 493-501, 1995.
3. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93*: 705-716, 1998.
4. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc.Natl.Acad.Sci.U.S.A, *94*: 3336-3340, 1997.
5. Sheng, H., Shao, J., Morrow, J. D., Beauchamp, R. D., and DuBois, R. N. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res., *58*: 362-366, 1998.
6. Cutler, N. S., Graves-Deal, R., LaFleur, B. J., Gao, Z., Boman, B. M., Whitehead, R. H., Terry, E., Morrow, J. D., and Coffey, R. J. Stromal production of prostacyclin confers an antiapoptotic effect to colonic epithelial cells. Cancer Res., *63*: 1748-1751, 2003.
7. Cao, Y. and Prescott, S. M. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J.Cell Physiol, *190*: 279-286, 2002.
8. DuBois, R. N., Shao, J., Tsujii, M., Sheng, H., and Beauchamp, R. D. G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. Cancer Res., *56*: 733-737, 1996.
9. Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett, W. C., and Speizer, F. E. Aspirin and the risk of colorectal cancer in women. N.Engl.J.Med., *333*: 609-614, 1995.
10. Thun, M. J., Namboodiri, M. M., Calle, E. E., Flanders, W. D., and Heath, C. W., Jr. Aspirin use and risk of fatal cancer. Cancer Res., *53*: 1322-1327, 1993.
11. Thun, M. J., Henley, S. J., and Patrono, C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J.Natl.Cancer Inst., *94*: 252-266, 2002.
12. Tuynman, J. B., Peppelenbosch, M. P., and Richel, D. J. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev.Oncol.Hematol., *52*: 81-101, 2004.
13. Tuynman, J. B., Buskens, C. J., Kemper, K., ten Kate, F. J., Offerhaus, G. J., Richel, D. J., and van Lanschot, J. J. Neoadjuvant selective COX-2 inhibition down-regulates important oncogenic pathways in patients with esophageal adenocarcinoma. Ann.Surg., *242*: 840-9, discussion, 2005.
14. Fux, R., Schwab, M., Thon, K. P., Gleiter, C. H., and Fritz, P. Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. Clin.Cancer Res., *11*: 4754-4760, 2005.
15. Soumaoro, L. T., Uetake, H., Higuchi, T., Takagi, Y., Enomoto, M., and Sugihara, K. Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. Clin.Cancer Res., *10*: 8465-8471, 2004.
16. Kapiteijn, E., Liefers, G. J., Los, L. C., Kranenbarg, E. K., Hermans, J., Tollenaar, R. A., Moriya, Y., van de Velde, C. J., and van Krieken, J. H. Mechanisms of oncogenesis in colon versus rectal cancer. J.Pathol., *195*: 171-178, 2001.
17. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
18. International Union Against Cancer. TNM Classification of Malignant tumours, 6 ed.New York: Wiley-Liss, 2003.

19. Nagtegaal, I. D., Kranenbarg, E. K., Hermans, J., van de Velde, C. J., and van Krieken, J. H. Pathology data in the central databases of multicenter randomized trials need to be based on pathology reports and controlled by trained quality managers. J.Clin.Oncol., *18*: 1771-1779, 2000.
20. E.C.de Bruin, C. J. H. van de Velde S. van de Pas I. D. Nagtegaal J. H. J. M. van Krieken M. J. E. M. Gosens L. T. C. Peltenburg J. P. Medema C. A. M. Marijnen. Intrinsic and radiotherapy-induced apoptosis in rectal cancer patients of the Dutch TME trial: no need for radiotherapy in intrinsically high apoptotic tumours. Clin.Cancer Res. 2006 Nov 1;12(21):6432-6.
21. Buskens, C. J., van Rees, B. P., Sivula, A., Reitsma, J. B., Haglund, C., Bosma, P. J., Offerhaus, G. J., van Lanschot, J. J., and Ristimaki, A. Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology, *122*: 1800-1807, 2002.
22. Landis, J. R. and Koch, G. G. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics, *33*: 363-374, 1977.
23. Xi, H., Baldus, S. E., Warnecke-Eberz, U., Brabender, J., Neiss, S., Metzger, R., Ling, F. C., Dienes, H. P., Bollschweiler, E., Moenig, S., Mueller, R. P., Hoelscher, A. H., and Schneider, P. M. High cyclooxygenase-2 expression following neoadjuvant radiochemotherapy is associated with minor histopathologic response and poor prognosis in esophageal cancer. Clin.Cancer Res., *11*: 8341-8347, 2005.
24. Choy, H. and Milas, L. Enhancing radiotherapy with cyclooxygenase-2 enzyme inhibitors: a rational advance? J.Natl.Cancer Inst., *95*: 1440-1452, 2003.
25. Marijnen, C. A., Nagtegaal, I. D., Mulder-Stapel, A. A., Schrier, P. I., van de Velde, C. J., van Krieken, J. H., and Peltenburg, L. T. High intrinsic apoptosis, but not radiation-induced apoptosis, predicts better survival in rectal carcinoma patients. Int.J.Radiat.Oncol.Biol.Phys., *57*: 434-443, 2003.
26. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl.Acad.Sci.U.S.A, *88*: 2692-2696, 1991.
27. Kikuchi, T., Itoh, F., Toyota, M., Suzuki, H., Yamamoto, H., Fujita, M., Hosokawa, M., and Imai, K. Aberrant methylation and histone deacetylation of cyclooxygenase 2 in gastric cancer. Int. J.Cancer, *97*: 272-277, 2002.
28. Sawada, R., Tsuboi, S., and Fukuda, M. Differential E-selectin-dependent adhesion efficiency in sublines of a human colon cancer exhibiting distinct metastatic potentials. J.Biol.Chem., *269*: 1425-1431, 1994.
29. Kakiuchi, Y., Tsuji, S., Tsujii, M., Murata, H., Kawai, N., Yasumaru, M., Kimura, A., Komori, M., Irie, T., Miyoshi, E., Sasaki, Y., Hayashi, N., Kawano, S., and Hori, M. Cyclooxygenase-2 activity altered the cell-surface carbohydrate antigens on colon cancer cells and enhanced liver metastasis. Cancer Res., *62*: 1567-1572, 2002.
30. Kishi, K., Petersen, S., Petersen, C., Hunter, N., Mason, K., Masferrer, J. L., Tofilon, P. J., and Milas, L. Preferential enhancement of tumor radioresponse by a cyclooxygenase-2 inhibitor. Cancer Res., *60*: 1326-1331, 2000.
31. Davis, T. W., O'Neal, J. M., Pagel, M. D., Zweifel, B. S., Mehta, P. P., Heuvelman, D. M., and Masferrer, J. L. Synergy between celecoxib and radiotherapy results from inhibition of cyclooxygenase-2-derived prostaglandin E2, a survival factor for tumor and associated vasculature. Cancer Res., *64*: 279-285, 2004.
32. Liao, Z., Komaki, R., Milas, L., Yuan, C., Kies, M., Chang, J. Y., Jeter, M., Guerrero, T., Blumenschien, G., Smith, C. M., Fossella, F., Brown, B., and Cox, J. D. A phase I clinical trial of thoracic radiotherapy and concurrent celecoxib for patients with unfavorable performance status inoperable/unresectable non-small cell lung cancer. Clin.Cancer Res., *11*: 3342-3348, 2005.

33. Govindan, R., McLeod, H., Mantravadi, P., Fineberg, N., Helft, P., Kesler, K., Hanna, N., Stoner, C., Ansari, R., and Fox, E. Cisplatin, fluorouracil, celecoxib, and RT in resectable esophageal cancer: preliminary results. Oncology (Williston.Park), *18*: 18-21, 2004.
34. Yamauchi, T., Watanabe, M., Kubota, T., Hasegawa, H., Ishii, Y., Endo, T., Kabeshima, Y., Yorozuya, K., Yamamoto, K., Mukai, M., and Kitajima, M. Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. Dis.Colon Rectum, *45*: 98-103, 2002.
35. Karnes, W. E., Jr., Shattuck-Brandt, R., Burgart, L. J., DuBois, R. N., Tester, D. J., Cunningham, J. M., Kim, C. Y., McDonnell, S. K., Schaid, D. J., and Thibodeau, S. N. Reduced COX-2 protein in colorectal cancer with defective mismatch repair. Cancer Res., *58*: 5473-5477, 1998.
36. Karnes, W. E., Jr. Implications of low COX-2 expression in colorectal neoplasms with defective DNA mismatch repair. J.Cell Biochem.Suppl, *34*: 23-27, 2000.
37. Gervaz, P., Bucher, P., and Morel, P. Two colons-two cancers: paradigm shift and clinical implications. J.Surg.Oncol., *88*: 261-266, 2004.
38. Iacopetta, B. Are there two sides to colorectal cancer? Int.J.Cancer, *101*: 403-408, 2002.

CHAPTER 6

Celecoxib inhibits growth of tumors in a syngeneic rat liver metastases model for colorectal cancer

P. de Heer, M.H. Sandel, G. Guertens, G. de Boeck, M.M. Koudijs, J.F. Nagelkerke, J.M.C. Junggeburt, E.A. de Bruijn, C.J.H. van de Velde, P.J.K. Kuppen

Submitted for publication

Abstract

In colorectal cancer 40% of patients will develop liver metastases. The present study was designed to evaluate the inhibitory effects of the COX-2 inhibitor celecoxib on the growth of colorectal cancer liver metastases in a syngeneic rat model, CC531. The effects of celecoxib on cell viability *in vitro* were evaluated by treatment of CC531 tumor cell cultures with celecoxib. *In vivo*, Wag/Rij rats were inoculated with CC531 tumor cells at two sites in the liver and treated with celecoxib starting one week before, or directly after tumor inoculation. Control rats were inoculated without treatment. Three weeks after tumor inoculation rats were sacrificed. Tumor size, immune cell infiltration and PGE_2 and celecoxib levels were determined.

CC531 tumors did not show COX-2 expression, tumor growth was significantly inhibited by celecoxib treatment in a dose dependent manner. Immune cell infiltration was decreased after celecoxib treatment, indicating that the immune system was not involved in preventing tumor growth. Celecoxib serum concentration starting at 0.84 µg/ml significantly inhibited the outgrowth of CC531 liver tumors. In contrast, *in vitro* concentrations of celecoxib of at least 12 µg/ml were needed to affect tumor cell viability, suggesting that the effect of celecoxib on tumor growth in vivo was not a direct cytotoxic effect.

Introduction

In colon cancer, surgical resection potentially offers cure of the disease. Prognosis is mainly dependent on the occurrence of local or distant metastases, which occur in approximately 40% of the patients[1]. Epidemiological studies have indicated a considerable reduction in risk of occurrence of colorectal carcinoma in patients with reported long-term Non Steroidal Inflammatory Drugs (NSAID) use[2]. In addition to the chemoprophylactic potential, chemotherapeutic effects of NSAIDs have been suggested and evaluated in *in vitro*, animal and clinical studies[3-5]. The mechanism by which NSAIDs reduce the risk of colorectal carcinogenesis is generally attributed to the inhibition of the arachidonic acid metabolism via the cyclooxygenase enzymes (COX). COX is a critical step in the synthesis of prostaglandins (PG) that affects cell proliferation, tumor growth and immune responsiveness[6,7]. Several isoforms of COX exist[8]. The isoform COX-2 is upregulated in many types of malignancies[9] and is responsible for prostaglandin E_2 (PGE_2) production by tumor cells. Several recent reports have suggested that COX-2 expression has an important role in haematogenous metastasis of colorectal carcinomas to the liver[10,11], however, the effects of COX-2 inhibition on the growth of established liver metastases remains unknown.

Tumor cells use various strategies to escape host immune surveillance, among others by impairing the effectivity of the host immune response[12]. Overproduction of PG and specifically PGE_2 by tumor cells results in direct down regulation of effector cell cytotoxicity, but also creates an abnormal balance between the T helper (TH)-1 and TH-2 response favouring the TH-2, hereby functionally blunting the host anti-tumor cellular immune response[13,14].

A recent animal study suggested that the inhibitory effect of COX-2 inhibitors on tumor growth is immunological and is dependent on the presence of B or T lymphocytes[15]. Given the immunomodulating nature of PGE_2 production by tumor cells via COX-2 it has been suggested that COX-2 inhibition can result in an increased anti-tumor immune response by facilitating infiltration[14-16].

The aim of the present study was to investigate the effects of the COX-2 inhibitor celecoxib on the growth of established liver metastases by use of the CC531 rat tumor model[17,18]. In addition we evaluated the effects of celecoxib treatment on prostaglandin production, immune cell infiltration and apoptosis in the liver metastases.

Material and Methods

Animals

Twenty Male Wag/Rij rats weighing approximately 245 g were used (Charles River, Zeist, The Netherlands). All animals were housed in the animal facility of the Leiden University Medical Center. The animals had free access to food and water. The weight of the animals was followed throughout the experiment to monitor their general health state. Principles of laboratory animal care were followed and, according to Dutch law, the Animal Welfare Committee of the Leiden University Medical Center approved the study.

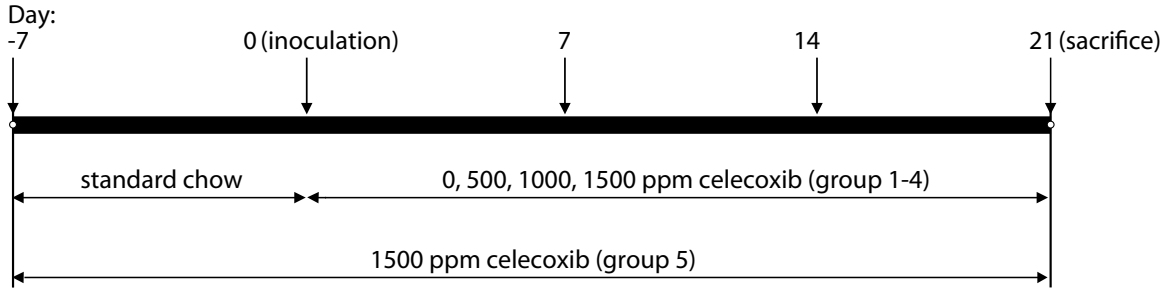
Cell culture and liver metastasis model

The colon adenocarcinoma cell line CC531 (1,2-dimethylhydrazine-induced) which is moderately differentiated and syngeneic to Wag/Rij rats[19] was used for tumor inoculation. Briefly, tumor cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine (Gibco, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate (complete medium). Tumor cells were harvested with a solution of 0.25% (w/v) EDTA and 0.25% (w/v) trypsin in HBSS (Sigma, St. Louis, MO, USA), washed three times in 0.9% (w/v) NaCl solution buffered with 1.4 mM phosphate (PBS) and adjusted to a suspension containing 1 x 10^6 viable (trypan blue exclusion test) tumor cells per ml PBS. For local liver tumor induction, 5 x 10^4 viable tumor cells (in 50 µl suspension) per site were injected subcapsulary into the upper lobe of the liver at 2 sites.

In vivo experimental design

Rats were randomly assigned to one of the following five groups: (1) control group, (2) celecoxib 500ppm starting at tumor inoculation, (3) celecoxib 1000ppm starting at tumor inoculation, (4) celecoxib 1500ppm starting at tumor inoculation (5) celecoxib 1500ppm starting one week before tumor inoculation (Figure 1). Two tumors were inoculated as described above in the liver at day 0. Tumors were allowed to grow for 21 days after which rats were sacrificed. Abdominal organs were evaluated for signs of toxicity. Liver tumors were separately enucleated from the surrounding liver parenchyma and measured. Blood samples were taken from all rats by aortal punction at time of sacrifice. Blood samples were allowed to coagulate and were centrifuged for 10 minutes at 13000 rpm (Beckman GS-6R centrifuge, Beckman Coulter, Fullerton, CA, USA); supernatants were collected and stored at -20°C until analysis. The cross sectional tumor area was used for analyses. This was determined using the formula: L x W x 0,25 x π in which L is maximum length and W is maximum width of the tumor[20].

Figure 1 Design of experiment with celecoxib treatment of CC531 tumors in a rat liver metastases model for colorectal cancer



Groups of 4 male Wag/Rij rats were fed 0, 500, 1000, or 1500 ppm celecoxib starting 7 days before (group 5) or directly after subcapsular tumor cell inoculation in the liver on day 0 (group 1-4). Rats were followed up for 21 days, after which they were sacrificed. After sacrifice, rat serum was collected and rat tumors were enucleated from the liver.

Medication

The COX-2 inhibitor celecoxib (SC-58635), obtained as a gift from Pfizer Pharmaceuticals, was incorporated into Altromin 1310 rat breeding diet by Altromin (Altromin Gesellschaft für Tierernährung mbH, Lage, Germany) at various concentrations. Rats were fed this diet according to experimental design as indicated in Figure 1.

Analysis of celecoxib concentrations in serum

A high-performance liquid chromatographic (HPLC) method was used and validated for the determination of celecoxib in serum. Ibuprofen was used as an internal standard. Blanc serum samples (250 µl) were spiked with celecoxib (range 80ng/ml - 6000 ng/ml) and Ibuprofen (2000 ng/ml) and used as calibrators and quality control samples. The limit of quantification was 100 ng/ml. Within-run and between-run precisions were less than 10 % and average accuracies were between 90 and 110 %. To 250 µl of serum, 50 µl internal standard work solution (10 µg/ml)[21] was added and the sample was mixed thoroughly. To precipitate the proteins, 1 ml of acetonitrile was added and the sample was vortexed again. After centrifugation, the supernatant was transferred to a glass tube and evaporated till dry. The residue was resuspended in 1 ml of the mobile phase[21] and filtered over a 0.45 µm PVDF HPLC-filter (Acrodisc, Waters Corporation) for HPLC injection (40 µl).

Separation was achieved on a Symmetry 300 C18 column (25 cm x 4.6 mm, 5 µm) (Waters, Milford, USA) connected to a Luna C18 guard column (4 mm x 3 mm, 5 µm) (Phenomenex, Torrance, USA). The mobile phase, which was filtered through a 0,20 µm nylon filter before use, consisted of an acetonitrile-water-acetic acid-triethylamine (47 : 53 : 0,1 : 0,03) mixture and was pumped at a flow rate of 1 ml/min. Celecoxib and Ibuprofen were detected by fluorescence detection. Emission and excitation wavelengths of Celecoxib and Ibuprofen were 280/340 and 253/300, respectively.

Analysis of PGE_2 concentrations in liver metastases and serum

Tumor and serum levels of PGE_2 were measured to analyse celecoxib activity in rats fed the control diets or diets supplemented with 500ppm, 1000ppm or 1500ppm. A competitive enzyme immunoassay (R&D Systems Inc., Minneapolis MM 55413, USA) was used for the determination of PGE_2 in serum and tumor tissue. The sensitivity of the PGE_2 assay was typically higher than 13 pg/ml. Each tissue sample (50-300 mg) was dried for surface moisture and accurately weighed. The sample was then homogenized in 1 ml of distilled water. After centrifugation, the supernatant was treated the same way as serum.

Immunohistochemical staining of CC531 liver metastases

Cryostat sections (Cryocut 3000, Leica, Nuss-loch, Germany) 5 µm thick were cut from the tumor tissue that was snap-frozen directly after resection, of the control group (group 1) and the group receiving celecoxib 1500ppm (group 4). Sections were air-dried for at least 16 hr at 60 °C, then fixed in acetone for 10 min and washed twice in PBS. All dilutions of antibodies and conjugates were performed with PBS containing 1% (w/v) bovine serum albumin (BSA, Boehringer, Mannheim, Germany). Immunohistochemistry for detection of tumor cell COX-2 expression was performed as described previously with a polyclonal anti-COX-2 antibody

(ALX-210-711, Alexis, San Diego, CA, USA, 1:300[22]). As negative controls sections were incubated with PBS instead the primary antibody. Immunohistochemical analysis of immune cell infiltration was performed as follows: The tissue sections were incubated for 30 min with a previously determined optimal concentration of protein-A-purified primary antibody. The monoclonal antibody (MAb) 3.2.3 IgG1 (a gift from Dr. W.H. Chambers, University of Pittsburgh Cancer Institute, Pittsburgh, PA) was used for detection of CD161A (NKR-P1A+, Natural Killer cells) cells, the MAb R73, anti-rat T-cell receptor (TCR) (a gift from Dr. Th. Hünig, University of Würzburg, Germany), was used for the detection of T cells. After incubation with the primary antibody, the sections were washed in PBS 3 times for 5 min, followed by two 30-min incubations with horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse Ig (dilution 1:100) and HRP-conjugated swine anti-rabbit Ig (dilution 1:50, both obtained from DAKO, Glostrup, Denmark) and subsequent washes in PBS. Visualization of immune complexes was performed by a 10-min incubation with a 3,3'-diaminobenzidine (DAB) substrate containing 1.8 x10E-3% (v/v) H_2O_2. A polyclonal rabbit anti-laminin antibody (Sigma-Aldrich) was used for the detection of laminin. After 3 wash steps with PBS, the sections were incubated for 30 min with HRP-conjugated swine anti-rabbit Ig (dilution 1:50, DAKO) for the detection of laminin. The immune complexes were visualized by a 12-min incubation step in a buffered TRIS-HCl (pH 7.6) solution containing, per 100 ml, (1) 40 mg 4-chloro-1-naphtol (Merck, Darmstadt, Germany) dissolved in 200 µl dimethylformamide (Baker, Deventer, The Netherlands) and 300 µl ethanol (Merck) and (2) 100 µl of a 30% (v/v) H_2O_2 solution (Merck). The sections were slightly counterstained using methyl green (Klinipath) and mounted using Kaiser's glycerine (Merck). Control sections (1 per tumor) were included in which both primary antibodies were omitted[21].

Quantification of immunostaining

After immunohistochemical staining slides were directly coded in order to blind the observer for tumor number or treatment group of the tumors. The number of tumor infiltrating R73+ and 323+ cells in tumor epithelium were estimated using a scoring method described by Menon et al[23]. In brief, an ocular grid, with a total surface area of 38 mm², was used at a 200x magnification to count all leukocytes that were located intraepithelially in 25 different randomly chosen tumor fields of the tissue section. Laminin was used to distinguish between intraepithelially, that is, leukocytes in direct contact with tumor cells, and intrastromally located leukocytes (figure 3A-C). This tumor compartment-specific analysis made it possible to calculate the number of leukocytes per tumor cell area (leukocytes/mm² tumor epithelium). The mean leukocyte infiltration of 25 fields per tumor section was calculated and defined as the intraepithelial leukocyte infiltration. After evaluation, the slides were unblinded for treatment group for further analyses.

Effects of celecoxib on CC531 cell viability *in vitro*

The cell viability was assessed by the mitochondrial function, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity as previously reported[24]. Briefly, cells were seeded in a 96-well plate and incubated with increasing concentrations of celecoxib (figure 4). After 72 hours, the cells were incubated with 0.5 mg/ml MTT (Sigma-

Aldrich) for 4 hours at 37°C. Subsequently, 100 µl SDS (10% (v/v) in 0.01 M HCl) was added, after which the absorbance was read at 590 nm, using a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). Stock solutions of the pure compound celecoxib were made in dimethyl sulphide (DMSO). A final DMSO concentration of 0.1% in medium was used in all in vitro experiments including control experiments.

Statistical analyses

Statistical analysis between groups was performed using the Fisher exact test. Correlations between variables were evaluated using Spearmans' rank analysis, Mann-Whitney, Kruskal-Wallis, or student's T-test. Values with $P<0.05$ were considered statistically significant. The Statistical Package for Social Sciences (SPSS) version 12.0 was used for all statistical analyses.

Results

General condition of rats

The body weights of rats fed the control diet or the experimental diets containing various levels of celecoxib were comparable throughout the study. There was no difference in animal behavior between the treatment groups. Animals experienced a slight weight loss after laparotomy for inoculation of CC531 tumor cells in the liver, but no rats lost more than 5% body weight. The initial tumor induction was successful in all rats and no rats died before the end of the experiment. After sacrifice of the animals no gross intra-abdominal changes were noted that would indicate toxicity.

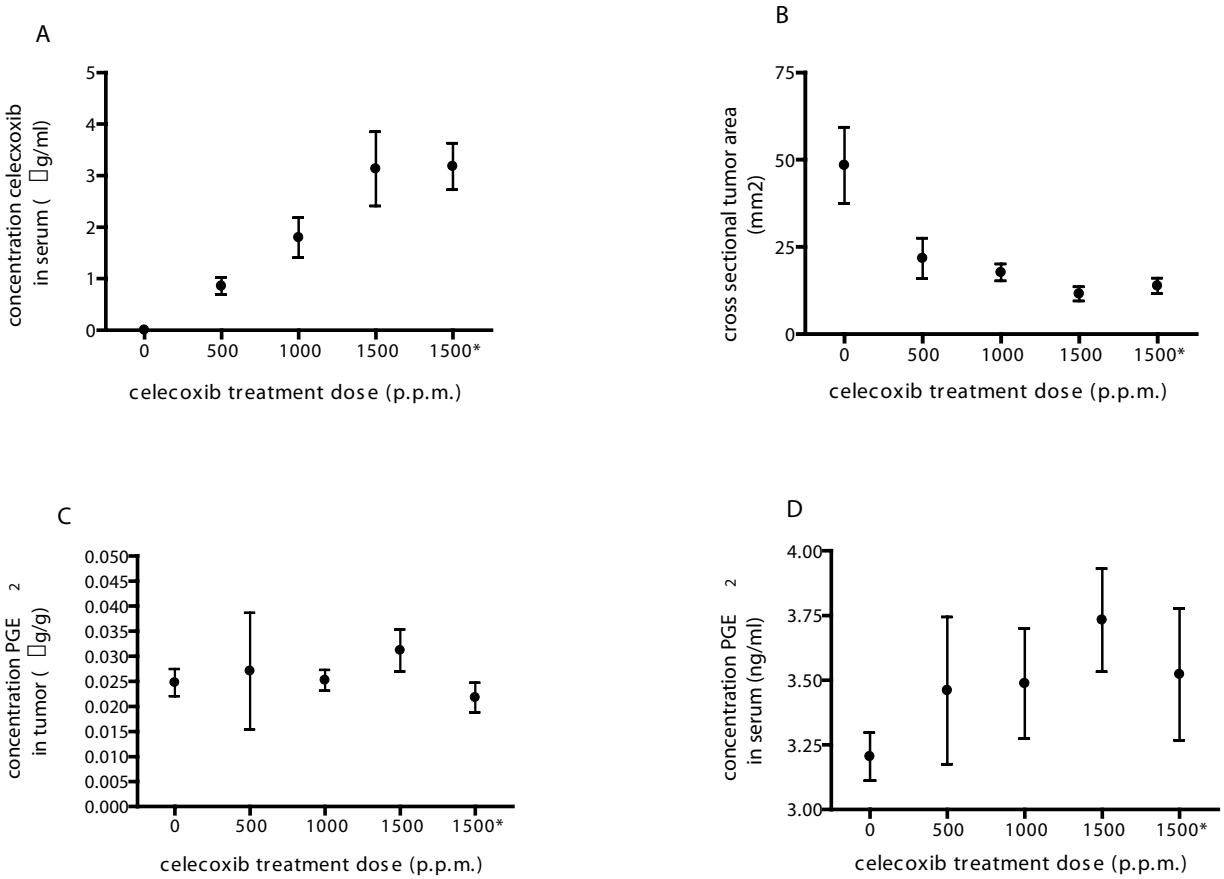
Serum celecoxib levels

To establish if administration of celecoxib to rat diet resulted in adequate levels of celecoxib in rat serum, serum samples were collected after sacrifice. Increased dosage of celecoxib in the rat chow showed a corresponding increase in serum celecoxib levels (figure 2A). Rats in the control group who were fed regular chow had undetectable levels. Steady-state serum levels were as follows: celecoxib 500ppm (group 2): 0.84 ± 0.33 µg/ml, celecoxib 1000ppm (group 3): 1.97 ± 0.77 µg/ml, celecoxib 1500ppm (group 4): 3.10 ± 1.44 µg/ml, celecoxib 1500ppm starting 1 week pre inoculation (group 5): 3.07 ± 0.91 µg/ml (figure 2A). Serum celecoxib levels in the present study were comparable with the 0.1-5.0 µM concentrations in cancer patients treated with celecoxib[4,25].

Effects of celecoxib treatment on liver metastasis growth

The effects of celecoxib administration on the tumor growth are summarised in figure 2B. Administration of celecoxib resulted in a significant dose dependent reduction of tumor size when compared to the rats that were fed control diet (group 1): Celecoxib 500ppm (group 2): p=0.04, celecoxib 1000ppm (group 3): p=0.02, celecoxib 1500ppm (group 4): p=0.006, celecoxib 1500 ppm starting one week before inoculation (group 5): p=0.007 (figure 2B) (Mann-Whitney). The administration of celecoxib 1 week before tumor cell inoculation did not significantly inhibit tumor growth compared to administration after inoculation (group 4) (p=0.28)

Figure 2



*celecoxib treatment started 7 days before tumor inoculation.
In all other groups treatment started at inoculation.

Figure 2 Effects of 21 days of celecoxib treatment on CC531 liver metastases and PGE2 serum and tumor level. All treatment groups consisted of 4 rats in each of which 2 tumors were inoculated subcapsularly in the liver. Rats received control diet, or a diet containing: celecoxib 500 ppm, celecoxib 1000 ppm, celecoxib 1500 ppm, starting at tumor inoculation, or celecoxib 1500 ppm, starting 7 days before tumor inoculation. Blood and tumors were obtained from rats after sacrifice. Serum celecoxib levels and serum and tumor PGE_2 levels were measured as described in the material and methods section. Values represent the mean and standard error. 2A: Serum celecoxib concentrations. 2B: Effects of celecoxib on tumor size (cross sectional tumor areas). 2C: PGE_2 concentrations in the tumors of the rats. 2D: PGE_2 concentrations in sera from the rats.

Effects of celecoxib treatment on serum and tumor PGE_2 levels

The effects of celecoxib on tumor and serum PGE_2 levels can be seen in figure 2C-D. No significant differences were found in tumor and serum PGE_2 levels between the treatment groups (p=0.32 and p=0.51 respectively, Kruskal-Wallis).

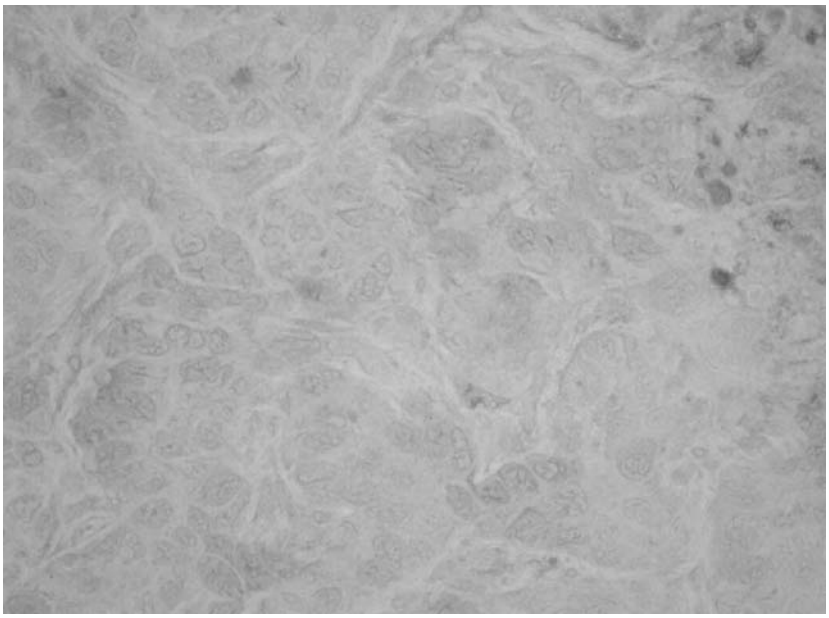


Figure 3 Fresh frozen tumor sections from CC531 stained with polyclonal rabbit antibodies against COX-2 (1:300) at 400x magnification. COX-2 expression is not visible in tumor epithelium. Surrounding tumor stroma shows light brown imunoreactivity. Infiltrating macrophages show positive COX-2 expression.

Tumor COX-2 expression

COX-2 expression in CC531 tumors is shown in figure 3: All CC531 tumor cells were negative for COX-2 expression. Surrounding tumor stroma showed light brown immunostaining, this was not affected by celecoxib treatment. Infiltrating macrofages showed to be positive for COX-2 and thus served as internal positive control for the test. All negative controls showed no immunoreactivity.

Effects of celecoxib treatment on infiltration of tumors by R73+ and 323+ cells

Previously, it was established that intraepithelial immune cells that are in direct contact with tumor target cells affect tumor growth[30]. Therefore, infiltration of intraepithelial immune cells in the tumor was evaluated. Figure 4A-D shows the effects of celecoxib treatment on the intraepithelial infiltration of R73+ (TCR-positive cells, T cells) and 323+ (CD161A-positive cells, Natural Killer cells) cells. Intraepithelial infiltration of R73+ and 323+ cells was significantly diminished in the 1500ppm celecoxib group (group 4) compared to the control group (group 1) ($p=0.01$ and $p=0.02$ respectively). Infiltration with R73+ cells was positively correlated with 323+ cell infiltration ($p=0.03$, Spearmans' rank analysis).

Effects of Celecoxib administration on tumor cell viability *in vitro*

In the present study we observed that concentrations of 0.84 μg/ml were sufficient to reduce tumor growth. When CC531 cells were exposed to celecoxib concentrations equal to *in vivo* concentrations after 72 hours, no effect on cell viability was observed. In vitro, treatment with concentrations of at least 12 μg/ml or higher were needed to inhibit cell growth (mean % cell

viability 86.7 ± 11.5, p=0.10) and exposure to 24 µg/ml celecoxib resulted in a significant inhibition of cell viability as compared to the control group (mean % cell viability 43.0 ± 3.7, p<0.0001, one sample t-test).

Figure 4

Figure 4A R73+ infiltration

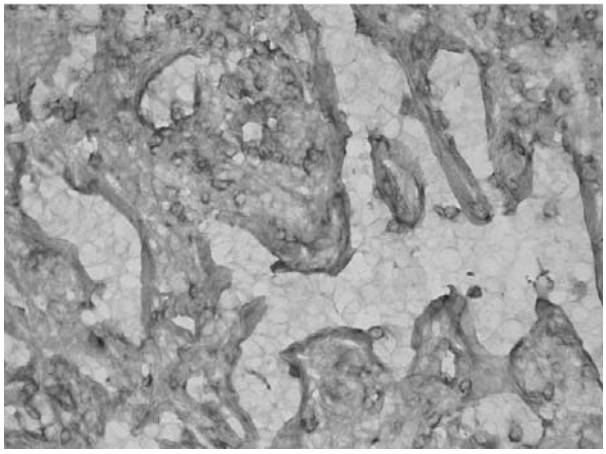


Figure 4B 3.2.3.+ infiltration

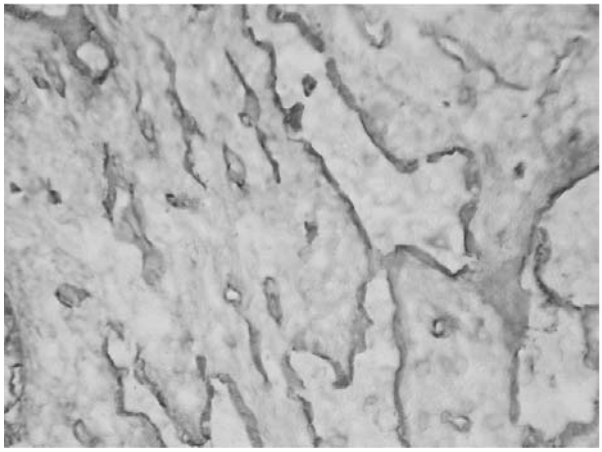


Figure 4C R73+ infiltration after celecoxib treatment

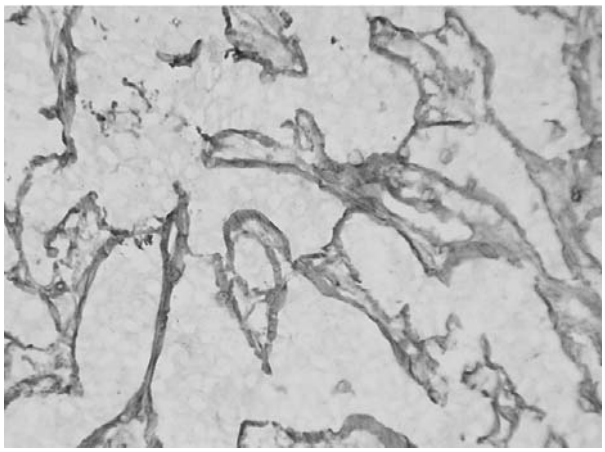
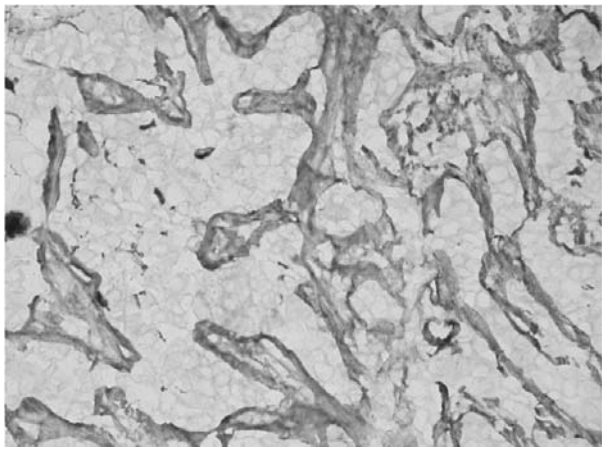


Figure 4C 3.2.3.+ infiltration after celecoxib treatment



A representative staining for T cell and NK cell infiltration of tumor sections from celecoxib-treated (1500ppm) and -untreated rats 21 days after tumor inoculation. Sections were double-stained with laminin and R73 (anti- TCR, 1:100, Fig. 4A, 4C) or 323 (anti-CD161A, 1:50, Fig. 4B, 4D) antibodies respectively. R73+ and 323+ cells were stained brown, as revealed by immunohistochemistry (see material and methods). The matrix protein laminin was stained blue, blank spaces represent tumor nodules, delineated by a laminin-containing basal-membrane-like structure. The majority of R73+ and 323+ cells were localized in the tumor stroma, few positive cells were found in the tumor nodules. (200x magnification)

Discussion

The current study demonstrates that treatment of rats with levels of celecoxib equal to therapeutical levels in humans[4,25], showed an inhibitory effect on the growth of liver metastases, even in a situation of low COX-2 activity. Recent RNA expression array data from a study by Germann et al. demonstrated that the CC531 cell line shows low COX-2 RNA expression[27]. The low COX-2 expression was confirmed in our study as tumor epithelium was negative for COX-2 immunostaining. In addition, PGE_2 serum and tumor levels were not affected by celecoxib treatment. Furthermore, the level of PGE_2, assumed to reflect COX-2 activity, was very low as compared to a similar study using MC-26 cell line, that showed a 2000-fold higher PGE_2 production in untreated COX-2 positive tumors[34].

In our model, increasing levels of celecoxib were associated with a corresponding decrease in tumor size. Celecoxib is known to have direct cytotoxic effect on tumor cells as well as indirect effects, in which the immune system and angiogeneis is involved[7]. Treatment of CC531 cells *in vitro* for 36 hours with concentrations of up to12 μg/ml (32μM) did not have any significant effect on cell viability while *in vivo* already 0,84 μg/ml significantly inhibited tumor growth, suggesting no direct effect of celecoxib on tumor cell viability *in vivo*. These observations are supported by a study by Williams et al. [29] Celecoxib may create an unfavourable host environment for tumor growth. Several environmental interactions that determine tumor growth have been described to be affected by celecoxib treatment, including the immune system[30-33]. Infiltration of cytotoxic T-cells, NK cells and leukocytes is associated with improved prognosis in several malignancies and tumor cells utilise various strategies to escape the host immune surveillance[23,26,34]. *In vitro* production of PGE_2 by COX-2 prevents activation of natural killer cells and T-cell mediated anti-tumor response, impairs the function of DC's and suppresses lymphocyte proliferation[14,35-37]. The before mentioned studies suggest that these effects can be reversed by selective COX-2 inhibition. A recent study indicates that, in addition to enhancement of lymphocyte accumulation in tumors by COX-2 inhibition[14,15], the anti-tumor effects of COX-2 inhibition are immunological and depend on the presence of lymphocytes in the tumor[15]. In the current study we quantified the immune cell infiltration: Surprisingly, we found a significant decrease in T-cell and NK-cell infiltration in tumors receiving celecoxib treatment, showing that the effect of celecoxib on tumor growth in our model can not be attributed to immune effector cells. A decrease in infiltration after treatment with NSAIDs or COX-2 inhibitors has been described in inflammatory processes as inflammatory bowel disease[38] and rheumatoid arthritis[39] and indicates that the effects of COX-2 inhibition on tumor growth is not mediated through an increased anti-tumor immune response.

Most studies evaluating the immunological effects of COX-2 inhibition in tumor growth were performed with COX-2 overexpressing tumors. However it is estimated that 25-30% of human colorectal cancer does not express the COX-2 enzyme[40]. The results from the current study indicate that effects of COX-2 inhibitors on tumors with low COX-2 activity are still significant, but independent of immune effector mechanisms.

References

1. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
2. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. Aspirin use and reduced risk of fatal colon cancer. N.Engl.J.Med., *325*: 1593-1596, 1991.
3. Waskewich, C., Blumenthal, R. D., Li, H., Stein, R., Goldenberg, D. M., and Burton, J. Celecoxib exhibits the greatest potency amongst cyclooxygenase (COX) inhibitors for growth inhibition of COX-2-negative hematopoietic and epithelial cell lines. Cancer Res., *62*: 2029-2033, 2002.
4. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N.Engl.J.Med., *342*: 1946-1952, 2000.
5. Reddy, B. S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K., and Rao, C. V. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res., *60*: 293-297, 2000.
6. Sheng, H., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R. J., Morrow, J., Beauchamp, R. D., and Dubois, R. N. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J.Clin.Invest, *99*: 2254-2259, 1997.
7. Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. Cyclooxygenase in biology and disease. FASEB J., *12*: 1063-1073, 1998.
8. DuBois, R. N. COX-2 in large bowel cancer: a one-sided story. Gut, *45*: 636-637, 1999.
9. Dannenberg, A. J., Altorki, N. K., Boyle, J. O., Dang, C., Howe, L. R., Weksler, B. B., and Subbaramaiah, K. Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. Lancet Oncol., *2*: 544-551, 2001.
10. Chen, W. S., Wei, S. J., Liu, J. M., Hsiao, M., Kou-Lin, J., and Yang, W. K. Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. Int.J.Cancer, *91*: 894-899, 2001.
11. Kakiuchi, Y., Tsuji, S., Tsujii, M., Murata, H., Kawai, N., Yasumaru, M., Kimura, A., Komori, M., Irie, T., Miyoshi, E., Sasaki, Y., Hayashi, N., Kawano, S., and Hori, M. Cyclooxygenase-2 activity altered the cell-surface carbohydrate antigens on colon cancer cells and enhanced liver metastasis. Cancer Res., *62*: 1567-1572, 2002.
12. Pardoll, D. Does the immune system see tumors as foreign or self? Annu.Rev.Immunol, *21*: 807-839, 2003.
13. Huang, M., Stolina, M., Sharma, S., Mao, J. T., Zhu, L., Miller, P. W., Wollman, J., Herschman, H., and Dubinett, S. M. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. Cancer Res., *58*: 1208-1216, 1998.
14. Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., Lee, J. C., and Dubinett, S. M. Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J.Immunol., *164*: 361-370, 2000.
15. DeLong, P., Tanaka, T., Kruklitis, R., Henry, A. C., Kapoor, V., Kaiser, L. R., Sterman, D. H., and Albelda, S. M. Use of cyclooxygenase-2 inhibition to enhance the efficacy of immunotherapy. Cancer Res., *63*: 7845-7852, 2003.
16. Rocca, B. and FitzGerald, G. A. Cyclooxygenases and prostaglandins: shaping up the immune response. Int.Immunopharmacol., *2*: 603-630, 2002.

17. Hagenaars, M., Zwaveling, S., Kuppen, P. J., Ensink, N. G., Eggermont, A. M., Hokland, M. E., Basse, P. H., van de Velde, C. J., Fleuren, G. J., and Nannmark, U. Characteristics of tumor infiltration by adoptively transferred and endogenous natural-killer cells in a syngeneic rat model: implications for the mechanism behind anti-tumor responses. Int.J.Cancer, *78*: 783-789, 1998.
18. van Duijnhoven, F. H., Tollenaar, R. A., Terpstra, O. T., and Kuppen, P. J. Locoregional Therapies of Liver Metastases in a Rat CC531 Coloncarcinoma Model Results in Increased Resistance to Tumour Rechallenge. Clin.Exp.Metastasis, *22*: 247-253, 2005.
19. Hagenaars, M., Ensink, N. G., Basse, P. H., Hokland, M., Nannmark, U., Eggermont, A. M., van de Velde, C. J., Fleuren, G. J., and Kuppen, P. J. The microscopic anatomy of experimental rat CC531 colon tumour metastases: consequences for immunotherapy? Clin.Exp.Metastasis, *18*: 189-196, 2000.
20. Marinelli, A., Dijkstra, F. R., van Dierendonck, J. H., Kuppen, P. J., Cornelisse, C. J., and van de Velde, C. J. Effectiveness of isolated liver perfusion with mitomycin C in the treatment of liver tumours of rat colorectal cancer. Br.J.Cancer, *64*: 74-78, 1991.
21. Guirguis, M. S., Sattari, S., and Jamali, F. Pharmacokinetics of celecoxib in the presence and absence of interferon-induced acute inflammation in the rat: application of a novel HPLC assay. J.Pharm.Pharm.Sci., *4*: 1-6, 2001.
22. Buskens, C. J., Sivula, A., van Rees, B. P., Haglund, C., Offerhaus, G. J., van Lanschot, J. J., and Ristimaki, A. Comparison of cyclooxygenase 2 expression in adenocarcinomas of the gastric cardia and distal oesophagus. Gut, *52*: 1678-1683, 2003.
23. Menon, A. G., Fleuren, G. J., Alphenaar, E. A., Jonges, L. E., Janssen-van Rhijn, C. M., Ensink, N. G., Putter, H., Tollenaar, R. A. E. M., van de Velde, C. J. H., and Kuppen, P. J. K. A basal membrane-like structure surrounding tumor nodules may prevent intra-epithelial leukocyte infiltration in colorectal cancer. Cancer Immunology Immunotherapy, 2003.
24. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J.Immunol.Methods, *65*: 55-63, 1983.
25. Raz, A. Is inhibition of cyclooxygenase required for the anti-tumorigenic effects of nonsteroidal, anti-inflammatory drugs (NSAIDs)? In vitro versus in vivo results and the relevance for the prevention and treatment of cancer. Biochem.Pharmacol., *63*: 343-347, 2002.
26. Menon, A. G., Janssen-van Rhijn, C. M., Morreau, H., Putter, H., Tollenaar, R. A., van de Velde, C. J., Fleuren, G. J., and Kuppen, P. J. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest, *84*: 493-501, 2004.
27. Germann, A., Dihlmann, S., Hergenhahn, M., Doeberitz, M. K., and Koesters, R. Expression profiling of CC531 colon carcinoma cells reveals similar regulation of beta-catenin target genes by both butyrate and aspirin. Int.J.Cancer, *106*: 187-197, 2003.
28. Yao, M., Kargman, S., Lam, E. C., Kelly, C. R., Zheng, Y., Luk, P., Kwong, E., Evans, J. F., and Wolfe, M. M. Inhibition of cyclooxygenase-2 by rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Res., *63*: 586-592, 2003.
29. Williams, C. S., Watson, A. J., Sheng, H., Helou, R., Shao, J., and Dubois, R. N. Celecoxib prevents tumor growth in vivo without toxicity to normal gut: lack of correlation between in vitro and in vivo models. Cancer Res., *60*: 6045-6051, 2000.
30. Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, B. M., Edwards, D. A., Flickinger, A. G., Moore, R. J., and Seibert, K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res., *60*: 1306-1311, 2000.
31. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and Dubois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93*: 705-716, 1998.

32. Tuynman, J. B., Buskens, C. J., Kemper, K., ten Kate, F. J., Offerhaus, G. J., Richel, D. J., and van Lanschot, J. J. Neoadjuvant selective COX-2 inhibition down-regulates important oncogenic pathways in patients with esophageal adenocarcinoma. Ann.Surg., *242*: 840-9, discussion, 2005.
33. Tuynman, J. B., Peppelenbosch, M. P., and Richel, D. J. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev.Oncol.Hematol., *52*: 81-101, 2004.
34. Ohno, Y., Ohno, S., Suzuki, N., Kamei, T., Inagawa, H., Soma, G., and Inoue, M. Role of cyclooxygenase-2 in immunomodulation and prognosis of endometrial carcinoma. Int.J.Cancer, *114*: 696-701, 2005.
35. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55*: 3785-3789, 1995.
36. Kojima, M., Morisaki, T., Uchiyama, A., Doi, F., Mibu, R., Katano, M., and Tanaka, M. Association of enhanced cyclooxygenase-2 expression with possible local immunosuppression in human colorectal carcinomas. Ann.Surg.Oncol., *8*: 458-465, 2001.
37. Sharma, S., Stolina, M., Yang, S. C., Baratelli, F., Lin, J. F., Atianzar, K., Luo, J., Zhu, L., Lin, Y., Huang, M., Dohadwala, M., Batra, R. K., and Dubinett, S. M. Tumor cyclooxygenase 2-dependent suppression of dendritic cell function. Clin.Cancer Res., *9*: 961-968, 2003.
38. Geboes, K. and Dalle, I. Influence of treatment on morphological features of mucosal inflammation. Gut, *50 Suppl 3*: III37-III42, 2002.
39. Kruithof, E., De Rycke, L., Roth, J., Mielants, H., Van den, B. F., De Keyser, F., Veys, E. M., and Baeten, D. Immunomodulatory effects of etanercept on peripheral joint synovitis in the spondylarthropathies. Arthritis Rheum., *52*: 3898-3909, 2005.
40. Yamauchi, T., Watanabe, M., Kubota, T., Hasegawa, H., Ishii, Y., Endo, T., Kabeshima, Y., Yorozuya, K., Yamamoto, K., Mukai, M., and Kitajima, M. Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. Dis.Colon Rectum, *45*: 98-103, 2002.

CHAPTER 7

Cutaneous and intra-abdominal abscess formation in rats following Radio Frequent Ablation of liver tumors in combination with celecoxib treatment

P. de Heer, M.H. Sandel, F.M. Speetjens, M.M. Koudijs, H. Putter, G.N. Ensink, C.J.H. van de Velde, P.J.K. Kuppen

In Vivo. 2006 May-Jun;20(3):373-5.

Abstract

Background: The present study evaluated the safety of treatment of colorectal liver metastases with Radio Frequency Ablation (RFA) in combination with high doses of the selective cyclooxygenase-2 inhibitor celecoxib.
Materials and Methods: The study was performed in the CC531 rat model for colorectal cancer. Rats were inoculated with CC531 tumor cells subcapsularly in the liver. Rats were randomized for treatment with celecoxib, RFA, or the combination thereof. Celecoxib treatment was started at tumor induction. At three weeks after tumor inoculation liver tumors were treated with RFA and the effects on rat health were monitored.
Results: Treatment that included RFA resulted in significantly ($p=0.003$) more deaths then sham-operated rats. Treatment that included celecoxib resulted in significantly increased cutaneous wound abscess formation after surgery ($p<0.0001$). In addition, the combination of celecoxib treatment with RFA resulted in intra-abdominal abscess formation ($p<0.0001$).
Conclusions: This study indicates that the use of high dose celecoxib in combination with RFA of liver metastases should be treated with caution when applied as anti-cancer treatment modality as additional side effects are induced.

Introduction

Epidemiological studies indicate that the long-term use of aspirin and other Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) reduces the risk of colorectal cancer by 40-50 percent[1]. Treatment with cyclooxygenase-2 (COX-2) specific NSAIDs has been shown to reduce polyp size and polyp number in FAP patients with a predisposition to colorectal adenoma and cancer[2]. The use of selective inhibitors in combination with additional treatment modalities is being investigated in clinical trials in colorectal cancer patients. Patients with metastases of colorectal cancer confined to the liver can benefit from Radio Frequency Ablation (RFA) if metastases are not surgically resectable[3]. Peri-operative treatment with selective COX-2 inhibitors is associated with impaired wound healing and anastomotic failure in animal models [4], but their effects in combination with RFA are unknown.
The aim of this in vivo study was to evaluate the effects and safety of celecoxib treatment in combination with RFA in an animal model. For this we used an in vivo colorectal cancer liver metastases rat model using the syngenic CC531 cell line [5].

Material and methods

In the present study principles of laboratory animal care according to Dutch law were followed. The Animal Welfare Committee of the Leiden University Medical Center approved the study.
Forty Wag/Rij rats (Charles River, Zeist, The Netherlands) were inoculated with CC531 tumor cells subcapsularly at two sites in the liver as previously described [6] and randomly assigned to celecoxib treatment, starting at the day of tumor inoculation, or no celecoxib treatment. For celecoxib treatment, celecoxib was mixed into standard chow at a concentration of 1500 mg/kg. In addition, in both groups rats were randomly assigned to treatment with RFA or a sham operation. RFA was performed on one of the two tumors in each rat at 21 days after tumor induction. This resulted in 4 treatment groups: 1, RFA + celecoxib; 2, sham + celecoxib; 3, RFA; 4, sham. RFA was applied using a RITA 1500x RF generator (RITA Medical Systems, Mountainview, CA, USA). A two cm expandable needle was inserted in the right tumor in the upper liver lobe and expanded to 1.5 cm, resulting in a lesion of 1.5 cm in diameter. Power output was set at 90 Watt, temperature was 90° Celsius and when this temperature was reached, an ablation of 2 minutes was performed. The tumor in the left liver lobe remained untreated as a negative control. All rats were operated under semi-sterile conditions. Sham operations were performed as a control. Weight of the rats and general health were monitored thrice weekly and rats were sacrificed on day 40 under general anesthesia. Data were entered into a statistical database (SPSS® version 12·0; SPSS, Chicago, Illinois, USA). Descriptive statistics were used for operative outcomes. The Fisher's exact test was used to test categorical variables. A P-value of < 0.05 was considered statistically significant.

Results

All rats were operated twice during the study. During the first operation tumors were inoculated subcapsularly in two separate liver lobes. Rats that were randomized for celecoxib treatment

received this drug in chow at a dose of 1500 mg per kg chow, this dose level was the highest used in a study by Reddy et al. and provided optimal inhibition of tumor growth [7]. Treatment with RFA or sham operation was performed three weeks after tumor induction. The weights of the rats were followed during the whole study and did not significantly differ among the treatment groups (p=0.76). The clinical outcomes of both operations are summarized in table 1.

Event of side effect	**Rats per treatment group (No. Of rats showing event/total no. Of rats)**				**Analysis of event occurrence**	
	1. RFA + celecoxib	2. sham + celecoxib	3. RFA	4. sham	p-value	Likelihood Ratio
post RFA/sham deaths	5/10	0/10	3/10	0/10	0.003 (RFA versus sham)	13,1
					p=0.01 (RFA + celecoxib versus RFA)	13.95
rats developing cutaneous abscesses	10/10	10/10	0/10	0/10	<0.0001 (celecoxib versus no celecoxib)	55. 5
rats developing intra-abdominal abscesses	10/10	0/10	0/10	0/10	<0.0001 (RFA + celecoxib versus any other treatment)	17.3

Table 1 Postoperative events registrated in rats randomized for liver tumor Radio Frequency Ablation or a sham operation, 3 weeks after induction of liver tumors as described in Material and Methods. Rats had previously been randomized to treatment with celecoxib 1500 mg/kg or no treatment that started immediately after tumor induction. None of the rats had died before RFA or sham treatment. Cutaneous and intra-abdominal abscesses were also present in rats that died following the RFA treatment.

No deaths occurred after tumor inoculation. Eight rats (20%) died after the second operation, of which 5 (12,5%) rats receiving celecoxib. The treatment with RFA resulted in significant more deaths (8 rats, 20%) as compared to sham operation, regardless of celecoxib treatment (0 rats)(p=0.003, Likelihood ratio: 13,1). There were significantly more deaths in the RFA-treated rats in the celecoxib group than in the groups that did not receive celecoxib (p=0.01, Likelihood ratio: 13.95). Rats in the celecoxib group developed intra-abdominal abscesses after RFA treatment (p<0.0001 Likelihood ratio: 17.3)(figure 1a, table 1). Significantly more rats in the celecoxib group compared to the groups that did not receive celecoxib developed cutaneous abscesses after surgery (p<0.0001 Likelihood ratio: 55. 5) (figure 2, table 1).

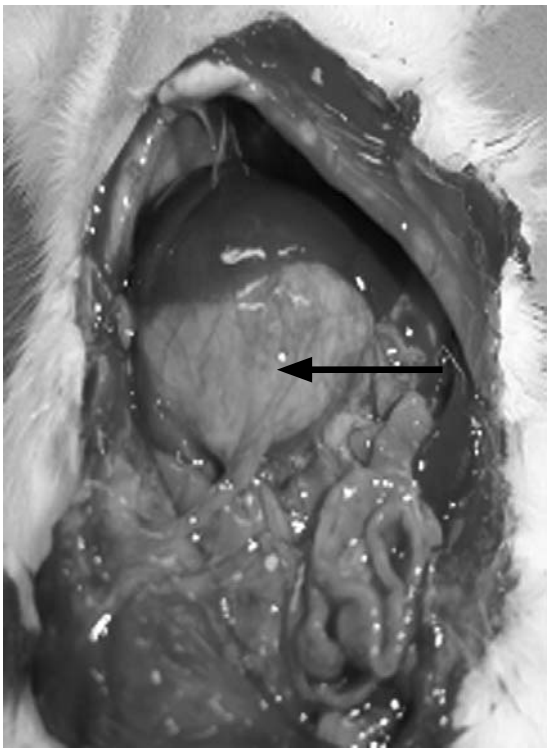


Figure 1a Intra-abdominal abscess covered with omentum after RFA treatment of one of two liver tumors in combination with celecoxib treatment (arrow).

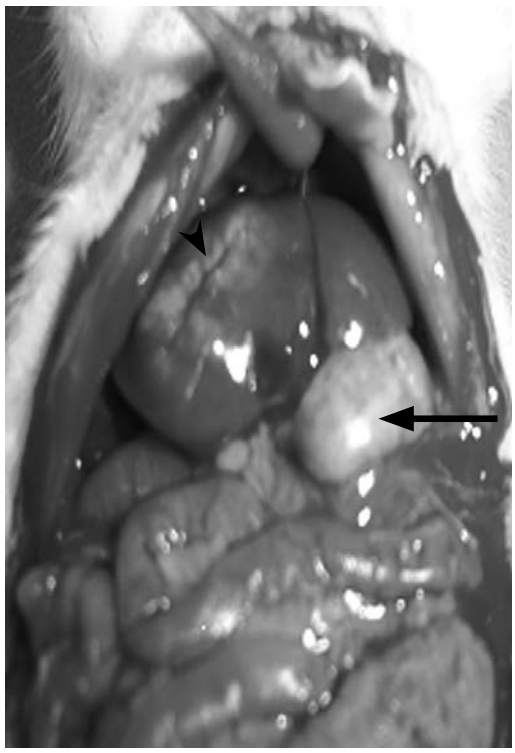


Figure 1b Rudimentary liver lobe after RFA treatment in a rat in the control group (group 2) (arrow), untreated tumor is visible in upper liver lobe (arrowhead).

Discussion

The use of COX-2 inhibitors in the treatment of inflammation and pain has increased in recent years as they are expected to avoid many of the complications associated with regular NSAIDs[8]. Furthermore, they show a potential benefit in prevention and treatment of various malignancies [9]. Clinical studies have employed the use of high dose COX-2 inhibitors in combination with other treatment modalities as COX-2 inhibition could potentially enhance the effectivity of such treatment, minimize side effects and decrease development of postoperative micrometastases [9]. However, recent studies showed that COX-2 inhibitors affect physiological processes at several levels, causing an increase in cardiovascular complications at high doses and affecting wound and anastomosis healing [4,10]. In this study treatment with celecoxib was associated with an increase in deaths after RFA treatment. Most notable in this study was the formation of cutaneous abscesses after surgery in all rats receiving celecoxib treatment and additional intra-abdominal abscess formation in rats that received celecoxib treatment in combination with RFA. RFA treatment has been associated with a complication rate of approximately 9%, including hepatic abscess formation[3]. The present study shows that the addition of celecoxib increases the incidence of complications following RFA treatment. Selective COX-2 inhibi-

tors have anti-inflammatory and anti-angiogenic properties [9]. Impairment of neovasculature of the post operative skin wounds and RFA lesions in combination with the suppression of an adequate immune response against opportunistic infections could be responsible for the side effects as seen in our study [4,9].

In summary, this study shows that use of high dose celecoxib in combination with surgery, and especially RFA of tumors, should be treated with great caution due to an increased risk of additional side effects.

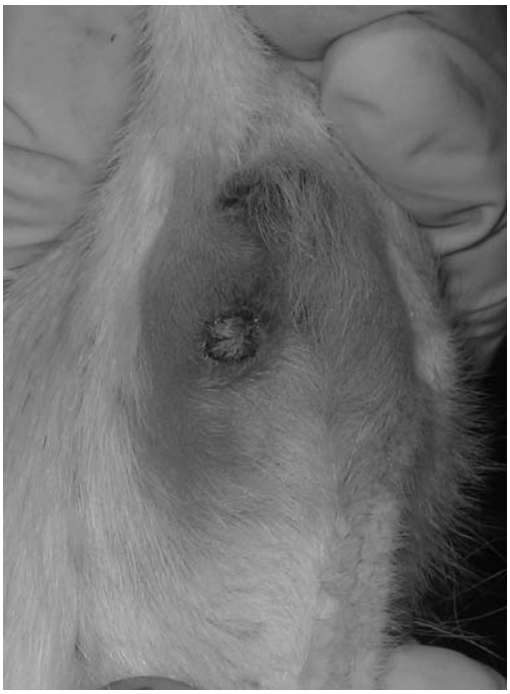


Figure 2 Presence of postoperative cutaneous abscesses in a rat that received celecoxib treatment.

References

1. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. Aspirin use and reduced risk of fatal colon cancer. N.Engl.J.Med., *325*: 1593-1596, 1991.
2. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N.Engl.J.Med., *342*: 1946-1952, 2000.
3. Jansen, M. C., van Duijnhoven, F. H., van Hillegersberg, R., Rijken, A., van Coevorden, F., van der, S. J., Prevoo, W., and van Gulik, T. M. Adverse effects of radiofrequency ablation of liver tumours in the Netherlands. Br.J.Surg., 2005.
4. Cahill, R. A., Sheehan, K. M., Scanlon, R. W., Murray, F. E., Kay, E. W., and Redmond, H. P. Effects of a selective cyclo-oxygenase 2 inhibitor on colonic anastomotic and skin wound integrity. Br.J.Surg., *91*: 1613-1618, 2004.
5. Hagenaars, M., Ensink, N. G., Basse, P. H., Hokland, M., Nannmark, U., Eggermont, A. M., van de Velde, C. J., Fleuren, G. J., and Kuppen, P. J. The microscopic anatomy of experimental rat CC531 colon tumour metastases: consequences for immunotherapy? Clin.Exp.Metastasis, *18*: 189-196, 2000.
6. van Duijnhoven, F. H., Tollenaar, R. A., Terpstra, O. T., and Kuppen, P. J. Locoregional Therapies of Liver Metastases in a Rat CC531 Coloncarcinoma Model Results in Increased Resistance to Tumour Rechallenge. Clin.Exp.Metastasis, *22*: 247-253, 2005.
7. Reddy, B. S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K., and Rao, C. V. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res., *60*: 293-297, 2000.
8. Silverstein, F. E., Faich, G., Goldstein, J. L., Simon, L. S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N. M., Stenson, W. F., Burr, A. M., Zhao, W. W., Kent, J. D., Lefkowith, J. B., Verburg, K. M., and Geis, G. S. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti- inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA, *284*: 1247-1255, 2000.
9. Gupta, R. A. and Dubois, R. N. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat.Rev.Cancer, *1*: 11-21, 2001.
10. Solomon, S. D., McMurray, J. J., Pfeffer, M. A., Wittes, J., Fowler, R., Finn, P., Anderson, W. F., Zauber, A., Hawk, E., and Bertagnolli, M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N.Engl.J.Med., *352*: 1071-1080, 2005.

CHAPTER 8

Summary and General Discussion

Published in part in Dutch Journal of Oncology. 2006 vol 3(1), 28-32

In the last decades major advances have been made in the treatment of colorectal cancer. The introduction of new surgical techniques and adjuvant and preoperative therapies has greatly improved clinical outcome of patients with colorectal cancer. Obviously, additional treatment to surgical resection may be of benefit for several patients, but will also lead to overtreatment in a large number of patients. A study by Moertel et al.[1] that evaluated the use of adjuvant 5-FU/LV for the treatment of colon cancer reported a survival benefit of 12% (49% in the treatment arm versus 37% in the control arm). Although these numbers clearly demonstrated the benefit of adjuvant 5-FU/LV, it still means that 37% of the patients will have no benefit from the chemotherapy. Comparable analyses have been conducted for rectal cancer; among which the Dutch TME trial, in which patients with resectable carcinoma of the rectum were randomized between TME surgery alone or preoperative radiotherapy (5x5Gy) followed by TME surgery. This study demonstrated a significant reduction of local recurrence rates from 11.4% to 5.8% by the addition of preoperative radiotherapy as compared to surgery alone[2]. This is an important improvement, but these numbers indicate that when 100 patients are treated 5.8% will still get a local recurrence despite the radiotherapy and 88.6% would not have had a local recurrence at all. Only 5.6% (11.4%-5.8%) of the patients will benefit from this additional treatment. So in order to prevent 1 local recurrence, 17 patients (94.4/5.6) are treated unnecessarily.

Chemotherapy and radiation therapy are associated with considerable morbidity. Several studies have evaluated the short- and long term morbidity of radiation therapy. Preoperative radiation therapy is associated with faecal incontinence, urgency and anal blood loss[3]. In addition to the general bowel dysfunction, an increase in venous thromboembolisms, pelvic fractures and sexual dysfunction have been reported[4,5]. The morbidity of adjuvant chemotherapy is equally considerable; general malaise, nausea, vomiting, diarrhea, temporary reduction in the production of blood cells by the bone marrow resulting in anemia, risk of bruising or bleeding and an increased risk of infection are common side effects[6]. These issues emphasize the need for finding predictive factors for tumor recurrence to exclude patients with a very high probability for cure with surgery alone from preoperative or adjuvant treatment. Equally, predictive factors are needed to distinguish patients who will respond to radiotherapy from those who will not.

A different situation is seen in colon cancers. At present, patients with a stage II colon cancer do not receive standard adjuvant treatment. Although a majority of patients will be cured by surgical resection alone, a significant minority (20%[7]) will ultimately relapse, suggesting the need to identify patients who may benefit from adjuvant therapy.

Currently, treatment allocation in colorectal cancer is based solely on tumor location and stage as these are so far the best prognostic factor at the time of surgery. However, tumor location is imprecise due to the distensible nature of the large bowel. Positive lymph nodes can easily be missed in routine HE- assessment In addition; patient outcome varies considerable within each stage. It is, therefore, of great importance to establish additional markers that are capable of predicting the clinical behavior of cancers of the large bowel to determine which treatment will optimally suit the patient. In the current thesis we have evaluated the use of tumor biological characteristics to predict clinical behavior in colorectal cancer as this may provide the possibility to adjust treatment, and accordingly provide patients with optimal therapeutic modalities. This will avoid both over treatment and unnecessary side effects in colorectal cancer patients.

Tumorigenesis is a multistep proces towards the progressive transformation of human cells into highly malignant derivatives. It has been suggested that the large number of cancer genotypes is a manifestation of several essential alterations in cell physiology that collectively determine malignant growth[8]. This includes, amongst others: self sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis and surviving environmental interactions, including the immune system. By evaluating several of these key processes in colorectal cancer, we aimed to predict the post treatment clinical behavior of tumors. We selected several markers with high potential for this purpose. As cell physiological processes on post-transcriptional level often determine clinical behavior, we focussed on biological marker at the level of protein expression and enzymatic activity in the tumor.

In **Chapter 2** we investigated expression levels of key regulators in cell motility FAK, Src, and paxillin in primary colorectal cancers and corresponding liver metastases. In this study, elevated expression of the individual markers FAK and paxillin expression did not significantly influence recurrence rates. Src expression had a significant impact on recurrence rates, and tumors showing elevated levels of both FAK and Src expression showed very unfavorable recurrence rates. Contrary to our expectations, the levels of expression of FAK and Src as well as paxillin in colon cancer were maintained in corresponding distant metastases. This indicates that colorectal liver metastases recapitulate the organization of their primary tumors and that the biological status of the primary tumor reflects its metastatic capacity.
The results from this study show that evaluating expression levels of markers that influence cell motility can be used to predict clinical behavior of tumors. This is supported by recent *in vitro* studies in which selective small molecular Src inhibitors and dominant negative deletion mutants of FAK act synergistically to promote colon tumor cell apoptosis[9]. Our study suggests that combined targeting of FAK and Src may be beneficial for the outcome of colon cancer and provide an opportunity for therapeutic intervention.

Apoptosis is at the convergence of several important pathways in the progression of colorectal cancer. In **Chapter 3 and 4** we investigated the prognostic relevance of apoptosis in colorectal cancer. Apoptosis in Chapter 3 was evaluated in 104 stage II and III colorectal cancer specimens by M30 immunostaining and in Chapter 4 by biochemical detection of caspase-3 activity in 117 stage III rectal cancer specimens from the Dutch TME trial and 47 preoperative biopsies with corresponding rectal cancer specimens. Colon and rectal cancer seem to have a different biologic behavior, at least with respect to apoptosis[10-12], and micro satellite instability[14]. In accordance with previous studies, a high apoptotic index was associated with an unfavourable prognosis in a mixed group of colorectal cancer. However, in a group of rectal cancers only, a high apoptotic index was associated with a favourable prognosis and low local recurrence rates. In addition Chapter 3 showed that the prognostic value of apoptosis is independent of MSI status of the tumor. The opposite effects of apoptosis on clinical behavior of tumors of the colon opposed to tumors of the rectum has been reported previously[16]. We hypothesize that the opposite impact of apoptosis on prognosis can be attributed to type of utilized surgical techniques. TME surgery versus blunt dissection of colon tumors and of rectal tumors pre-TME surgery era

results in differences in recurrence rates and clinical behavior and, therefore, could result from differences in induction of apoptosis. Further studies are required to investigate the biological mechanisms underlying the impact of apoptosis on clinical behaviour of colorectal cancer.
The clinical value of apoptosis detection was most apparent in rectal cancer; Chapter 4 showed that patients with high levels of apoptosis show very low local recurrence rates. The study demonstrated that by evaluating apoptosis in preoperative biopsies, rectal cancer patients could be selected in which preoperative radiotherapy is redundant.

Chapter 5 evaluated the prognostic value of COX-2 expression in 1530 irradiated and non-irradiated tumor specimens obtained from the Dutch TME trial, to investigate whether there is a clinical rationale for the use of COX-2 inhibitors in rectal cancer. The study showed that COX-2 expression did not have an impact on survival or local recurrence rates in non-irradiated rectal cancers. However, preoperative radiotherapy was shown to change COX-2 expression and high COX-2 expression after irradiation was associated with apoptosis resistance and high distant recurrence rates. As COX-2 expression and subsequent prostaglandin production can be reduced by COX-2 inhibitors[17] these data suggest that the addition of COX-2 inhibitors to preoperative radiotherapy may improve patient prognosis in rectal cancer.
Having shown a clinical rationale for COX-2 inhibitors to reduce distant recurrences in rectal cancer, we set out to investigate the effectiveness of COX-2 inhibitors to treat distant recurrences by the use of the CC531 rat colorectal liver metastases model. In **Chapter 6** Wag/Rij rates were inoculated with two liver tumors by subcapsular injection of CC531 tumor cells and were treated with various doses of celecoxib, a selective COX-2 inhibitor starting one week before, or directly after inoculation. Control rats were inoculated without treatment. The COX-2 inhibitor reduced tumor growth in a dose dependent manner. Doses sufficient to reduce CC531 growth *in vivo*, did not affect growth or induce apoptosis in CC531 cells *in vitro*. These results show that COX-2 inhibitors could be used to reduce growth of liver metastases of colorectal cancers. Secondly, these results suggest that COX-2 inhibition does not work by direct cytotoxicity against tumor cells, but by creating an unfavourable environment for tumor growth.
In **Chapter 7** treatment of liver metastases with celecoxib was combined with Radiofrequency Ablation (RFA), a surgical technique that is widely used in a clinical setting. In this study the safety of combining the two treatment modalities was evaluated. Rats were randomized for treatment with celecoxib, RFA, or the combination thereof. Celecoxib treatment was started at the time of tumor induction. At 3 weeks after tumor inoculation, the liver tumors were treated with RFA and the effects on rat health were monitored. Treatment that included celecoxib resulted in significantly increased cutaneous wound abscess formation after surgery. In addition, the combination of celecoxib treatment with RFA resulted in intra-abdominal abscess formation. This study indicates that the use of celecoxib in combination with RFA of liver metastases should be treated with caution when applied as anti-cancer treatment modality as additional side effects are induced.
Since the 1990s, the pharmaceutical industry has been developing agents that specifically inhibit the COX-2 enzyme without affecting COX-1 activity. The selective targeting of COX-2 by these agents has increased the gastro-intestinal safety[18,19] as compared to nonselective NSAIDs. However, the selective COX-2 inhibitors have been subject to scrutiny after an increase in the

risk of thrombotic events was demonstrated in long-term, high dose use of this medication in two large intestinal polyp prevention trials[20,21]. In addition, a recent study reported that an increase in risk of myocardial infarction is apparent with short-term, low dose use of the COX-2 inhibitor rofecoxib, but not with celecoxib[22]. The safety concerns of rofecoxib were so high that the drug was immediately removed from the market and numerous trials evaluating the effectivity of COX-2 inhibitors in the treatment of colorectal cancer were terminated. The ACTION trial, mentioned in the introduction of this thesis, was among the trials that were terminated, although the investigators felt that the potential benefits of adjuvant celecoxib treatment outweighed the increase in cardiovascular events with long-term celecoxib use. First, it should be underlined that the increase in cardiovascular side effects is unacceptable in chemopreventive strategies for healthy individuals, which is in sharp contrast to the chemotherapeutic setting in patients with cancer. It must be taken into consideration that patients with stage III colon carcinoma have a 30% chance of disease recurrence in 3 years[23] making an increase in cardiovascular events of 2.4%, as was reported with celecoxib[24] use, acceptable in the adjuvant setting. Furthermore, the ACTION study excluded all patients with a history of cardiovascular disease, providing a patient selection that will optimally benefit from this treatment option. In addition, the well-known substantial cardiovascular risk of 5-FU is considered as acceptable, even in adjuvant setting. Also with bevacizumab/ avastin, a drug with considerable cardiovascular side effects, several trials for stage III CRC have recently been initiated. Recent reports suggest that the use of celecoxib at the relatively low doses of 200 or 400 mg daily seem to be fairly well tolerated and the results from a large polyp prevention trial using celecoxib apparently did not reveal any difference in thrombotic events between the placebo and 400mg per day treated group[25]. Recently published increased insights in the working mechanisms of COX-2[26,27] have shifted the attention from cardiovascular side effects and the following landslide of litigation, to the potential of COX-2 inhibitors as an addition to the treatment of colorectal cancer. Studies from the current thesis indicate a beneficial role for COX-2 inhibitors in the treatment of rectal cancer and liver metastases. We feel an intervention study adding COX-2 inhibitors to preoperative radiotherapy is desired in light of our findings (Chapter 5), but with caution as the results from our chapter 7 indicate that the addition of COX-2 inhibitors to surgical techniques can result in an increase in unwanted side effects.

Concluding remarks

From identifying prognostic biological markers to integrating such markers into the clinical practice is obviously a long way. A PubMed search using the search terms: "Biological marker AND colorectal cancer AND prognosis" came up with 1977 hits (March 2007). The clinical practice teaches us that exactly none of them are used on a daily basis in colorectal cancer to determine patient prognosis or response to therapy. The disappointing performance of markers that were initially shown to have a strong association with outcome may be in part because a marker that is strongly associated with outcome may not be effective for predicting those who are likely and those who are not likely to have the outcome[28]. In order to determine the classification ability of markers that have shown to be associated with clinical outcome, the issue of sensitivity and specificity needs to be addressed.

Figure 1

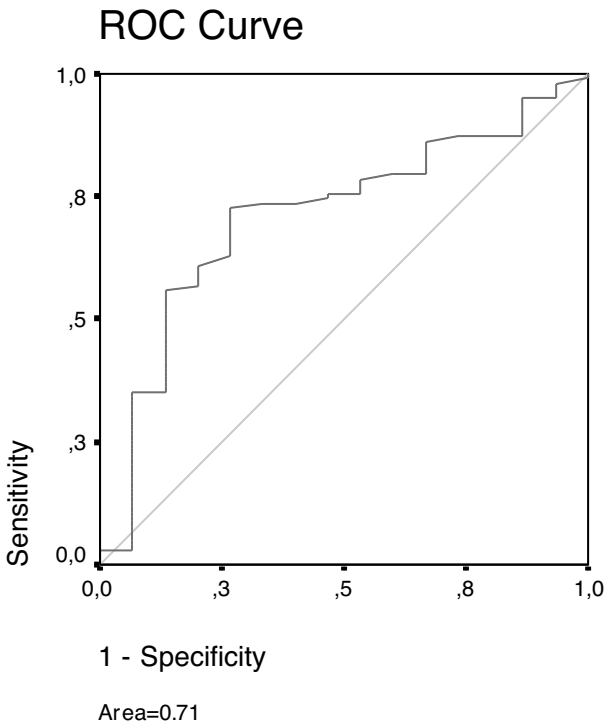


Figure 1 Receiver Operating Characteristic (ROC) curve of caspase-3 activity with regard to local recurrence rates. The area under the curve is 0.71.

The most powerful prognostic marker described in this thesis was caspase-3 activity in rectal cancer. Patients with a tumor with a low caspase-3 activity had a hazard ratio of 7.5 with regard to the risk for local recurrences (**Chapter 4**). We assessed whether determination of caspase-3 activity can be used as a clinical test. The accuracy of a test depends on how well the test separates the group being tested into those with and without the events in question. The accuracy of a test is measured by the area under the Receiver Operating Characteristic (ROC) curve. When evaluated as continuous variable in an ROC curve (figure 1) the predictive value of caspase-3 activity is not impressive. The area under the curve was 0.71, which denotes a "fair" accuracy of the test (in the medical practice a test is usually accepted if the area under the curve is higher than 0.80). However, the ROC curve includes sensitivity and specificity, so we determined both (table 1); Determination of low caspase-3 activity (defined as activity below the median) showed to be a test with a low sensitivity (0.22), but with a very high specificity (0.97). This means that determination of caspase-3 is not a good test to predict whether patients will have a high risk of local recurrence, but a very good test to identify patients with a low risk of local recurrence. In order to exclude patients from treatment, which was the case with determination of caspase-3 activity, a very high specificity is desirable.

Table 1

	Event (Positive)	No event (Negative)	Total
Low capase-3 activity (positive)	13	45	58
High capase-3 activity (negative)	2	57	59

Sensitivity: $\frac{\text{positive}}{\text{total}} = \frac{13}{58} = 0.22$ Specificity: $\frac{\text{negative}}{\text{total}} = \frac{57}{59} = 0.97$

Table 1 Sensitivity and specificity of caspase-3 activity with regard to local recurrence rates. Determination of low caspase-3 activity is not a sensitive test to predict local recurrences (0.22), but a highly specific test to identify patients with a low risk of local recurrence (0.97).

Previous studies have noted that a marker with a hazard ratio of as high as 3 will be a poor predictor of risk[29]. The focus of future studies should, therefore, not only be on determination of individual biological markers, but also on the integration of various individual markers with clinical and radiological parameters to ultimately construct a prognostic scoring system that incorporates clinical, clinicopathological and biological tumor features.

The advantages of assessing risk factors for local recurrence and poor survival by preoperative imaging are increasingly recognised. Relevant predictive factors for survival and local recurrences after rectal cancer surgery that can be accurately predicted with modern imaging techniques are tumor height (distance of the tumor from the anus), tumor stage, involved or threatened circumferential resection margin (CRM), and nodal disease. The advantage of an intrinsic high soft tissue contrast resolution combined with new technical developments (faster acquisitions, dedicated external coils, contrast agents etc.) has made magnetic resonance imaging (MRI) the most promising technique for local staging of rectal cancer[30-33]. The results of a systematic review of all published single center study data so far clearly shows that MRI performs very well in predicting the CRM in rectal cancer surgery[34]. Harisinghani et al. studied USPIO MRI in patients with resectable prostate cancer and the node-by-node analysis in 334 lymph nodes showed a sensitivity of 91 % for USPIO MRI[35,36].

Combination of prognostic biological markers with, for instance, preoperative radiological assessment will improve the sensitivity and specificity to a level by which clinical decisions can be based on the prognostic profile of the patient. In order to be able to implement this model into the clinical practice, addional prospective studies will have to be conducted in the future.

Future directions

To preoperatively determine the biological profile of tumors, biopsies will have to be evaluated. As these are taken routinely in a clinical workup they are ideal for routine assessment. In chapter 4 of the current thesis we have shown the feasibility of this approach. A next step in determining a prognostic profile based on biological and radiological markers should be the validation of the results in an independent study. Several studies in the current thesis were based on tumor material from the Dutch TME trial. Recently, a similar large international rectal cancer trial randomizing between TME surgery, short course preoperative radiotherapy followed by TME surgery versus selective long course post-operative radiotherapy, completed pa-

tient inclusion. This study, the MRC CR07, collected tumor material for translational research and included quality-controlled pre-operative imaging. Once sufficient follow-up data from this trial are available, it would provide the ideal data set to validate the results of the current thesis. The third and last step in implementing our findings in the clinical practice should be the prognostic evaluation of the clinical significance of biological and radiological markers. The departments of Radiology and Surgery of the University of Maastricht are currently conducting the MRI-RectUM study, a prognostic study in which patients will be allocated to short term pre-operative radiotherapy, long term chemoradiation or no preoperative therapy, based on their profile as determined by radiological imaging. The study will use the TME study as control arm. Implementation of tumor biological parameters in this trial is feasible and is currently in preparation, as tumor material will be collected throughout the study.

The current thesis has provided insight in the value of biological markers in colorectal cancer, and for some markers in particular, in determining patient prognosis and in selecting patients for preoperative therapy. More knowledge of biological mechanisms that control clinical tumor behavior is necessary to develop a model in which biological markers can be combined with sensitive clinical techniques like radiological imaging to accurately predict clinical behavior of tumors. We feel that this approach will lead to the treatment of the future for colorectal cancer patients: tailor-made treatment.

References

1. Moertel, C. G., Fleming, T. R., Macdonald, J. S., Haller, D. G., Laurie, J. A., Goodman, P. J., Ungerleider, J. S., Emerson, W. A., Tormey, D. C., Glick, J. H., and . Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N.Engl.J.Med., *322*: 352-358, 1990.
2. Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., Leer, J. W., and van de Velde, C. J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N.Engl.J.Med., *345*: 638-646, 2001.
3. Peeters, K. C., van de Velde, C. J., Leer, J. W., Martijn, H., Junggeburt, J. M., Kranenbarg, E. K., Steup, W. H., Wiggers, T., Rutten, H. J., and Marijnen, C. A. Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients--a Dutch colorectal cancer group study. J.Clin.Oncol., *23*: 6199-6206, 2005.
4. Holm, T., Singnomklao, T., Rutqvist, L. E., and Cedermark, B. Adjuvant preoperative radiotherapy in patients with rectal carcinoma. Adverse effects during long term follow-up of two randomized trials. Cancer, *78*: 968-976, 1996.
5. Marijnen, C. A., Kapiteijn, E., van de Velde, C. J., Martijn, H., Steup, W. H., Wiggers, T., Kranenbarg, E. K., and Leer, J. W. Acute side effects and complications after short-term preoperative radiotherapy combined with total mesorectal excision in primary rectal cancer: report of a multicenter randomized trial. J.Clin.Oncol., *20*: 817-825, 2002.
6. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
7. Zaniboni, A. and Labianca, R. Adjuvant therapy for stage II colon cancer: an elephant in the living room? Ann.Oncol., *15*: 1310-1318, 2004.
8. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100*: 57-70, 2000.
9. van Nimwegen, M. J., Verkoeijen, S., van Buren, L., Burg, D., van de Water, B. Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. Cancer Res., *65*: 4698-4706, 2005.
10. Hilska, M., Collan, Y. U., VJ, O. L., Kossi, J., Hirsimaki, P., Laato, M., and Roberts, P. J. The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. Dis. Colon Rectum, *48*: 2197-2208, 2005.
11. Jonges, L. E., Nagelkerke, J. F., Ensink, N. G., van der Velde, E. A., Tollenaar, R. A., Fleuren, G. J., van de Velde, C. J., Morreau, H., and Kuppen, P. J. Caspase-3 activity as a prognostic factor in colorectal carcinoma. Lab Invest, *81*: 681-688, 2001.
12. Marijnen, C. A., Nagtegaal, I. D., Mulder-Stapel, A. A., Schrier, P. I., van de Velde, C. J., van Krieken, J. H., and Peltenburg, L. T. High intrinsic apoptosis, but not radiation-induced apoptosis, predicts better survival in rectal carcinoma patients. Int.J.Radiat.Oncol.Biol.Phys., *57*: 434-443, 2003.
13. Hilska, M., Collan, Y. U., VJ, O. L., Kossi, J., Hirsimaki, P., Laato, M., and Roberts, P. J. The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. Dis. Colon Rectum, *48*: 2197-2208, 2005.
14. Soreide, K., Janssen, E. A., Soiland, H., Korner, H., and Baak, J. P. Microsatellite instability in colorectal cancer. Br.J.Surg., *93*: 395-406, 2006.
15. Kapiteijn, E., Liefers, G. J., Los, L. C., Kranenbarg, E. K., Hermans, J., Tollenaar, R. A., Moriya, Y., van de Velde, C. J., and van Krieken, J. H. Mechanisms of oncogenesis in colon versus rectal cancer. J.Pathol., *195*: 171-178, 2001.

16. Hilska, M., Collan, Y. U., VJ, O. L., Kossi, J., Hirsimaki, P., Laato, M., and Roberts, P. J. The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. Dis. Colon Rectum, *48*: 2197-2208, 2005.
17. Tuynman, J. B., Buskens, C. J., Kemper, K., ten Kate, F. J., Offerhaus, G. J., Richel, D. J., and van Lanschot, J. J. Neoadjuvant selective COX-2 inhibition down-regulates important oncogenic pathways in patients with esophageal adenocarcinoma. Ann.Surg., *242*: 840-9, discussion, 2005.
18. Silverstein, F. E., Faich, G., Goldstein, J. L., Simon, L. S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N. M., Stenson, W. F., Burr, A. M., Zhao, W. W., Kent, J. D., Lefkowith, J. B., Verburg, K. M., and Geis, G. S. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA, *284*: 1247-1255, 2000.
19. Bombardier, C., Laine, L., Reicin, A., Shapiro, D., Burgos-Vargas, R., Davis, B., Day, R., Ferraz, M. B., Hawkey, C. J., Hochberg, M. C., Kvien, T. K., and Schnitzer, T. J. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. N.Engl.J.Med., *343*: 1520-8, 2, 2000.
20. Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanas, A., Konstam, M. A., and Baron, J. A. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. N.Engl.J.Med., *352*: 1092-1102, 2005.
21. Solomon, S. D., McMurray, J. J., Pfeffer, M. A., Wittes, J., Fowler, R., Finn, P., Anderson, W. F., Zauber, A., Hawk, E., and Bertagnolli, M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N.Engl.J.Med., *352*: 1071-1080, 2005.
22. Levesque, L. E., Brophy, J. M., and Zhang, B. Time variations in the risk of myocardial infarction among elderly users of COX-2 inhibitors. CMAJ., *174*: 1563-1569, 2006.
23. Labianca, R., Beretta, G., Gatta, G., de Braud, F., and Wils, J. Colon cancer. Crit Rev.Oncol.Hematol., *51*: 145-170, 2004.
24. Solomon, S. D., McMurray, J. J., Pfeffer, M. A., Wittes, J., Fowler, R., Finn, P., Anderson, W. F., Zauber, A., Hawk, E., and Bertagnolli, M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N.Engl.J.Med., *352*: 1071-1080, 2005.
25. DuBois, R. N. The COX-2 story: is any drug completely "safe?". Gastroenterology, *130*: 6, 2006.
26. Castellone, M. D., Teramoto, H., Williams, B. O., Druey, K. M., and Gutkind, J. S. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science, *310*: 1504-1510, 2005.
27. Clevers, H. Colon cancer--understanding how NSAIDs work. N.Engl.J.Med., *354*: 761-763, 2006.
28. Alonzo, T. A. Standards for reporting prognostic tumor marker studies. J.Clin.Oncol., *23*: 9053-9054, 2005.
29. Pepe, M. S., Janes, H., Longton, G., Leisenring, W., and Newcomb, P. Limitations of the odds ratio in gauging the performance of a diagnostic, prognostic, or screening marker. Am.J.Epidemiol., *159*: 882-890, 2004.
30. Brown, G., Radcliffe, A. G., Newcombe, R. G., Dallimore, N. S., Bourne, M. W., and Williams, G. T. Preoperative assessment of prognostic factors in rectal cancer using high-resolution magnetic resonance imaging. Br.J.Surg., *90*: 355-364, 2003.
31. Bissett, I. P., Fernando, C. C., Hough, D. M., Cowan, B. R., Chau, K. Y., Young, A. A., Parry, B. R., and Hill, G. L. Identification of the fascia propria by magnetic resonance imaging and its relevance to preoperative assessment of rectal cancer. Dis.Colon Rectum, *44*: 259-265, 2001.
32. Beets-Tan, R. G., Beets, G. L., Vliegen, R. F., Kessels, A. G., Van Boven, H., De Bruine, A., von Meyenfeldt, M. F., Baeten, C. G., and van Engelshoven, J. M. Accuracy of magnetic resonance

imaging in prediction of tumour-free resection margin in rectal cancer surgery. Lancet, *357*: 497-504, 2001.

33. Beets-Tan, R. G. and Beets, G. L. Rectal cancer: review with emphasis on MR imaging. Radiology, *232*: 335-346, 2004.
34. Lahaye, M. J., Engelen, S. M., Nelemans, P. J., Beets, G. L., van de Velde, C. J., van Engelshoven, J. M., and Beets-Tan, R. G. Imaging for predicting the risk factors--the circumferential resection margin and nodal disease--of local recurrence in rectal cancer: a meta-analysis. Semin. Ultrasound CT MR, *26*: 259-268, 2005.
35. Saksena, M. A., Saokar, A., and Harisinghani, M. G. Lymphotropic nanoparticle enhanced MR imaging (LNMRI) technique for lymph node imaging. Eur.J.Radiol., 2006.
36. Rockall, A. G., Sohaib, S. A., Harisinghani, M. G., Babar, S. A., Singh, N., Jeyarajah, A. R., Oram, D. H., Jacobs, I. J., Shepherd, J. H., and Reznek, R. H. Diagnostic performance of nanoparticle-enhanced magnetic resonance imaging in the diagnosis of lymph node metastases in patients with endometrial and cervical cancer. J.Clin.Oncol., *23*: 2813-2821, 2005.

Nederlandse samenvatting

Het carcinoom van de dikke darm en endeldarm (colorectale carcinoom) is een veel voorkomende doodsoorzaak in westerse landen. In Nederland wordt de diagnose jaarlijks bij 8600 mensen gesteld en overlijden er 4400 mensen aan de gevolgen van de ziekte.
Chirurgische verwijdering van de tumor met bijbehorende lymfeklieren en eventuele levermetastasen is de belangrijkste pijler van de behandeling. De behandeling van het colorectale carcinoom heeft de laatste decennia sterke ontwikkelingen doorgemaakt. De introductie van nieuwe chirurgische technieken (ondermeer de Totale Mesorectale Excisie (TME) bij rectumturmoren), preoperatieve radiotherapie en adjuvante chemotherapie hebben gezorgd voor een grote verbetering in overleving. Echter, pre- en post-operatieve therapieën bij chirurgische resectie zijn werkzaam bij sommige, maar zal tot overbehandeling leiden bij de overgrote meerderheid van patiënten. Bovendien kunnen therapieën als radiotherapie en chemotherapie tot aanzienlijke bijwerkingen leiden. Preoperatieve radiotherapie, zoals die gegeven wordt bij het rectum carcinoom, kan leiden tot incontinentie, continue aandrang, anale bloedverlies en impotentie. Adjuvante chemotherapie kan ondermeer leiden tot misselijkheid, braken, diarree en tijdelijke afname van de productie van bloedcellen door het beenmerg. Deze bijwerkingen benadrukken het belang van het vinden van factoren die klinisch gedrag van tumor en patiënt kunnen voorspellen en die gebruikt kunnen worden in een beslismodel om patiënten te selecteren die baat hebben bij aanvullende therapieën bij chirurgie.
Op het moment wordt er op basis van tumorstadiëring en locatie van de tumor in de darm beslist welke operatie een patiënt zal ondergaan en welke adjuvante therapie de patiënt krijgt toegewezen. Echter, vanwege de rekbaarheid van de darm, is de bepaling van tumorlocatie onnauwkeurig. Lymfeklieren worden na resectie van de tumor door een patholoog beoordeeld op de aanwezigheid van tumorcellen. Deze kunnen echter bij de beoordeling gemakkelijk worden gemist. Hierdoor lopen patiënten met hoog-stadium-tumoren, die onterecht als laag-stadium worden aangemerkt, kans niet adequaat behandeld te worden.
Dit proefschrift richt zich op het onderzoek naar biologische eigenschappen van de tumor om klinisch gedrag te kunnen voorspellen. Het voorspellen van klinisch gedrag verschaft de mogelijkheid een op de patiënt toegespitste behandeling te kunnen bieden waardoor onderbehandeling enerzijds en onnodige bijwerkingen door overbehandeling anderzijds kunnen worden vermeden.

Het ontstaan van een colorectale carcinoom is een stapsgewijs proces van maligne ontaarding van gezonde cellen tot carcinoom. Recent is gesuggereerd dat de grote hoeveelheid genetische veranderingen die betrokken zijn bij het ontstaan van kanker uitingen zijn van enkele essentiele processen. Deze processen zijn onder meer: autonomie van groeisignalen, ongevoeligheid voor groeiremmende signalen, ongevoeligheid voor apoptose (geprogrammeerde celdood), aanhoudende vaatnieuwvormingen, invasieve groei en ontduiking van beschermende afweercellen.

Door het evalueren van enkele van deze belangrijke processen in maligne ontaarding van darm epitheel cellen proberen wij het klinische beloop van een patiënt na resectie van de primaire tumor te voorspellen. Hiertoe hebben wij enkele markers geselecteerd met een grote potentiële voorspellende waarde.

In **hoofdstuk 2** hebben wij de expressie van enkele belangrijke regulators van motiliteit van tumorcellen onderzocht in primaire tumoren en gepaarde levermetastasen: FAK, Src en paxillin. Deze zogenaamde receptorkinases zijn betrokken bij signaaltransductie van de extracellulaire matrix naar het intracellulaire cytoskelet. De expressie van deze markers verschilt van tumor tot tumor wat een verschil in functie en activiteit per tumor suggereert. In dit hoofdstuk beschrijven wij dat de toegenomen expressie van FAK en paxillin in tumoren geen invloed heeft op de prognose van patiënten. Echter patiënten met hoge expressie van Src en een combinatie van FAK en Src hadden een zeer hoge kans op recidief van de tumor. Er was geen toename van FAK, Src of paxillin expressie in levermetastasen vergeleken met de primaire tumor. De resultaten van deze studie laten zien dat het evalueren van markers die celmotiliteit beïnvloeden, kunnen worden gebruikt om klinisch gedrag te voorspellen. Onze resultaten suggereren dat gecombineerde aanpak van FAK en Src een gunstig effect zou kunnen hebben in de behandeling van het colorectale carcinoom. Dit wordt ondersteund door een recente studie in cellijnen waarbij remmers van Src en FAK synergistisch werken om apoptose in cellijnen te induceren.

In hoofdstuk 3 en 4 hebben wij de prognostische waarde van apoptose in colorectale tumoren onderzocht. Apoptose in **hoofdstuk 3** werd onderzocht in 104 stadium II en III patiënten door middel van een immunohistochemische kleuring met M30 antilichamen. In **hoofdstuk 4** werd apoptose bepaald door biochemische detectie van caspase-3 (een pre-apoptotisch enzym) in 117 stadium III patiënten uit de Nederlandse TME studie en 47 preoperatieve biopsieën en de primaire tumor van gepaarde onbestraalde patiënten. De aanwezigheid van een hoog aantal apoptotische cellen in colorectale tumoren was geassocieerd met een slechte overleving van de patiënt. Echter in rectumtumoren uit de TME studie was veel apoptose juist geassocieerd met een laag lokaal recidief percentage en een goede overleving. Deze schijnbaar tegenstrijdige bevindingen zijn waarschijnlijk te verklaren door verschillen in operatiemethoden die in de patiëntgroepen in de twee hoofdstukken zijn gehanteerd. Patiënten in hoofdstuk 3 kregen een stompe dissectie voor hun rectumcarcinoom dat geassocieerd is met hoge lokaal recidief percentages, maar waarbij de operatie minder lang en ingrijpend is. Patiënten uit hoofdstuk 4 waren geopereerd door middel van TME-chirurgie, wat een ander recidiefgedrag heeft, maar waarbij meer apoptose wordt geïnduceerd. Wij hypothetiseren dat de verschillen in de prognostische betekenis van apoptose in de twee studies worden veroorzaakt door verschillen in operatiemethoden en daaruit voorkomende verschillen in inductie van celdood en recidiefgedrag van de tumor. De klinische waarde van bepaling van apoptose was het meest duidelijk in hoofdstuk 4. Deze studie liet zien dat patiënten kunnen worden geselecteerd voor preoperatieve radiotherapie door middel van bepaling van caspase-3 activiteit in preoperatieve biopten.

Hoofdstuk 5 evalueert de prognostische waarde van een enzym dat betrokken is bij de prostaglandineproductie en maligne ontaarding van epitheliale cellen: COX-2. Wij onderzochten

1530 bestraalde en onbestraalde rectumtumoren op COX-2 expressie door middel van een immunohistochemische kleuring op tissue micro arrays. Om de effecten van radiotherapie op COX-2 expressie te beoordelen, evalueerden wij COX-2 expressie in 20 preoperatieve biopten en corresponderende bestraalde tumoren. COX-2 expressie had geen effect op overleving in onbestraalde patiënten. Wij zagen echter dat radiotherapie effect had of COX-2 expressie: na bestraling nam de COX-2 expressie gemiddeld toe. Hoge COX-2 expressie na bestraling bleek geassocieerd te zijn met resistentie tegen radiotherapiegeïnduceerde apoptose, met een toegenomen kans op afstandsmetastasen, slechtere overleving, maar niet met een lokaal recidief. Het gebruik van COX-2 remmers kan COX-2 expressie en daaropvolgende prostaglandineproductie in tumoren verlagen. Onze resultaten suggereren dat de toevoeging van COX-2 remmers aan de behandeling van het rectumcarcinoom de kans op afstandsmetastasen zou kunnen verkleinen en de overleving van deze patiënten verlengen.

Gezien de aanwijzingen voor het gebruik van COX-2 remmers om afstandsmetastasen te voorkomen, hebben wij in **hoofdstuk 6** onderzocht of metastasen met COX-2 remmers effectief kunnen worden behandeld in een dierexperiment. Hierbij hebben wij gebruik gemaakt van een diermodel waarbij wij metastasen in levers van ratten induceerden door middel van injectie van ratcolontumorcellen. Ratten werden behandeld met oplopende doseringen celecoxib, een selectieve COX-2 remmer, in hun voer. Als controle kreeg een groep ratten voer zonder medicatie. Groei van de metastasen werd geremd op een dosis- afhankelijke wijze, waarbij het niet uitmaakte of celecoxib vóór, of na injectie van de tumor werd gegeven. De doseringen die leidden tot reductie in tumorgroei *in vivo* waren niet voldoende om reductie van groei in *vitro* te induceren. Deze resultaten laten zien dat COX-2 remmers kunnen worden gebruikt om de groei van levermetastasen van colorectale tumoren te remmen. Bovendien werken COX-2 remmers niet door directe cytotoxiciteit tegen tumorcellen, maar eerder door het creëren van een ongunstige omgeving voor tumorcellen om in te groeien.

In **hoofdstuk 7** van dit proefschrift is het gebruik van COX-2 remmers in de behandeling van levermetastasen, gecombineerd met radio frequente ablatie (RFA). RFA wordt op grote schaal gebruikt in de kliniek bij het behandelen van levermetastasen. In dit experiment werd de veiligheid van het combineren van de twee behandelingsmethoden onderzocht. Levermetastasen werden geïnduceerd door middel van injectie van ratcolontumorcellen, waarna direct werd gestart met celecoxib behandeling. Na 3 weken werden ratten geopereerd en behandeld met RFA, vervolgens werden de effecten op de gezondheid van de ratten geëvalueerd. Behandeling met celecoxib leidde tot wondabcessen in behandelde ratten. De combinatie van celecoxib en RFA leidde tot vorming van intra-abdominale abcessen en sterfte in ratten. Deze studie laat zien dat het gebruik van celecoxib in combinatie met RFA leidt tot ongewenste bijeffecten en met voorzichtigheid moet worden gebruikt in de behandeling van levermetastasen van colorectale tumoren.

De uitkomsten van de verschillende experimenten en onderzoeken worden samen besproken in **hoofdstuk 8**. De resultaten van onze studies laten zien dat onderzoek naar biologische eigenschappen van de tumor om klinisch gedrag te kunnen voorspellen haalbaar is en relevante

gegevens voor de behandeling van colorectale tumoren oplevert. Het voorspellen van klinisch gedrag verschaft de mogelijkheid een op de patiënt toegespitste behandeling te kunnen bieden. Toekomstig onderzoek zal zich richten op het uitbreiden van biologische markers en het valideren van onze resultaten in onafhankelijke series. De combinatie van detectie van biologische markers met bestaande en experimentele klinische parameters zoals preoperatieve bepaling van lymfekliermetastasen en bepaling van tumorlocatie door MRI om klinisch gedrag van tumoren te voorspellen, zal de negatief- en positief voorspellende waarde van deze bepalingen sterk doen toenemen. De laatste stap in het implementeren van onze bevindingen in de klinische praktijk zal vervolgens prospectieve evaluatie van biologische markers moeten zijn.
De resultaten van dit proefschrift wijzen erop dat de bepaling van tumor biologische markers een waardevolle bijdrage kan leveren aan het voorspellen van het klinische beloop van colorectale tumoren en aan het voorspellen van respons op behandeling. De combinatie van tumorbiologische markers met technieken zoals preoperatieve radiologische beeldvorming, verschaft de behandelende arts een hulpmiddel wat zal leiden tot de therapie van de toekomst voor colorectale kankerpatiënten: een op maat gemaakte therapie.

List of abbreviations

AA	Arachidonic Acid
APC	Adenomatous Polyposis Coli
CI	Confidence Interval
CIN	Chromosomal Instability
COX	Cyclooxygenase
DCC	Deleted in colorectal cancer
DFS	Disease Free Survival
FAK	Focal Adhesion Kinase
FAP	Familial Adenomatous Polyposis
HR	Hazard Ratio
MIN	Microsatellite Instability
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
MSI	Micro Satellite Instability
NK cells	Natural Killer cells
OS	Overall Survival
PGE2	Prostaglandin E2
RFA	Radiofrequency Ablation
SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
TCR	T cell receptor
TH cells	T helper cells
TME	Total Mesorectal Excision
5-FU/LV	5-Fluorouracil/Leukovorin
TNF	Tumor Necrosis Factor
TNM	Tumor Node Metastasis
TTR	Time to Recurrence
USPIO	Ultrasmall particles of iron oxide

List of publications

Quality of life in adults following bone marrow transplantation during childhood. *Bone Marrow Transplant 2004 Feb; 33(3):329-36*: Helder DI, Bakker B, de Heer P, van der Veen F, Vossen JM, Wit JM, Kaptein AA

Aspirine en COX-2 remmers in de oncologie. *Vorderingen en Praktijk; cursusboek Boerhaave Cursus 2003 Dec, 78-85*: de Heer P, CJH van de Velde

ACTION-2/PETACC-5 studie: Pan-Europese fase III studie naar het adjuvante gebruik van de selectieve Cyclooxygenase-2 remmer Celecoxib (Celebrex®) met chemotherapie bij patiënten met een curatief gereseceerd stadium III colon carcinoom. *Nederlands Tijdschrift voor de Oncologie 2004 Dec; 1(6), 243-245:* de Heer P, CJH van de Velde

Cutaneous and intra-abdominal abscess formation in rats following radio frequently ablation of liver tumors in combination with celecoxib treatment. *In Vivo 2006 May-Jun;20(3):373-5:* de Heer P, Sandel MH, Speetjens FM, Koudijs MM, Putter H, Ensink GN, Van de Velde CJ, Kuppen PJ

Onderzoek naar selectieve COX-2 remmers in de oncologie: stilte na de storm. *Nederlands Tijdschrift voor Oncologie 2006 vol 3(1), 28-32:* de Heer P, Van de Velde CJ, Kuppen PJ

COX-2 expression in rectal cancer is of prognostic significance in patients receiving preoperative radiotherapy. *Clinical Cancer Research 2007 May 15;13(10):2955-60:* de Heer P, Gosens MJEM, de Bruin EC, Dekker-Ensink NG, Putter H, Marijnen CM, van den Brule AJC, van Krieken JHJM, Rutten HJ, Kuppen PJK, van de Velde CJH

Apoptosis is a poor prognostic factor in colorectal cancer. *Submitted for publication* de Heer P, Speetjens FM, Ensink NG, Aalbers, RIJM, Asselbergs CPE, Putter H, Tollenaar RAEM, Morreau H, van de Velde CJH, Kuppen PJK

Celecoxib inhibits growth of tumors in a syngeneic rat liver metastases model for colorectal cancer. *Submitted for publication:* de Heer P, Sandel MH, Guertens G, de Boeck G, Koudijs MM, Nagelkerke JF, Junggeburt JMC, de Bruijn EA, van de Velde CJH, Kuppen PJK

Combined expression of the non-receptor protein tyrosine kinases FAK and Src in primary colorectal cancer is associated with tumor recurrence and metastasis formation. *Submitted for publication:* de Heer P, Koudijs MM, van de Velde CJH, Aalbers RIJM, Tollenaar RAEM, Putter H, Morreau J, van de Water B, Kuppen PJK

Caspase-3 activity predicts local recurrence in rectal cancer. *Clinical Cancer Research, in press:* de Heer P, de Bruin EC, Klein-Kranenbarg E, Aalbers RIJM, Marijnen CAM, Putter H, de Bont HJ, Nagelkerke JF, van Krieken JHJM, Verspaget HW, Kuppen PJK, van de Velde CJH

The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. *Cellular Oncology in press,* Mesker WE , Junggeburt JMC, Szuhai K , de Heer P, Morreau H, Tanke HJ, Tollenaar RAEM

Epigenetic Silencing of *Cyclooxygenase-2* Affects Clinical Outcome in Gastric Cancer. *Journal of Clinical Oncology in press:* de Maat MFG, van de Velde CJH, Umetani N, de Heer P, Putter H, van Hoesel AQ, Meijer GA, van Grieken NC, Kuppen PJK, Bilchik AJ, Tollenaar RAEM, Hoon DSB

Nawoord

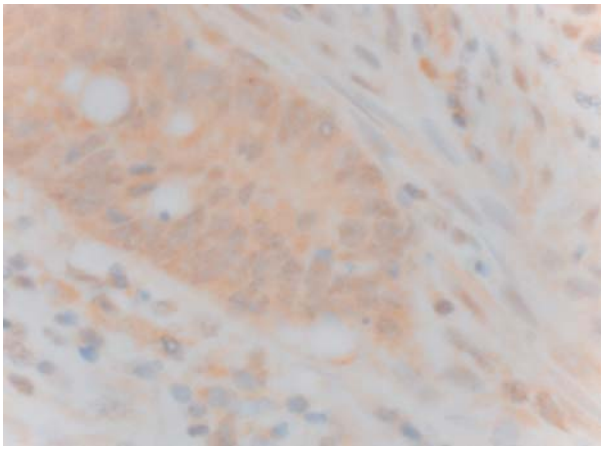
Een woord van dank aan allen zonder wier ontbaatzuchtige hulp en ondersteuning dit proefschrift nooit to stand zou zijn gekomen. Ik ben veel verschuldigd aan de toegewijde wetenschappers, laboranten, artsen en verpleegkundigen van de vakgroep chirurgische oncologie van het LUMC met wie ik de laatste jaren met groot genoegen heb samengewerkt. Vier groepen zou ik in dit verband met name willen noemen. Ten eerste, de staf en medewerkers van het lab SOLIT en mijn collega's op J10 en P01. Ten tweede, de samenwerkingsverbanden buiten de vakgroep oncologie; Marleen Goosens, Elza de Bruin, Jurriaan Tuynman en Ernst de Bruijn ben ik ten zeerste erkentelijk voor hun enthousiasme en het mede opzetten van enkele studies. Ten derde, Dick Richel, Jan Smeets en de medewerkers van het European Organisation for Research and Treatment of Cancer, die een cruciale rol hebben gespeeld bij het opzetten van de ACTION/PETACC-5 studie. En ten vierde, de medewerkers van het datacentrum heelkunde. Met elkaar schiepen zij een professionele en stimulerende werkomgeving die maakte dat tijd die ik aan dit onderzoek heb mogen wijden voorbij is gevlogen.
Verder wil ik al mijn vrienden bedanken die zich tijdens het onderzoek zeer betrokken hebben gevoeld. Ijsbrand Theunissen wil ik bedanken voor zijn hulp bij de lay-out en drukken van het proefschrift, mijn paranimfen Paul de Heer en Michiel de Maat bedank ik voor hun hulp en correcties en het telkens weer vragen of ze al iets moeten doen.
Tot slot wil ik mijn broer, mijn ouders en Kyra bedanken voor hun nimmer aflatende, steun, begrip, liefde en geduld; zonder jullie had ik dit proefschrift nooit kunnen realiseren.

Curriculum Vitae

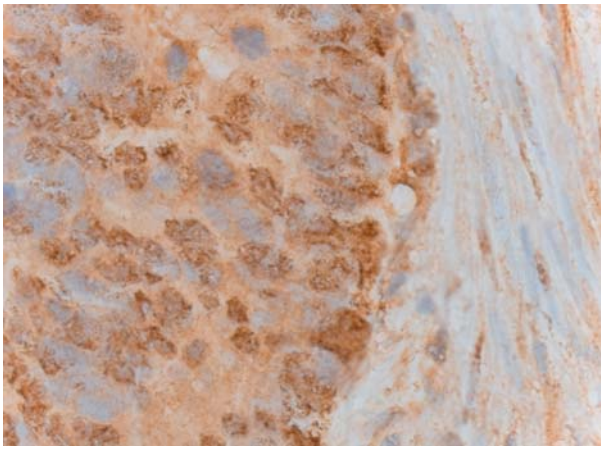
The author of this thesis was born on 7 May, 1976 in Hong Kong. He grew up in Beijing, Washington D.C., Singapore and the Netherlands and graduated from VWO at the Huygens Lyceum in Voorburg in 1994. In the same year he started studying medicine at the University of Leiden. During his medical training he conducted research at the department of pediatric oncology of the said university. From December 1999 to May 2000 he worked for several months as a researcher at the department of gynaecology of the Groote Schuur Hospital in Cape Town, South Africa. Both projects sparked his interest in oncological research. After graduation from medical school in 2000, he commenced with his intership and medical rotations in June 2000. After obtaining a medical degree in June 2002 he started working at the Surgical Oncology research group of the department of Surgery at the Leiden University Medical Center as a researcher and project manager of the PETACC-5 trial. The research on prognostic factors in colorectal cancer resulted in the current thesis. After completing the research for this thesis in 2006 he worked as AGNIO in the St Lucas Andreas Hospital in Amsterdam. He commenced with his surgical residency in January 2007 and is currently working in the Groene Hart Hospital, Gouda, the Netherlands (head: Dr. R. Ottow).

Chapter 2

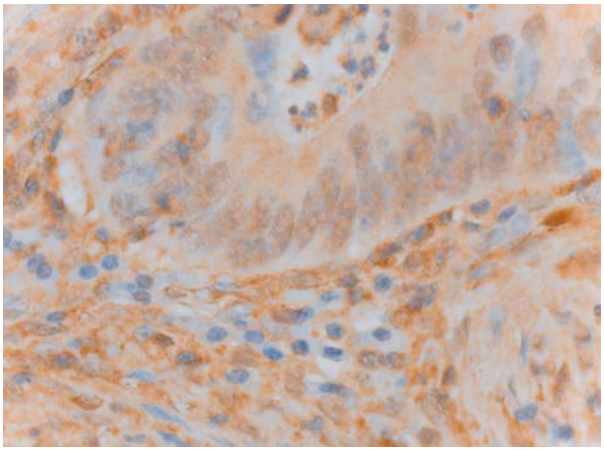
Figure 1



A



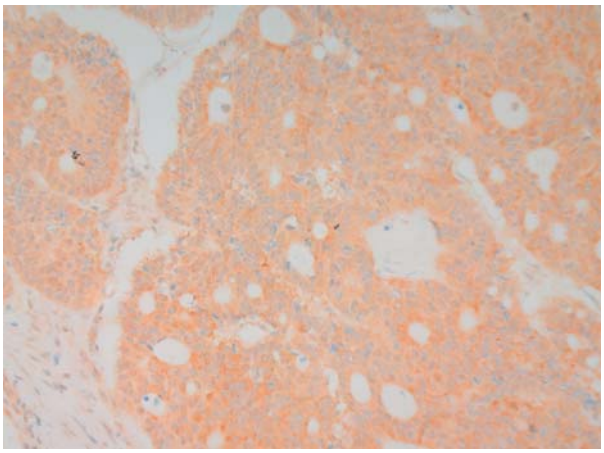
B



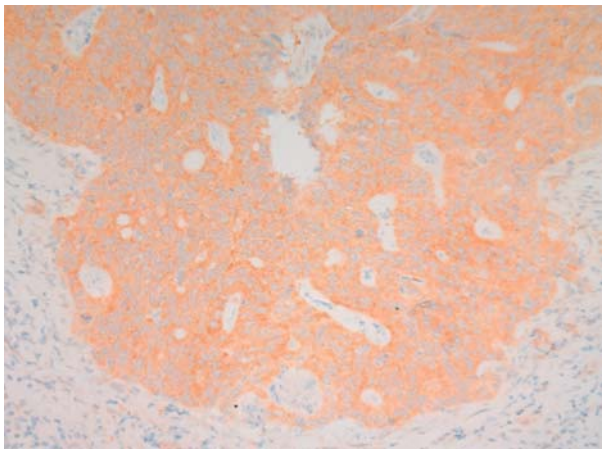
C

Figure 1 Immunohistochemical staining patterns of FAK, Src and paxillin. Tumor specimens were immunohistochemically stained with antibodies against FAK (Fig. 1A), Src (Fig 1B), or paxillin (Fig. 1C.) as described in Materials and Methods. Pictures were taken at 40 times magnification.

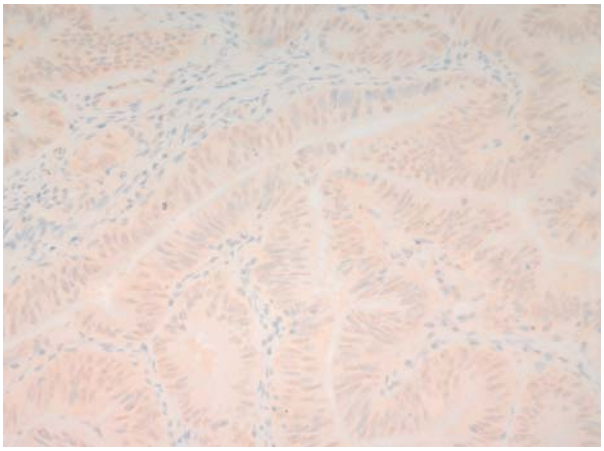
Figure 2



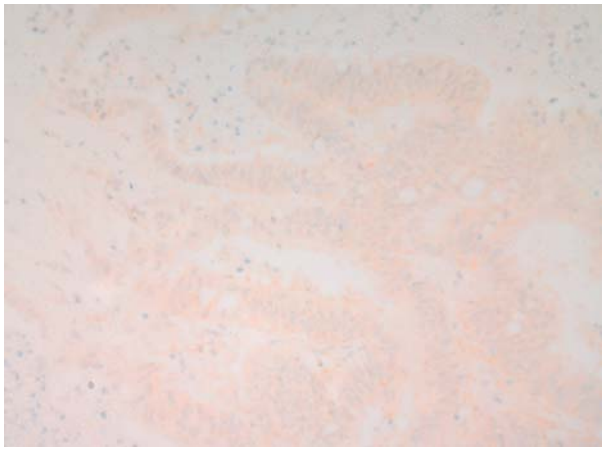
A1



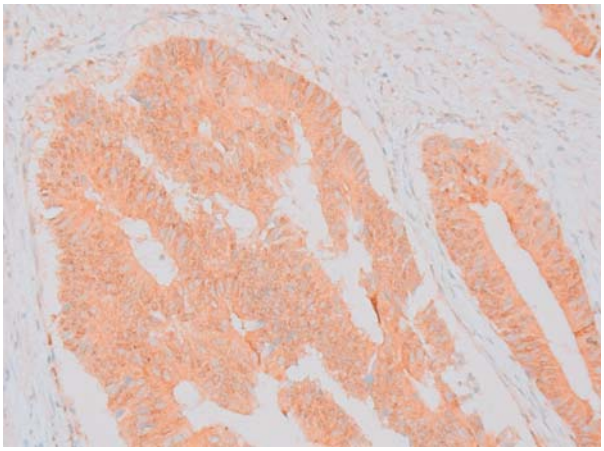
A2



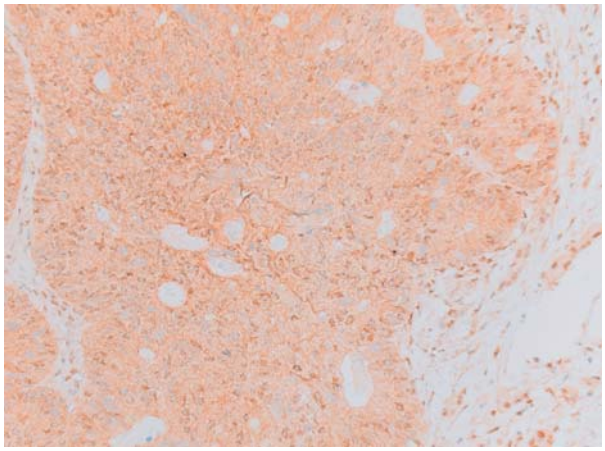
A3



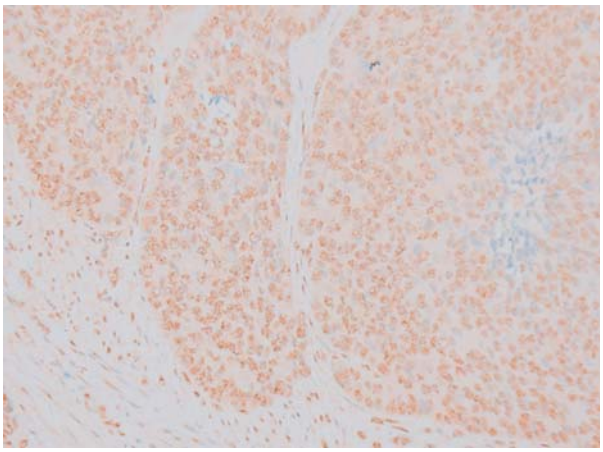
A4



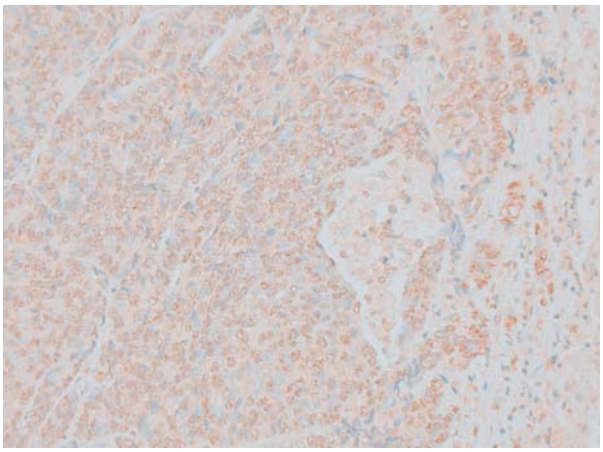
B1



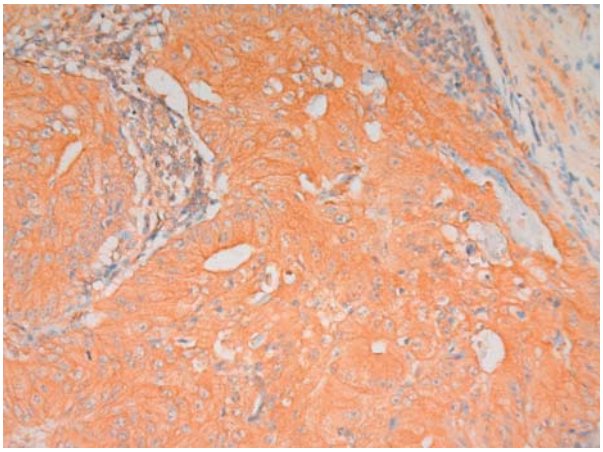
B2



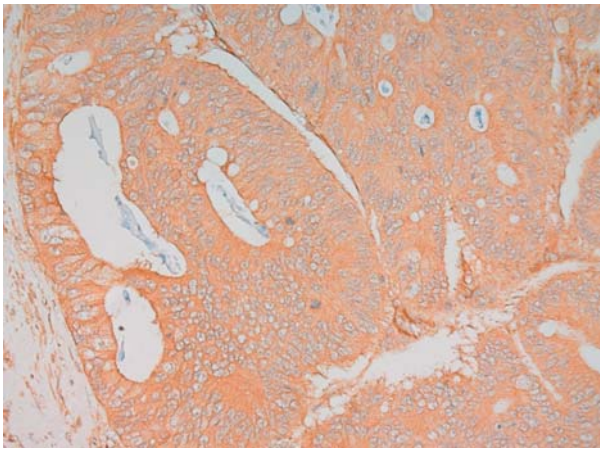
B3



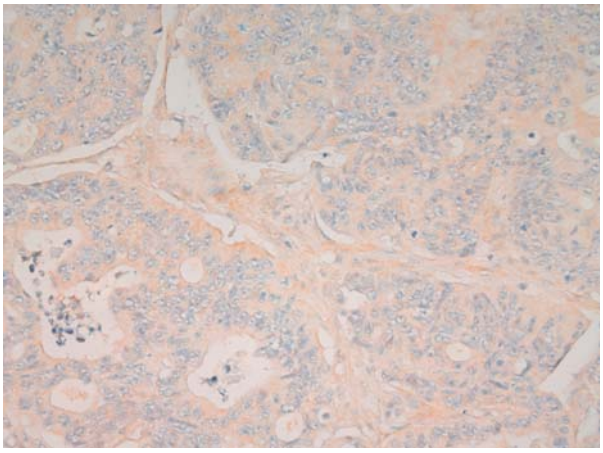
B4



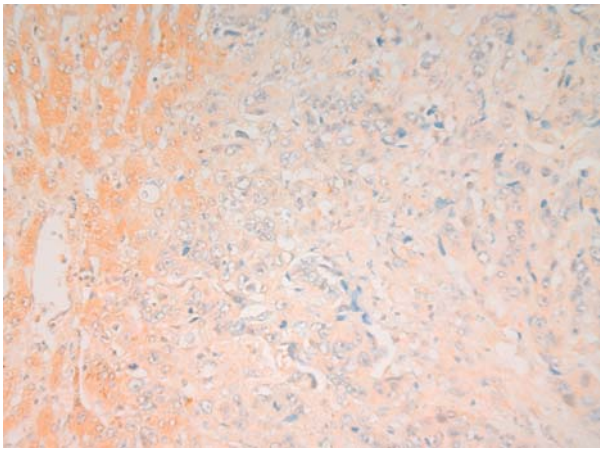
C1



C2



C3

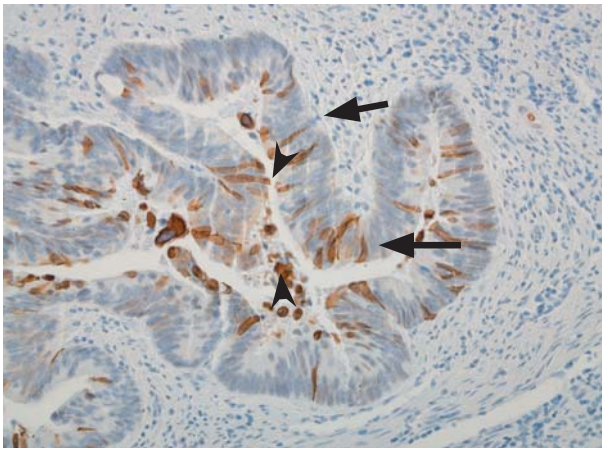


C4

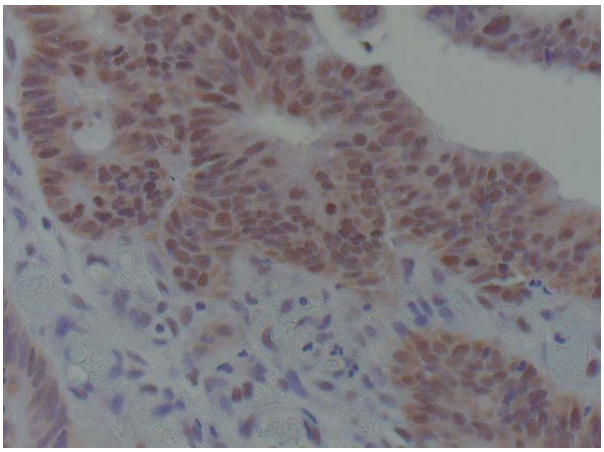
Figure 2 Immunohistochemical staining patterns of FAK, Src and paxillin expression in 68 primary colorectal cancers and corresponding liver metastases. Paired samples were stained with antibodies against FAK (Fig 2A1-A4), Src (Fig 2B1-B4) or paxillin (Fig 2C1-C4). Pictures were taken at 20 times magnification. Figures 2ABC: strong FAK (A), Src, (B) or paxillin (C) expression in primary tumor (1) and corresponding liver metastasis (2) and weak FAK (A), Src, (B) and paxillin (C) expression in primary tumor (3) and corresponding liver metastasis (4).

Chapter 4

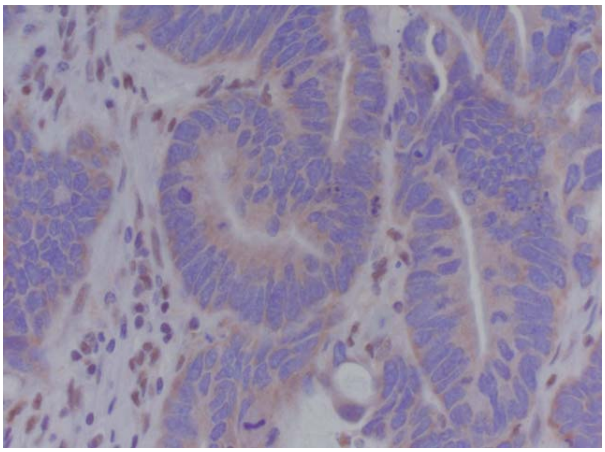
Figure 1



A



B

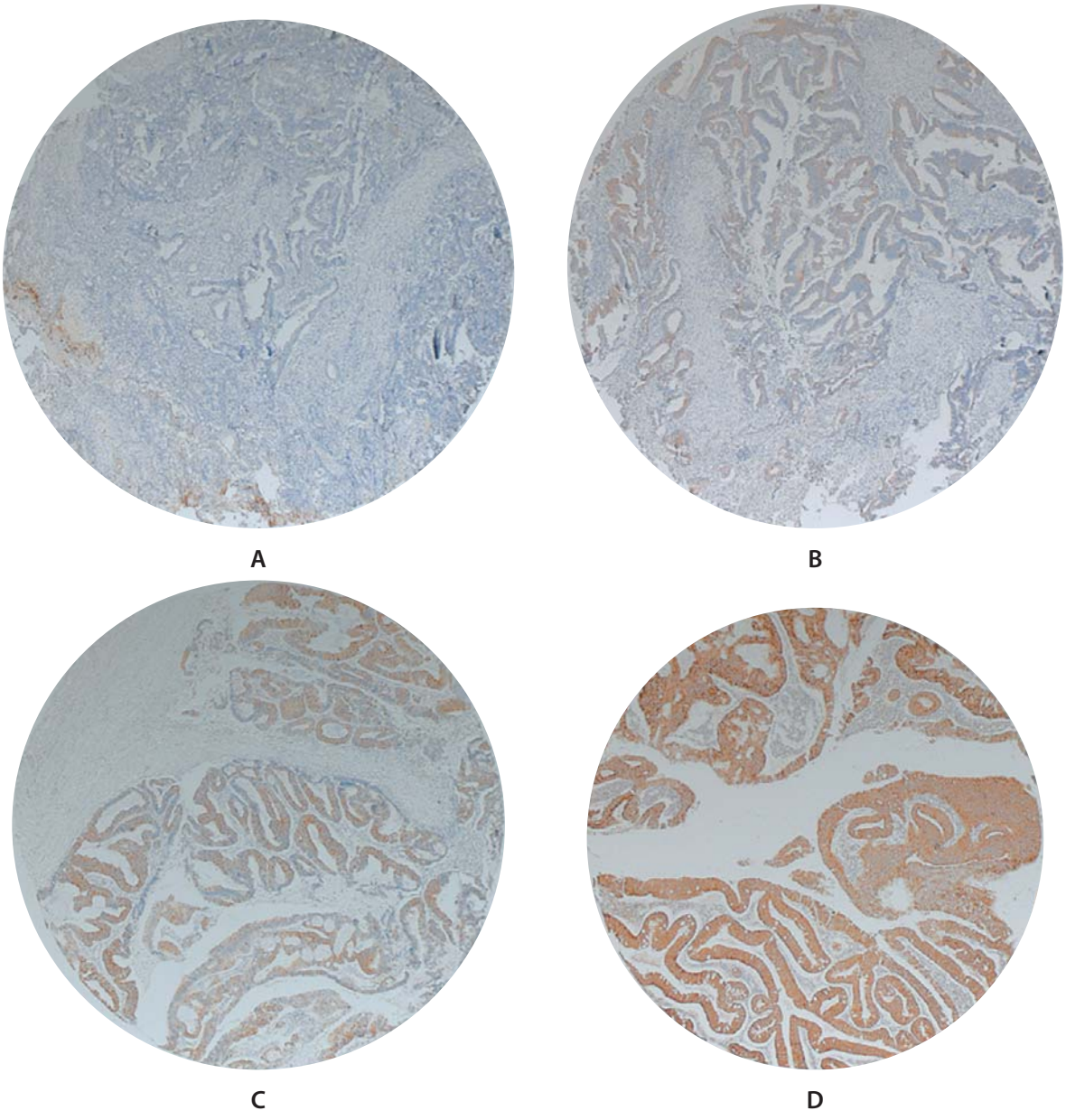


C

Representative immunohistochemical staining of apoptotic tumor cells, and nuclear PMS2 expression in colorectal cancer specimens. **Figure 1A** Apoptotic tumor cells were detected with M30 monoclonal antibody. Arrows indicate examples of apoptotic epithelial cells. Arrowheads indicate apoptotic bodies, these were not counted. **B** MSI colon adenocarcinoma with loss of PMS2 expression. Nuclear staining of non-epthelial cells serves as internal positive control. **C** adenocarcinoma with normal nuclear PMS2 expression.

Chapter 5

Figure 1 Representative staining of COX-2 expression in tissue micro array cores from the 1231 rectal cancer specimens evaluated in this study.



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 cytoplasmatic staining (score 1). Figure 1C: moderate to strong granular cytoplasmatic staining (score 2). Figure 1D: strong intensity of the staining (score 3).

Chapter 6

Figure 3

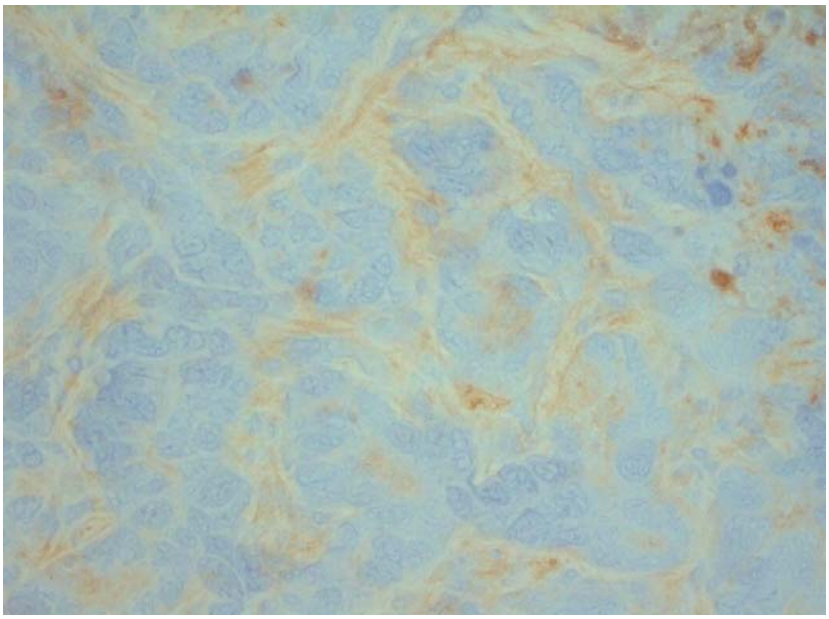


Figure 3 Fresh frozen tumor sections from CC531 stained with polyclonal rabbit antibodies against COX-2 (1:300) at 400x magnification. COX-2 expression is not visible in tumor epithelium. Surrounding tumor stroma shows light brown imunoreactivity. Infiltrating macrophages show positive COX-2 expression.

Figure 4

Figure 4A R73+ infiltration

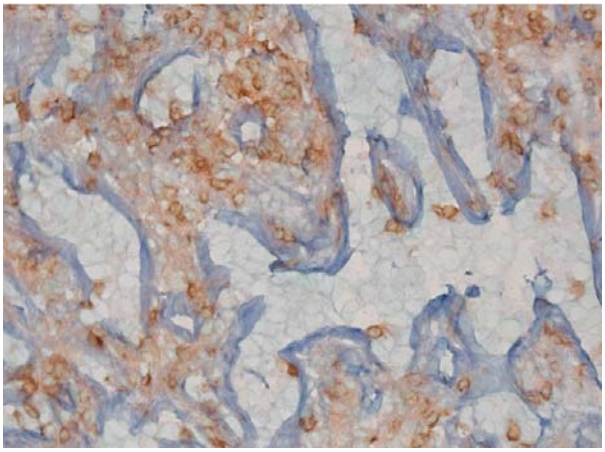


Figure 4B 3.2.3.+ infiltration

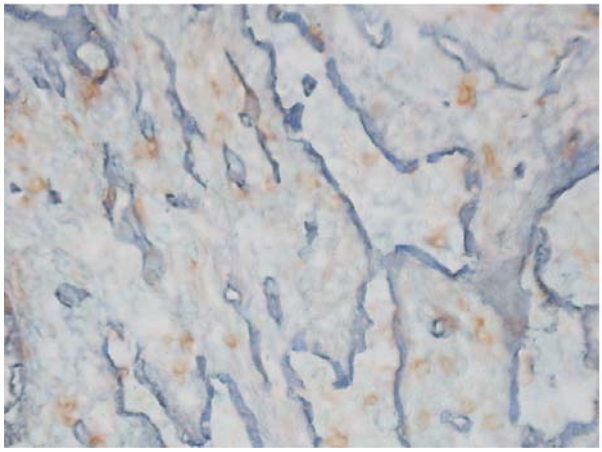


Figure 4C R73+ infiltration after celecoxib treatment

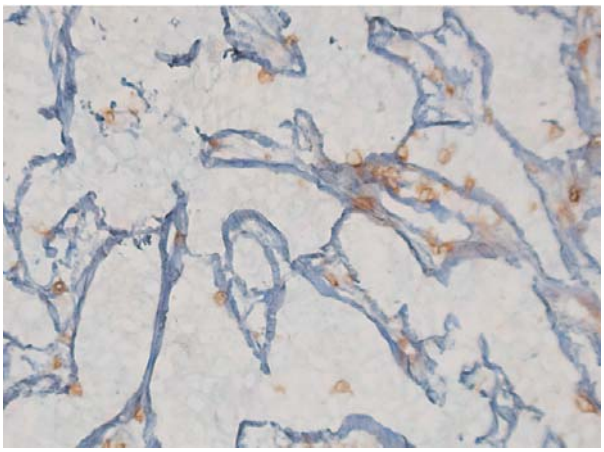
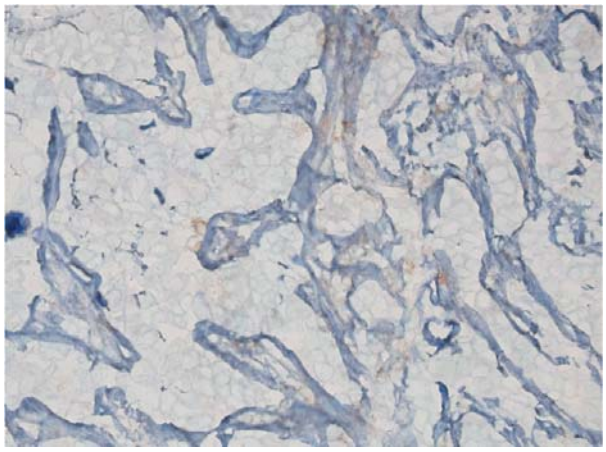


Figure 4C 3.2.3.+ infiltration after celecoxib treatment



A representative staining for T cell and NK cell infiltration of tumor sections from celecoxib-treated (1500ppm) and -untreated rats 21 days after tumor inoculation.
Sections were double-stained with laminin and R73 (anti- TCR, 1:100, Fig. 4A, 4C) or 323 (anti-CD161A, 1:50, Fig. 4B, 4D) antibodies respectively. R73+ and 323+ cells were stained brown, as revealed by immunohistochemistry (see material and methods). The matrix protein laminin was stained blue, blank spaces represent tumor nodules, delineated by a laminin-containing basal-membrane-like structure. The majority of R73+ and 323+ cells were localized in the tumor stroma, few positive cells were found in the tumor nodules. (200x magnification)

Chapter 7

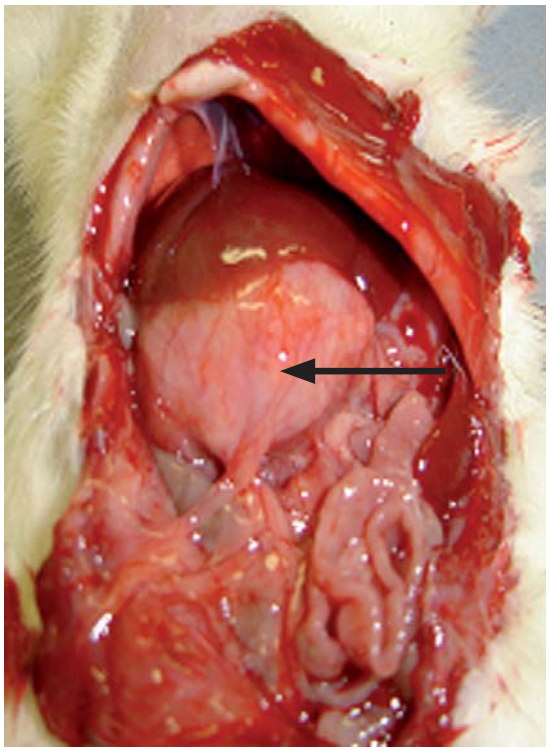


Figure 1a Intra-abdominal abscess covered with omentum after RFA treatment of one of two liver tumors in combination with celecoxib treatment (arrow).

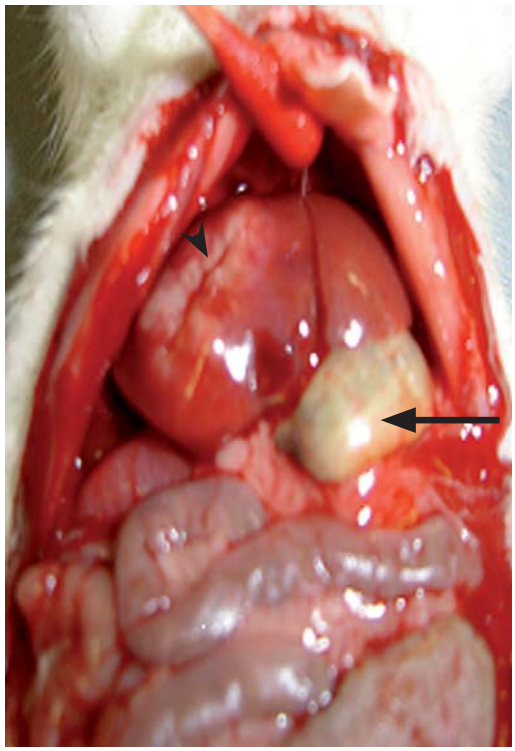


Figure 1b Rudimentary liver lobe after RFA treatment in a rat in the control group (group 2) (arrow), untreated tumor is visible in upper liver lobe (arrowhead).

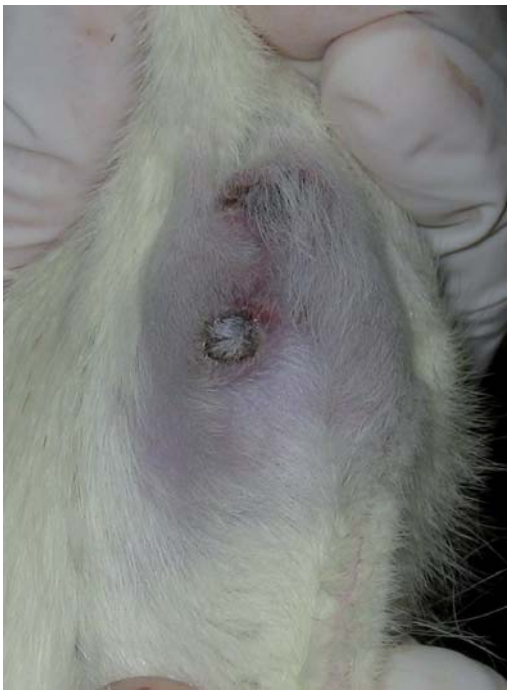


Figure 2 Presence of postoperative cutaneous abscesses in a rat that received celecoxib treatment.