Clinical, pathological and molecular prognostic factors in colorectal cancer

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ISBN: 978-90-77595-66-4

Layout and printing: Optima Grafische Communicatie, Rotterdam, The Netherlands
Cover illustration: Operatiekamer St. Elisabeth Ziekenhuis te Leiden ca. 1934
(Uit de collectie oude ansichtkaarten van Jorien M. Willems)

The printing of this thesis was financially supported by: Jeroen Bosch Academie, Raad van Bestuur VieCuri Medisch Centrum en Sanofi-Aventis Netherlands B.V.

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus Prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 23 maart 2017
klokke 15.00 uur

door

Frans Jeroen Vogelaar

geboren te Leidschendam

in 1975

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1

Introduction

GENERAL INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cancer cause of death globally, accounting for approximately 1.2 million new cases and 600000 deaths per year. Incidence is low at ages younger than 50 years, but strongly increases with age. Median age at diagnosis is about 70 years in developed countries. (1) In 2014 more than 13000 patients were diagnosed with CRC in the Netherlands. (2) Due to a rapidly aging population in the Netherlands it can be expected that the number of patients with CRC increases the coming decades. (3) Also the Dutch population based screening program for CRC, started in 2014, will cause an increase of incidence in the coming years. Five-year survival is over 90% when the disease is detected in an early stage (stage I), compared with <10% for CRC with distant metastases (stage IV). (2)

Since the 1990s, fluorouracil (FU)-based adjuvant chemotherapy has been used to reduce the risk of tumor recurrence and improve survival in colon cancer. (4) Postoperative treatment is universally recommended for patients with stage III disease.

Conversely, while all stage III patients have metastases in regional lymph nodes, only about 50% develop recurrent metastatic disease. Thus, about 50% of colorectal patients with nodal metastases visible by microscopy remain disease-free. Limited therapeutic benefit in some, but not all, patients with stage III disease probably reflects stage heterogeneity, in which some patients have clinically aggressive metastases. (5)

Adjuvant treatment in patients who are lymph node-negative by histopathology (stage II) is still controversial with small survival benefits in only some clinical trials. (6;7) However, it remains uncertain whether patients with stage II colon cancer derive sufficient benefit from adjuvant chemotherapy to justify its associated toxicity, inconvenience, and costs. The indications for using or not using adjuvant therapy are generally discussed with patients who have a higher risk of recurrence, because it is believed that they could derive larger absolute benefits from postoperative chemotherapy than patients at low risk of recurrence. (8) Definition of high-risk stage II colon cancer patients in the Dutch guideline is based on the guideline of the American Society of Clinical Oncology (ASCO). (6) Current treatment protocols recommend adjuvant treatment only to stage II patients with high-risk features e.g. T4 stage, bowel perforation or clinical bowel obstruction, inadequate lymph node sampling, poorly differentiated histology or (lymph)angioinvasion.

While histopathologic assessment of lymph nodes is a core element of colorectal cancer staging algorithms, the prognostic value of lymph node metastases is restricted. (5)

This highlights the need for approaches that detect occult tumor cells and define their prognostic and predictive value, to identify colorectal cancer patients who derive the greatest benefit from therapy. (5)

In an attempt to gain insights into prognosis in CRC, this thesis focuses on factors that play a role in the detection or behaviour of occult tumor cells. Especially clinical, pathological and molecular factors of CRC will here be discussed.

MINIMAL RESIDUAL DISEASE

Minimal residual disease (MRD) is defined by the presence of circulating tumor cells in the blood (CTC), disseminated tumor cells in bone marrow (DTC) or disseminated (isolated) tumor cells (ITC) or micrometastases in lymph nodes not found in conventional staging procedures. (9) Unless a curative oncological resection of the tumor, it seems that in a large fraction of patients MRD is present at the time of surgery, but these tumor cells escape detection by traditional hematological, pathological and radiological evaluation. (10)

CTC are circulating cells in the bloodstream originating either from the primary tumor or distant metastases. They can be found in the central venous blood compartment, the peripheral blood compartment, the tumor draining veins, the portal venous system or within the arterial blood system. Most of these cells will not have the capacity to survive in the bloodstream or to form distant metastases as they will ultimately undergo cell death by apoptosis or will die due to shearing forces within the blood-stream.

DTC are tumor cells, which can be found in the bone marrow or lymph nodes. Most research about DTC originates from patients with breast cancer and is usually assessed on biopsies from the bone marrow of the iliac crest. Tumor cells in the bone marrow often enter a dormant state and can eventually be reactivated after several years of dormancy. DTC are thought to be one of the key elements in late disease recurrence. (11;12) DTC in lymph nodes are called isolated tumor cells (ITC). Micrometastases and ITC in lymph nodes are tumor cells in lymph nodes that can be detected by conventional hematoxylineosin (HE) staining, although routine staging done by the pathologist show limited sensitivity. These occult tumor cells can also be found by using molecular techniques, such as staining with tumor specific antibodies or reverse-transcriptase polymerase chain reaction (RT-PCR). (13)

Most data on the prognostic value of DTCs are available for breast cancer and document an association between the presence of DTCs at primary surgery and subsequent metastatic relapse. Also persistence of DTCs after adjuvant therapy in breast cancer patients increases the risk of subsequent relapse and death. (11)

CLINICAL, PATHOLOGICAL AND MOLECULAR PROGNOSTIC FACTORS

Treatment choices of CRC nowadays are influenced by the tumor, node and metastasis (TNM) classification of the Union for International Cancer Control (UICC). (14) The most significant prognostic marker of risk of disease recurrence in colorectal cancer is metastatic tumor cells in regional lymph nodes. (15) While histopathological examination is the standard approach, imprecision in staging by standard light microscopy signifies important methodological limitations. Possible under-treatment or over-treatment of some patients groups might arise when using the TNM staging system for treatment allocation. (16)

A systematic review concluded that the number of lymph nodes evaluated after surgical resection was positively associated with survival of patients with stage II and stage III colon cancer. These results support consideration of the number of lymph nodes evaluated as a measure of the quality of colon cancer care. (17)

Other clinical and pathologic features have been identified and used to identify high risk node-negative patients. These include patients with bowel obstruction at presentation, perforation of the colon at the tumor site, poor histologic grade, and peritumoral lymphovascular involvement. (6)

Sentinel lymph node mapping

At the end of the 20th century it was found that molecular detection of micrometastases in lymph nodes is a prognostic tool in stage II colorectal cancer. (18) Because molecular assessment of all retrieved lymph nodes is time-consuming and impractical, further research started to focus on sentinel lymph node mapping. Sentinel lymph nodes (SLNs) are the lymph nodes that have the highest potential to harbor metastasis due to the fact that they are directly in communication with the location of the tumor. Different studies showed that ex vivo sentinel lymph node mapping is an easy and feasible technique for ultra-staging CRC patients. (19)

Molecular markers

The increased understanding of the molecular biology in colorectal carcinogenesis has revealed several promising biomarkers. (20) Nowadays, in the era of personalized cancer medicine, identifying mutations within patient tumors might play an important role in defining high risk colon cancer patients. It has been shown, for example, that molecularly defined subpopulations with node-positive tumors have survival estimates comparable to patients with low-risk node-negative disease. (21) Molecular prognostic factors can have major relevance for stage II disease allowing the identification of subsets of patients with lower or higher risk of relapse and for whom adjuvant chemotherapy could be avoided or favored, respectively. Several prognostic biomarkers have been described

in recent studies of early stage colon cancer of which Microsatellite Instability (MSI), *KRAS* and *BRAF* and *PIK3CA* are the most well-known. (8;16)

Tumor-stroma ratio

Although several high-risk pathological characteristics are now recognised as important determinants of survival, particularly in node negative disease, it is now clear that other host and tumor characteristics may similarly determine oncological outcome. Components of the tumor microenvironment, such as the tumor-associated stroma, has been identified as an important determinant of progression in a number of solid cancers. The stroma facilitates the survival and proliferation of neoplastic cells and promotes epithelial–mesenchymal transition (EMT), and local and metastatic dissemination. Consistent with this scheme an increase in the proportion of tumor-stroma has been associated with poorer survival in a number of solid cancers, including CRC. Assessment of the proportion of tumor-stroma using routine pathological specimens may act as a surrogate for tumor-stroma activity and its subsequent effect on survival. (22;23)

REGIONAL ANESTHESIA AND MRD

Surgery remains the mainstay of treatment for malignant tumors; however, surgical manipulation leads to a significant systemic release of tumor cells. Whether these cells lead to metastases is largely dependent on the balance between the aggressiveness of the tumor cells and the anticancer immune response of the body. Surgical stress per se, anesthetic agents and administration of opioid analgesics perioperatively can compromise immune function and might shift the balance towards progression of minimal residual disease. Regional anesthesia techniques provide perioperative pain relief; they therefore reduce the quantity of systemic opioids and of anesthetic agents used. Additionally, regional anesthesia techniques are known to prevent or attenuate the surgical stress response. (24) It can be hypothesized that interventions aimed at reducing exposure to immunosuppressive factors would improve patient outcomes after a potentially curative cancer resection.

AIM AND OUTLINE OF THIS THESIS

In this thesis clinical, pathological and molecular factors are described that influence the prognosis of colorectal cancer patients.

Disseminated tumor cells in bone marrow of colorectal cancer patients are studied in chapter 2 and 3. Chapter 2 describes the results of a study in patients undergoing

surgical resection of colorectal liver metastases. The clinical impact of disseminated tumor cells in bone marrow is assessed, using two different detection methods: reverse transcription-polymerase chain reaction (RT-PCR) and using immunocytochemical staining for cytokeratin (CK-ICC). The correlation of disseminated tumor cells in bone marrow and tumor-stroma ratio was studied in chapter 3 as well as the prognostic value of both entities in a cohort of primary colorectal cancer patients. Chapter 4 aims to identify high-risk factors in stage II colonic cancer patients related to oncological outcome and investigate whether the number of high risk factors present relates to outcome. Chapter 5 describes a novel molecular technique to analyze (sentinel) lymph nodes in colon cancer patients: one-step nucleic acid amplification (OSNA). The diagnostic value of OSNA for sentinel lymph nodes in colon cancer patients will be assessed. A cohort of node-negative colon cancer patients that did not receive adjuvant chemotherapy was studied in chapter 6. The prognostic value of Microsatellite Instability (MSI), *KRAS*, *BRAF* and *PIK3CA* mutations was obtained in this study. The role of regional anesthesia, especially epidural analgesia, on cancer survival is subject of chapter 7 and 8. A review of the literature about this topic will be described. Furthermore the association of survival and epidural anesthesia is described as a part of a large retrospective study of colon cancer patients. In chapter 9 the findings and the possible clinical implications of all presented studies are summarized. Future perspectives for research and clinical management for colorectal cancer are discussed.

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2

Clinical impact of different detection methods for disseminated tumor cells in bone marrow of patients undergoing surgical resection of colorectal liver metastases: a prospective follow-up study

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BMC Cancer. 2010 Apr;20;10:153.

ABSTRACT

Introduction Large number of patients with colorectal liver metastasis show recurrent disease after curative surgical resection. Identification of these high-risk patients may guide therapeutic strategies. The aim of this study was to evaluate whether the presence of disseminated tumor cells in bone marrow from patients undergoing surgical resection of colorectal liver metastases can predict clinical outcome.

Methods Sixty patients with colorectal liver metastases were planned for a curative resection between 2001 and 2007. All patients underwent bone marrow aspiration before surgery. Detection of tumor cells was performed using immunocytochemical staining for cytokeratin (CK-ICC) combined with automated microscopy or indirectly using reverse transcription-polymerase chain reaction (RT-PCR).

Results Disseminated tumor cells were found in 15 of the 46 patients (33%) using CK-ICC and in 9 of 44 of the patients (20%) using RT-PCR. Patients with negative results for RT-PCR had a significant better disease-free survival after resection of their liver metastases (p=0.02). This group also showed significant better overall survival (p=0.002). CK-ICC did not predict a worse clinical outcome.

Conclusions The presence of disseminated tumor cells in bone marrow detected using RT-PCR did predict a worse clinical outcome. The presence of cells detected with CK-ICC did not correlate with poor prognosis.

INTRODUCTION

Over the past decades surgical resection has evolved as the first choice of treatment for colorectal liver metastases because it is a relatively safe and potentially curative procedure. (1;2) The reported 3-year survival of patients after surgical resection of colorectal liver metastasis ranges from 57% to 73%. (3;4) However, even after curative surgical resection, a high percentage of patients show recurrent disease, either in the liver or extra hepatic, within a relatively short period of time after surgical treatment (5) caused by minimal residual disease (MRD). (6) These high-risk patients might benefit from additional systemic treatment. (7) Currently available prognostic factors are insufficient to select patients at risk for tumor progression. (8) Therefore the need for additional methods for the selection of high-risk patients is evident.

On a clinical level, the Memorial Sloan-Kettering Cancer Center Clinical Risk Score (MSKCC-CRS) is a frequently used tool to predict the risk for recurrence and tumor progression. (4) On a cellular level, disseminated tumor cells (DTCs) might also give this prognostic information. DTCs can be detected in blood and bone marrow of patients with various epithelial malignancies either directly, using immunocytochemical staining combined with automated microscopy (CK-ICC), or indirectly using reverse transcription-polymerase chain reaction (RT-PCR). (6;9;10) Automated microscopy is proven to be an accurate method for pathological evaluation of tumor cells in blood and bone marrow. (11)

Currently, most data on the prognostic value of DTCs are available for breast cancer. Recent meta-analysis (12) showed that the presence of DTCs in bone marrow was predictive for the development of distant metastases in breast cancer. Tumor cell persistence in bone marrow also showed to be an independent prognostic factor for subsequent breast cancer survival. (13)

However, in colorectal cancer the results are more controversial. Different groups describe a positive association between DTCs in bone marrow and an increased recurrence rate or reduced survival while other found no association between DTCs and prognostic factors. (6) Therefore, the clinical meaning of DTC detection in colorectal cancer is still open for debate both for CK-ICC and RT-PCR. (14-19)

The aim of this prospective study is to evaluate whether the presence of disseminated tumor cells in bone marrow from patients undergoing surgical resection of colorectal liver metastases is associated with worse overall survival and shorter disease-free survival.

METHODS

Patients

Between October 2001 and November 2007, a total of 180 consecutive patients with colorectal liver metastases were scheduled for surgical therapy. Only the patients planned for curative resection were included in this study. Other types of surgery or diagnoses were excluded: surgery combined with radio frequency ablation (RFA) (n=25), RFA alone (n=23), isolated liver perfusion (n=64), liver perfusion prior to surgery (n=2), benign disease (n=2), other malignancy (n=4). Overall, 60 patients planned for resection of the liver metastases were included for analysis. Twenty four of them were diagnosed in 2001-2004 and 36 were included in 2005-2007. Approval from all the local Ethical Committees for this study was granted and informed written consent was obtained from all patients. All patients underwent a preoperative abdominal computed tomography (CT) to confirm metastatic disease confined to the liver. Eligibility and exclusion criteria for the scheduled treatment and criteria for disease progression within the liver according to the WHO guidelines have been previously published. (1;20-23) During follow-up, CT-scans of the liver were made at 4, 8 and 12 months after surgery and then after every 12 months until 3 years after surgery. The patients who did not undergo any intervention or showed disease progression which could not be surgically treated, were referred to a medical oncologist for further treatment. All patients underwent bone marrow aspiration under general anesthesia just prior to surgery and all patients were followed up until June 2008.

Bone marrow aspiration

5-10 ml of bone marrow was aspirated from both sides of the anterior iliac crest of all included patients. Before inserting the needle in the anterior iliac crest, an incision was made into the overlying skin to prevent contamination with skin epithelial cells. Mononuclear cells were isolated from bone marrow by ficoll gradient centrifugation and aliquoted to isolate RNA to use for the RT-PCR or to make cytospin-slides to stain with ICC.

Immunocytochemistry and automated microscopy (CK-ICC)

The cytospin slides were stained with primary antibodies A45-B/B3, directed against cytokeratins 8, 18 and 19 or with isotype control antibodies directed against an irrelevant antigen, MOPC21, as a negative control staining. A detailed protocol has been published before by Pantel et al. (24) This staining resulted in a red precipitate in the cytoplasm of cytokeratin 8, 18 and 19-positive cells. The slides were counterstained with hematoxylin to visualize nuclear morphology. The stained slides were analysed using the ARIOL SL-50 automated microscope®. One slide stained for cytokeratin and one

negative control slide were analyzed per patient. The features of this system have been previously published. (25)

Combining ICC with automated microscopy, cytokeratin-positive cells were confirmed by a independent pathologist and categorized based on morphological criteria according to the guidelines of the European ISHAGE Working Group for Standardization of Tumour Cell Detection. (26) Candidate tumor cells and apoptotic cells were cells that did not meet all criteria for a positive cell but could not be unambiguously defined as normal. A patient was considered positive if at least one tumor cell, candidate tumor cell or apoptotic cell was found, all verified by an independent pathologist.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the mononuclear cells by Trizol reagent. Random primed cDNA was synthesized from 1 µg of total RNA using the 1st strand cDNA synthesis kit for RT-PCR (AMV). cDNA samples were five times diluted to 100 µl to diminish pipetting variation. Primers and probes for the marker CK20 were selected with Primer Express®v1.5 software. The low-copy housekeeping gene porphobilinogen deaminase was used as an internal control. For each patient two RNA samples resulting in cDNA samples were processed. Five micro-liters of cDNA were used per amplification. For all PCRs the same PCR conditions were used. Per reaction 300nM of each primer was used.

PCR samples were considered positive if the threshold cycle was less than 55. The threshold cycle reflects the PCR cycle number at which the fluorescence generated within a reaction crosses the threshold (background noise). The threshold cycle is inversely proportional to the copy number of the target template i.e. the higher the template concentration, the lower the threshold cycle measured. Bone marrow from a patient was considered positive if at least one of the PCR samples was positive after duplo analysis.

Statistical analysis

Frequencies were described as mean (SD), or median (range) in case of a non-normal distribution. The mortality of the patients with positive RT-PCR was compared with the subjects with negative RT-PCR using Cox regression adjusted for sex and age. The same analysis was done for CK-ICC. Hazard ratio's (HR) were calculated by Cox regression analysis for disease-related survival and disease-free survival. Disease-related survival was considered from the day of liver metastases-surgery to the day of death due to disease or censored at most recent follow-up visit. Patients who did not undergo resection of the liver metastases, who showed extra-hepatic disease at the time of surgery or who died after the operation due to complications or none disease-related causes were excluded from disease-related survival analyses. Disease-free survival was considered from the day of surgery to the day of recurrence or censored at most recent follow-up. The association between mortality and ICC or RT-PCR was visually depicted with a

Kaplan-Meier survival curve. All analyses were performed with SPSS for Windows (version 16.0, SPSS Inc, Chicago, Ill). P-values of less than 0.05 were considered statistically significant.

RESULTS

The study population comprised of 42 males and 18 females. No complications of bone marrow aspiration were reported. Overall, 27% of the patients (16/60) did not undergo the planned surgical treatment because of the presence of extra-hepatic disease (n=9), the high number of metastases (n=5) or the location of the metastasis to the portal vein (n=2). The median (range) follow-up time from the date of diagnosis of the primary tumor was 40.1 (7.6-96.3) months. 21 patients (35%) died during follow-up: 17 patients due to disease progression, one patient because of complications during surgery and 3 patients because of other none disease-related causes. Median (SE) disease-free survival of all the patients was 12.1 (1.9) months and the median (SE) overall survival was 23.5 (1.8) months. One year survival was 93% and 3-year survival 72%.

RT-PCR

Bone marrow samples of 16 patients could not be analysed with RT-PCR because of the low amount of harvested mononuclear cells. RT-PCR positivity was found in 9 of 44 of the patients (20%). A positive RT-PCR test was seen in 6 of 32 patients (19%) who underwent a surgical resection compared to 3 of 12 inoperable patients (25%). Characteristics of the patients analyzed with RT-PCR and CK-ICC are shown in Table I.

CK-ICC

Due to a low number of harvested mononuclear cells, bone marrow samples of 14 colorectal liver metastases patients could not be analysed using ICC combined with automated microscopy to identify disseminated tumor cells. 15 of the 46 patients (33%) had a positive ICC test. ICC bone marrow analysis resulted in the presence of tumor cells in 13 of 37 patients (35%) who underwent a curative resection and 2 of 9 patients (22%) who finally underwent no surgical resection (only abdominal exploration).

MSKCC-CRS

Primary tumor stage showed no correlation with the detection of tumor cells in bone marrow. High risk score-patients (3-5) according to the MSKCC-CRS showed disseminated tumor cells (RT-PCR) in 31% compared to 15% in the low risk group (0-2). CK-ICC positivity was found in 21% of the high-risk score-patients compared to 38% of the low risk score-patients.

Table 1. Characteristics of the patients analyzed with RT-PCR and CK-ICC.

	RT-PCR		CK-ICC	
	positive N=9	negative N=35	positive N=15	negative N=31
Male	5 (56)	26 (74)	12 (80)	20 (65)
Age (years), mean (se)	63.4 (3.7)	60.9 (1.3)	62.9 (2.3)	60.2 (1.6)
TNM stage of primary tumor				
1	1 (11)	3 (9)	2 (13)	1 (3)
2	1 (11)	6 (17)	2 (13)	4 (13)
3	3 (33)	9 (26)	5 (33)	8 (26)
4	4 (45)	17 (49)	6 (40)	18 (58)
Time span between PT and LM (months)				
< 12	6 (67)	20 (57)	8 (53)	23 (74)
> 12	3 (33)	15 (43)	7 (47)	8 (26)
Preoperative systemic chemotherapy				
No	4 (44)	25 (71)	12 (80)	18 (58)
Yes	5 (56)	10 (29)	3 (20)	13 (42)
Serum CEA level				
< 200 ug/l	5 (55)	30 (86)	13 (87)	25 (81)
> 200 ug/l	1 (11)	3 (9)	1 (7)	2 (7)
not assessed	3 (33)	2 (5)	1 (7)	4 (14)
No. of liver metastases				
1	3 (33)	14 (40)	5 (33)	12 (39)
> 1	6 (67)	21 (60)	10 (67)	19 (61)
Diameter of liver metastases (cm)				
< 5	7 (77)	28 (80)	12 (80)	26 (84)
> 5	2 (33)	7 (20)	3 (20)	5 (16)
MSKCC clinical risk score				
0-2 (low)	4 (44)	24 (69)	12 (80)	20 (65)
≥ 3 (high)	5 (56)	11 (31)	3 (20)	11 (35)
Death	6 (67)	10 (29)	5 (33)	14 (45)

Data presented in number (%), unless otherwise stated.
*Abbreviations; PT, primary tumor; LM, liver metastases; CEA, carcino embryonic antigen; MSKCC, memorial sloan-kettering cancer center.

Pre-operative chemotherapy

Bone marrow of patients receiving neo-adjuvant chemotherapy was CK-ICC positive in 19% versus 40% in patients not receiving chemotherapy. RT-PCR positivity was found in 33% of the patients with chemotherapy compared to 14% of the patients without chemotherapy. Combining the two techniques (CK-ICC and/or RT-PCR positive), 57% of bone marrow was negative in patients who received chemotherapy and 54% in patients

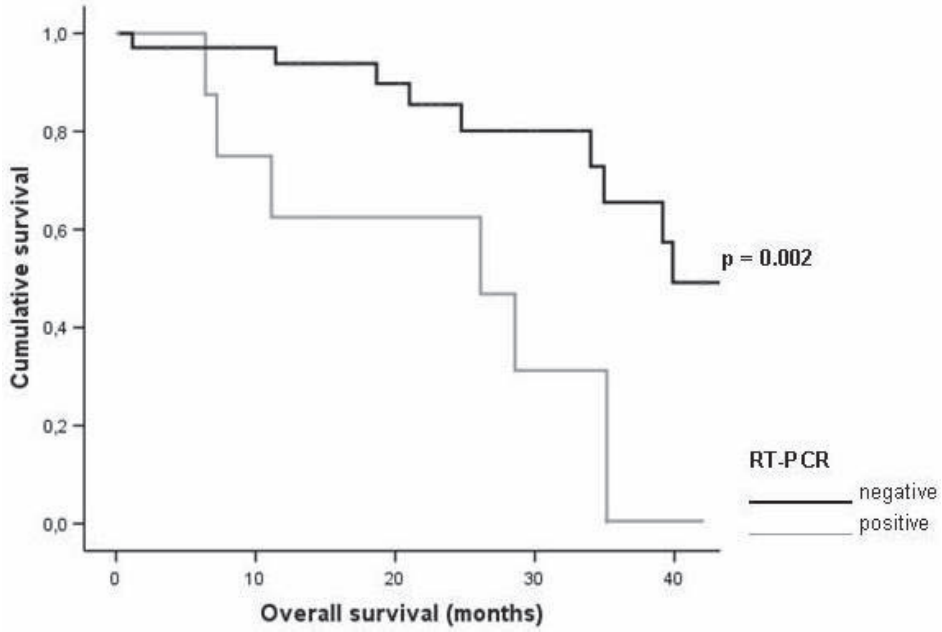

Figure 1. Graphical representation of the relationship between reverse transcription-polymerase chain reaction (RT-PCR) status and overall survival in subjects after colorectal liver metastases surgery.

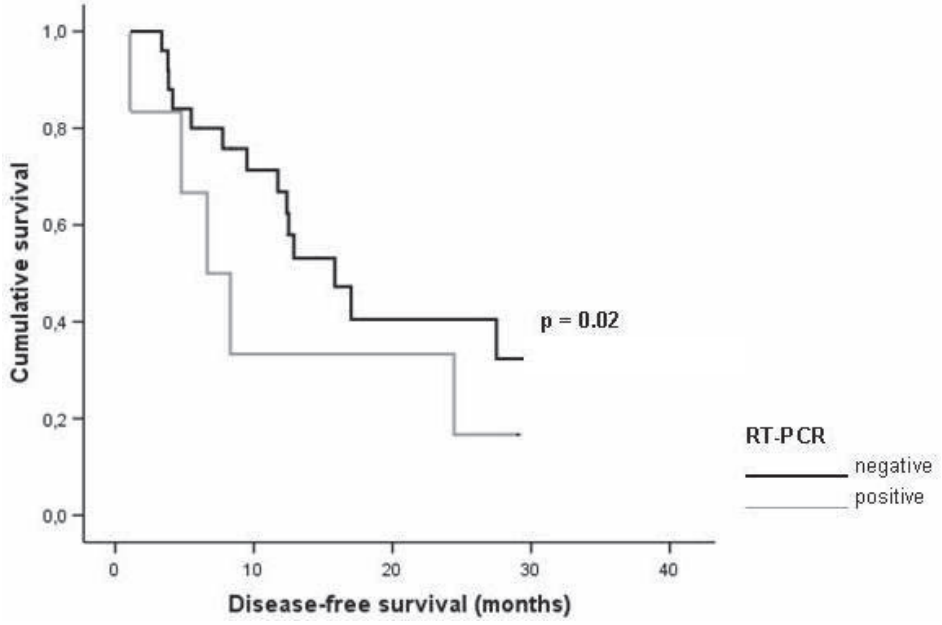

Figure 2. Graphical representation of the relationship between reverse transcription-polymerase chain reaction (RT-PCR) status and disease-free survival in subjects after colorectal liver metastases surgery.

without pre-operative chemotherapy. Survival in the group receiving pre-operative chemotherapy was better than in the non chemotherapy group (p=0.02). This was also seen when patients who did not undergo resection were excluded from this analysis (p=0.03).

Survival

Patients with RT-PCR negative bone marrow had a significant better overall survival (figure 1); HR 6.40, 95% CI 1.93-21.16, p=0.002. This group also showed significant better disease-free survival after resection of their liver metastases (figure 2); HR 4.11, 95% CI.1.33-12.58, p=0.02.

CK-ICC positive bone marrow showed no significant difference in overall survival after resection (p=0.24) neither a significant difference in disease-free survival (p=0.86).

Combined RT-PCR and/or ICC positive analysis did not show any overall survival difference (p=0.68) neither a difference in disease-free survival (p=0.60). The low risk group according to the MSKCC-CRS (0-2) had a better overall survival (HR 3.32, 95%CI 1.14-9.67, p= 0.03) compared to the high MSKCC-CRS (3-5) patients. All HR were adjusted for age and sex.

When adjusted for MSKCC-CRS score, also a significant better overall survival was found in RT-PCR negative patients; HR 5.42, 95% CI.1.53-19.18, p=0.009.

DISCUSSION

In our study, disseminated tumor cells found in bone marrow by RT-PCR have prognostic value in patients scheduled for surgical resection of colorectal liver metastases. Patients with positive bone marrow by RT-PCR had an increased risk of cancer related mortality. Also adjusted for the MSKCC risk score, RT-PCR positivity showed a significant worse overall survival which reflects its additional medical value. In contrast, ICC did not predict outcome in these patients.

In our study the patients with positive RT-PCR bone marrow showed worse overall and disease-free survival after liver metastasis surgery. Similar results were found by *Koch et al.* (15) This study investigated bone marrow samples from 25 patients with colorectal liver metastases who underwent surgical resection and showed a positive RT-PCR test to be an independent prognostic factor for recurrence-free survival. The percentage RT-PCR positive bone marrow of the patients in our study (20.5%), is comparable with other studies, showing 16-27% RT-PCR positivity. (15;18;19)

In contrast to RT-PCR, a positive ICC did not predict worse overall- and disease-free survival. Studies from *Bjornland et al* and *Schoppmeyer et al* had the same conclusion. (14;17) In contrast, DTCs detected with the CK-ICC in breast cancer patients are found to be of major prognostic significance. (27) It might be argued that the bone marrow in

breast cancer patients not just reflects the metastatic load but is also a preferred site for metastases outgrowth. This may relate to the possibility that the bone marrow compartment offers a more fertile microenvironment for breast cancer cells than for colorectal cancer cells. (28)

ICC positivity in bone marrow in our study is 32.6%. Differences in percentages of ICC positivity in colorectal cancer are reported in literature; *Schoppmeyer et al* found 55% CK positive cells in bone marrow (17), *Cohen et al* found 9.5-34% ICC positivity (29) and *Bjornland et al* found 8% ICC positivity. (14)

The two methods used in our study to detect DTCs in liver metastatic colorectal cancer showed different results. A possible explanation is the hypothesis that only a small subset of tumor cells has the capacity to proliferate extensively and to outgrow to new tumors as increasing evidence supports (30;31). Alternative detection methods, therefore may find disseminated cells that differ in their tumor initiating capacities. Another explanation may be that ICC detects only intact cells, while RT-PCR can also detect fragments of cells that are degraded in the circulation. Intact cells then may be considered biologically irrelevant (thus left alone by the immune system). Cells that would have been attacked by the immune system but escaped in distant organs may find a niche there to evolve into clinically manifest disease. Although highly speculative this same phenomenon has been described in minimal residual disease detection in lymph nodes (8), where RT-PCR is also more prognostic then ICC.

Earlier report from *Vlems et al* showed no positive bone marrow in 12 of the 20 patients who underwent chemotherapy and the authors therefore suggest chemotherapy prevents shedding or accelerates clearance of disseminated tumor cells. (18) However, we found no influence of preoperative chemotherapy on bone marrow positivity.

Possible limitations and strength

The clinical relevance of the molecular detection of DTCs is possibly restricted by tumor cell heterogenity and differing sensitivity and specificity of each specific detection method. This may explain inconclusive findings from previous disseminated tumor cell studies. Despite the small number of patients, to the best of our knowledge our study is the largest study comparing both techniques, CK-ICC and RT-PCR, in patients with metastatic colorectal cancer.

CONCLUSIONS

The presence of disseminated tumor cells in bone marrow of patients with colorectal cancer detected with RT-PCR does predict a worse clinical outcome in our study. Positive ICC did not find to have this predictive value. In the future, a more detailed and also

functional analysis of the cells found in bone marrow of colorectal cancer patients may help in therapy selection and may give better prognostic information and as such contribute to patient management.

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3

Are disseminated tumor cells in bone marrow and tumor-stroma ratio clinically applicable for patients undergoing surgical resection of primary colorectal cancer? The Leiden MRD study

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Cell Oncol. 2016 Dec;39(6):537-44.

ABSTRACT

Introduction Current TNM staging does not appropriately identify high-risk colorectal cancer (CRC) patients. The aim of this study was to evaluate whether the presence of disseminated tumor cells (DTCs) in the bone marrow (BM) and the presence of stroma in the primary tumor, i.e., the tumor-stroma ratio (TSR), in patients undergoing surgical resection of primary CRC provides information relevant for disease outcome.

Methods Patients with primary CRC (n = 125), consecutively admitted for curative resection between 2001 and 2007, were included in the study. All patients underwent BM aspiration before surgery. Detection of tumor cells was performed using immunocytochemical staining for cytokeratin (CK-ICC). The TSR was determined on diagnostic H&E stained sections of primary tumors.

Results DTCs were detected in the BM of 23/125 patients (18%). No association was found between BM status and overall survival (HR 0.97 (95%CI 0.45-2.09), $p = 0.93$). Also, no significant difference was found in their 5-year survival rate (resp. 72% and 68% for BM-positive versus BM-negative patients). The TSR was found to be associated with a worse overall survival (HR 2.16, 95% CI 1.02-4.57, $p = 0.04$) with 5-year survival rates of 84% versus 62% for stroma-low and stroma-high patients, respectively. No relation was found between the presence of DTCs and TSR.

Conclusions Our data indicate that the presence of DTCs in the BM of CRC patients is not associated with disease outcome. The TSR was, however, found to be associated with a worse overall survival, which indicates that for CRC the tumor microenvironment plays an important role in its behavior and prognosis.

INTRODUCTION

There is a need for predictors of recurrence of disease after resection of colorectal cancer (CRC) with curative intent. If tumor recurrence can be identified early enough, potentially curative reoperations may be considered. Moreover, selective adjuvant treatment may be administered to patients at a high risk of recurrence.

In CRC the prognosis and indication for adjuvant therapy is mainly based on TNM staging. Although this histopathological approach is of paramount importance in cancer classification, for most CRC patients with stage II disease who are classified as standard risk, there are no additional markers to further refine risk assessment or to predict adjuvant chemotherapy benefit. On the other hand, subgroups of stage III CRC patients may not require postoperative adjuvant chemotherapy and may, thus, be prevented from chemotherapy toxicity and side effects. (1) This implies that current staging does not appropriately identify all high-risk patients, especially in the group of lymph node-negative cases (those presumed to have localized disease only), of which 25% does exhibit recurrence of disease. (2)

Available data strongly support the view that disseminated tumor cells (DTCs) may serve as a candidate biomarker suitable for prognostication in (colorectal) cancer. (3;4) Bone marrow (BM) appears to be a common homing site for carcinomas derived from different organs, and may serve as a reservoir of DTCs with the capacity to metastasize to other distant organs. (4-6) In breast cancer, for example, the presence of DTCs is being considered as an independent prognostic factor for reduced survival. (7) There are several methods to detect DTCs (8), of which immunocytochemical staining for cytokeratin (CK-ICC) is the most frequently used and most standardized technique. Earlier studies have indicated that the clinical importance of the detection of DCTs in the BM of CRC patients is still a matter of debate. (3;9)

Before tumor cells become DTCs, they escape from their microenvironment. Nowadays, there is an increasing appreciation of the importance of the tumor microenvironment, including the stromal compartment, in the process of tumorigenesis. This compartment facilitates the survival and proliferation of neoplastic cells and promotes epithelial-mesenchymal transition (EMT), as well as local and metastatic dissemination. Moreover, the stroma of each tumor is different in terms of quantity and cellular composition. In recent years, the tumor-stroma has gained interest in the clinic with regard to patient prognosis and its potential to affect therapy responses. In colon cancer patients the tumor-stroma ratio (TSR) has been identified in several studies as an important prognosticator of disease-free and overall survival. (10-13)

The aim of this study was to determine the clinical importance of DTCs in the BM of patients with primary CRC using immunocytochemical staining for cytokeratin (CK-ICC). We also compared the CK-ICC data to the TSR data. In addition, we evaluated possible relationships between the TSR and DTCs.

METHODS

Patients

A total of 125 consecutive patients were enrolled in the study between May 2001 and November 2007. Only patients with primary CRC (TNM stage I-III) planned for curative resection in the participating hospitals were included. Patients who died within 2 months after surgery were excluded. After surgery all patients underwent routine clinical examination, liver ultrasonography and CEA testing. Forty-two patients with stage III (27/38) or IV (15/18) disease received systemic adjuvant chemotherapy. Follow-up was carried out in all cases and completed until June 2014. Approval of the local Medical Ethical Committees for this study was provided and informed written consent was obtained from all patients.

Bone marrow aspirations

From all patients 5-10 ml BM was aspirated from both sides of the anterior iliac crest under general anesthesia prior to surgery. Before inserting the needle in the anterior iliac crest, an incision was made into the overlying skin to prevent contamination with skin epithelial cells. Mononuclear cells were isolated from the BM by ficoll gradient centrifugation and aliquoted for the preparation of cytospin-slides for immunocytochemistry.

Immunocytochemistry and automated microscopy

The cytospin-slides were stained with primary antibodies directed against cytokeratins 8, 18 and 19 (A45-B/B3; Micromet AG, Munich, Germany), or with isotype antibodies directed against an irrelevant antigen as a negative control (MOPC21; BD Pharmingen, Erembodegem, Belgium). A detailed cytokeratin immunocytochemical (CK-ICC) staining protocol has been published before by Pantel *et al.* (14) CK-ICC staining results in a red precipitate in the cytoplasm of cytokeratin-positive cells. The slides were counterstained with haematoxylin (Mayer's Hemalaum; Merck, Darmstadt, Germany) to visualize the nuclei. The slides were analyzed using an ARIOL SL-50 automated microscope® (Applied Imaging Corporation, San Jose, CA). One slide stained for cytokeratin and one negative control slide were analyzed per patient. Detailed features of this approach have been reported before. (15)

By combining CK-ICC with automated microscopy, cytokeratin-positive cells were confirmed by an independent pathologist and categorized into tumor cells, candidate tumor cells, apoptotic cells or hematopoietic cells, based on morphological criteria according to the guidelines of the European ISHAGE Working Group for Standardization of Tumour Cell Detection [16]. Candidate tumor cells and apoptotic cells were denoted as cells that did not meet all criteria for a positive tumor cell, but could not unambiguously be defined as being normal. A patient was considered positive if at least one tumor cell, one candidate tumor cell or one apoptotic cell was found.

Control samples

To validate our technique, we performed CK-ICC staining on BM derived from 20 breast cancer patients and from 29 individuals that were operated because of a benign disease without any evidence of a malignancy until June 2014.

Determination of the tumor-stroma ratio

Histopathological examination entailed routine microscopic analysis of 5 µm H&E stained sections of the primary tumor as reported before [10]. The slides were selected from the most invasive part of the tumor (i.e., the slides used in routine pathology to determine the T-status), as indicated in the pathology reports, and analyzed by conventional microscopy. In case the pathology information could not be retrieved, all available tumor slides were collected and analyzed (1). Areas with the largest amount of stroma were selected using a 2.5x or a 5x objective. Areas in which both tumor and stromal tissue were present were selected using a 10x objective, after which the final TSR score was determined. Tumor cells should be present at all borders of the image field(s) to be selected. Two observers (GvP, WM) estimated the TSR in a blinded manner. A third independent pathologist was decisive in case of an inconclusive score or a lack of consensus. Scoring percentages were given *per* tenfold (10%, 20%, 30% etc.) *per* image-field. Since rectal cancer patients are pre-operatively treated with radiotherapy, which influences the intra-tumor-stroma, only tissues from colon cancer patients were evaluated for TSR.

Statistical analyses

Frequencies were described as mean plus standard deviation (SD), or median plus range in case of a non-normal distribution. Patients that were found to have synchronous metastases, defined as metastases found during operation or within 3 months, were excluded from the disease-free survival analyses. The recurrence rate of the patients with a positive CK-ICC score was compared to those with a negative CK-ICC score using Cox regression adjusted for sex and age. Hazard ratios (HRs) were calculated by Cox regression analysis for overall survival and disease-free survival. Overall survival was considered from the day of primary tumor surgery to the day of death or censored at the most recent follow-up date. Disease-free survival was considered from the day of surgery to the day of recurrence, or to the day of death, or censored at the most recent follow-up date. Associations between BM or TSR status and survival were depicted in Kaplan-Meier survival curves. Stroma-high was defined as > 50% stroma and stroma-low as ≤ 50% stroma. All analyses were performed using SPSS for Windows (version 23.0, IBM SPSS Inc, Chicago, Ill). *P*-values < 0.05 were considered statistically significant.

RESULTS

The study population comprised 125 patients (65 females and 60 males). No complications of BM aspiration were reported. The patient and tumor characteristics of the study population are listed in Table 1. The median follow-up time from the date of diagnosis of the primary tumor was 6.5 years (range: 0-12 years). Of the 125 patients, 38 developed recurrent disease during follow-up at either single sites (liver (n=20), lung (n=5), peritoneum/lymph node (n=3), bone (n=1)), or at multiple sites (n=9).

Table 1. Clinicopathological characteristics of the study population.

	All patients N = 125	CK-ICC positive N = 23	CK-ICC negative N = 102	*P* value
Gender				
Male	60 (48)	15 (65)	45 (44)	0.055
Female	65 (52)	8 (35)	57 (56)	
Age (years)[a]	69 (41-90)	68 (45-79)	69 (41-90)	0.694
Hospital				
University	67 (54)	17 (74)	50 (49)	0.025*
Affiliated	58 (46)	6 (26)	52 (51)	
Location primary tumor				
Colon	108 (87)	18 (78)	90 (88)	0.175
Rectum	17 (13)	5 (22)	12 (12)	
TNM stage				
I	22 (18)	5 (22)	17 (17)	
II	47 (38)	7 (30)	40 (39)	0.596
III	38 (30)	6 (26)	32 (31)	
IV	18 (14)	5 (22)	13 (13)	
TSR[b]				
Stroma-low	57 (59)	9 (50)	48 (61)	0.282
Stroma-high	40 (41)	9 (50)	31 (39)	
Number of LNs[a]	14 (1-33)	12 (1-20)	14 (1-33)	0.188
Follow-up (years)[a]	6.5 (0-12)	7.0 (0-12)	5.8 (0-12)	0.851
Death	41 (33)	8 (35)	33 (32)	0.501

Data stated in number (%), unless otherwise stated.
[a]Stated in median (range)
[b]Only colon cancer patients evaluated (total N = 97)
Abbreviations: CK-ICC; cytokeratin immunocytochemistry, TNM; tumor-node-metastasis, TSR; tumor-stroma ratio, LNs; lymph nodes

Prevalence of DTCs in BM

In 23 of the 125 patients (18%) disseminated tumor cells (DCTs) were found in the BM using CK-ICC. Table 1 shows, next to the clinical parameters of the patients included in the study, the percentage of BM-positive patients per TNM stage. We found that the percentages of BM-positive patients per stage did not differ significantly. Three patients developed bone metastases of which two were classified as BM-positive. The presence of DCTs in the BM was not found to be associated with TSR ($p = 0.28$).

Control groups

In Tables 2 and 3 the patient characteristics of both control groups are depicted. In 9 of the 20 patients (45%) operated because of breast cancer, CK-ICC positive cells were detected in the BM, whereas in 2 of the 29 patients (7%) operated because of benign disease, CK-ICC positive cells were detected in the BM.

Table 2. Characteristics of the breast cancer control group.

Breast cancer patients	N = 20
Age (years), median (range)	56 (40-74)
Stage	
I	14
II	3
III	3
CK-ICC positive	**9**

Abbreviation: CK-ICC; cytokeratin immunocytochemistry

Table 3. Characteristics of the benign control group.

Benign disease	N = 28
Sigmoid resection (diverticulitis)	5
Bowel resection (inflammatory bowel disease)	13
Bowel resection (tubulovillous adenoma)	3
Cholecystectomy	4
Benign stenosis duodenum	1
Hernia repair	2
CK-ICC positive	**2**

Abbreviation: CK-ICC; cytokeratin immunocytochemistry

DTCs and survival

Patients with a CK-ICC negative BM were not found to exhibit a significantly better overall survival (OS) than those with CK-ICC positive cells in the BM (Fig. 1a); HR 0.97 (95%CI 0.45-2.09), $p = 0.93$. No significant difference was found in the five year survival rate of

both groups (68% and 72%, respectively). Also, the disease-free survival (DFS) did not show a significant difference between the BM-positive and BM-negative patient groups (Fig. 1b); HR 0.80 (95%CI 0.35-1.82), $p = 0.59$.

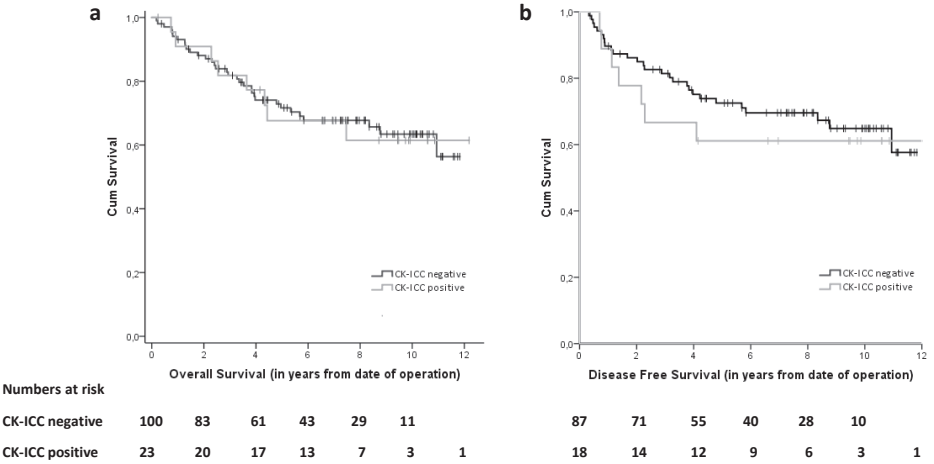

Fig 1 Kaplan-Meier survival curves for CK-ICC negative and CK-ICC positive patients: overall survival (**a**) and disease-free survival (**b**) in patients after surgery for primary colorectal cancer.

Subgroup analyses

In the lymph node-negative (stage I-II) patients, no significant difference was observed between the BM-negative and BM-positive cases in OS or DFS (Fig. 2); HR 0.85 (95%CI 0.24-3.04), $p = 0.80$ and HR 1.83 (95%CI 0.66-5.09), $p = 0.25$, respectively. Also in the group of elderly patients (> 70 years), no significant difference was found between the BM-negative and BM-positive cases in OS (n = 58; $p = 0.25$) or DFS (n = 49; $p = 0.24$). Adjustment for sex, age, tumor location and chemotherapy did not change the results for any of these survival analyses.

TSR and survival

Five out of 108 pre-selected patients could not be analyzed due to a poor quality of the histological material and of 6 patients material was not available, leaving H&E sections from 97 patients for TSR analysis. Fifty-seven of these sections (59%) were scored as stroma-low and 40 (41%) as stroma-high. Patients with a high stroma percentage within the primary tumor showed a trend towards a worse OS in an univariate analysis; HR 1.84 (95%CI 0.89-3.82), $p = 0.10$, with a 5 year survival rate of 84% versus 62% in those with a low stroma percentage (Fig. 3). After adjustment for sex, age and chemotherapy, we found that TSR serves as a prognostic factor for a worse OS in patients with a high TSR; HR 2.16 (95%CI 1.02-4.57), $p = 0.04$. In case of DFS no significant difference between

stroma-low and stroma-high patients was observed. The five year DFS rates were 76% and 71% for the stroma-low and stroma-high cases, respectively.

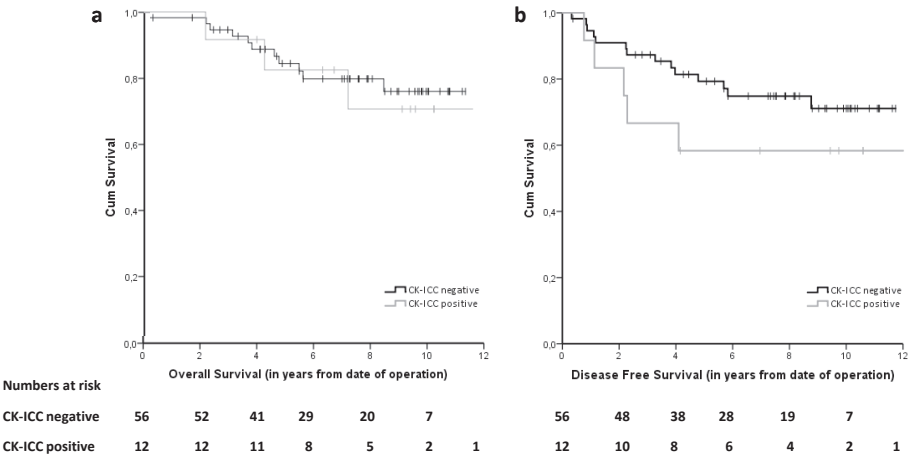

Fig 2 Kaplan-Meier survival curves for CK-ICC negative and CK-ICC positive patients: overall survival (**a**) and disease-free survival (**b**) in lymph node-negative patients after surgery for primary colorectal cancer

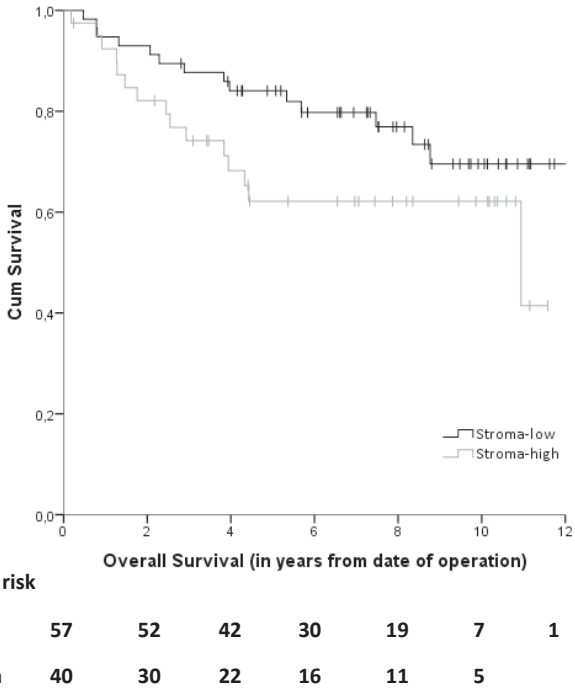

Fig 3 Kaplan-Meier overall survival curve for stroma-low and stroma-high patients after surgery for primary colon cancer

DISCUSSION

In nearly one-fifth of the patients with CRC, including early stage cases, we found DTCs in the BM. These DTCs, detected by CK-ICC, were not found to impose a significant impact on the prognosis of patients that underwent resection of the primary tumor. The idea that a high amount of stroma in the primary tumor may be related to the presence of DTCs was not confirmed by our study. We did find, however, that a high stroma-percentage was associated with a worse overall survival in the CRC patients included in this study.

Results obtained by others aimed at assessing the prognostic impact of DTCs in primary CRC cases have so far been conflicting. The amount of positive DTCs in BM were e.g. found to range from 24% to 64%. (6; 17-22) These differences in (mostly) cytokeratin stained cells may reflect the use of different techniques. Flatmark *et al.* (23) found that the use of two different techniques (immunomagnetic selection with an anti-EpCAM antibody and CK-ICC) resulted in a minimal detection overlap although, surprisingly, the results of both methods were found to be associated with disease outcome in distinct CRC prognostic subgroups. The main conclusion from their study was that the presence of DTCs in BM may serve as a prognostic biomarker for curatively resected CRC patients.

In some studies, the presence of DTCs was found to be associated with a shorter disease-free survival (18;24), whereas in other studies no prognostic relevance of DTCs in the BM was noted. (6;19;21) Steinert *et al*. (6) detected the same amount of CK-ICC positive cells throughout all CRC TNM stages, and Lindemann *et al*. (18) failed to find a significant difference between lymph node-positive and -negative patients, although they did find that the detection of tumor cells in the BM may serve as an independent determinant of relapse. In both studies immunocytochemistry was used for BM analysis. Leinung *et al*. (17) found an increased percentage of CK-ICC positive cells in the BM in rectal cancer patients compared to colon cancer patients. In 2010, Rahbari et al. published a meta-analysis (in which the above described studies were also included) to assess whether hematogeneous DTCs represent a prognostic factor in patients with CRC. They found a strong prognostic effect of circulating tumor cells in the peripheral blood but, in accordance with our findings, this effect was not found for DCTs in the BM. (25)

A pooled analysis of micro-metastases in the BM of breast cancer patients (7) revealed that the presence of DTCs in the BM was predictive for the development of distant metastases after long term follow-up. Tumor cell persistence in BM was also found to be an independent prognostic factor for subsequent breast cancer survival. (26) In contrast with breast cancer, in which bone is one of the most preferential metastatic target sites affecting more than half of the patients during the course of their disease, the incidence of bone metastases in CRC is rare. (27;28) Three of our 125 patients developed skeletal metastases during follow-up, two of which were BM-positive. In 18% of the patients DTCs

were detected in the BM, but this does not seem to reflect the presence of skeletal metastases. In our control group of 20 breast cancer patients, we found in 45% of the cases DTCs in the BM. This result is in accordance with other studies reporting DTC occurrences in the BM of 13% to 50% in early stage breast cancer patients, and increases in patients with metastatic breast cancer of up to 70%. (29) In 2 of 29 non-carcinoma control cases we found CK-ICC positive DTCs in the BM. This finding is not novel. Depending on the antibody used, CK-positive cells in BM have been found in up to 5.5% of individuals without a known malignancy. (29) Therefore, it may be questioned whether all disseminated epithelial cells in the BM are truly malignant cells. Although earlier characterization of these cells appeared to be difficult, a malignant phenotype has been suggested. (20;30) So, the option must be considered that these cells are indeed tumor cells, but that these cells may not all have the capacity to further metastasize. On the other hand, it has recently been found in an experimental model of colon cancer induced by male cancer cells injected into female nude mice that disseminated 'tumor' cells (i.e., epithelial-like cells) may be composed of two different populations, one originating from the cancer (cytokeratin-positive, Y chromosome-positive) and one originating from the resident BM cells. (31)

The clinical relevance of the molecular detection of DTCs appears to be restricted by both tumor cell heterogeneities and technical limitations. (32) Genomic analyses at single cell level have shown that DTCs detected with anti-cytokeratin antibodies frequently exhibit heterogeneous tumor-specific aberrations, particularly in patients without overt metastases. (33) Many tumors are known to undergo an extended period of 'dormancy', but little is known about the mechanisms underlying the 'awakening' of the dormant tumor cells, which may ultimately lead to the formation of metastases. The steady state of dormancy may be disturbed by changes in both DTCs and their surrounding microenvironment. (33) Tumor cells in the BM of CRC patients may be in the "wrong" environment and, as such, be kept in the dormant state. (21) Future efforts towards comprehensive genomic analysis of DTCs may provide a deeper understanding of the clinically relevant biology of DTCs. (34)

For many years, tumor stromal formation or desmoplasia was considered to be a passive bystander of tumorigenesis and tumor progression. However, during the last 10 years, attention has shifted towards the tumor microenvironment and it is now well established that tumor-stroma plays an important role in cancer initiation and progression. The tumor-stroma interacts with nonmalignant cells as well as with malignant cells during different stages of tumorigenesis, ranging from tumor onset to invasion and metastasis. (35;36) TSR is a relatively simple to apply and cheap cell-based parameter, which is a strong quality. Multiple other studies have confirmed significant prognostic implications of the TSR not only in colon cancer, but also in other solid tumors. (37-42)

Further research using a larger prospective cohort should bring this parameter closer to implementation in the TNM classification system.

CONCLUSIONS

DTCs were frequently detected in the BM of CRC patients, but its presence (as based on CK-ICC) did not predict a worse clinical outcome. In the future, a more precise molecular characterization and functional analysis of DTCs in the BM may provide a definite clue to its possible prognostic impact. In the meantime, a pooled analysis of multi-institutional studies on DTCs in the BM of CRC patients may provide more solid information on its prognostic impact. We found that TSR was associated with a worse overall survival in colon cancer patients. Considering its simplicity and availability for conventional clinical pathology, TSR may serve as a new prognostic histological biomarker after its prognostic value has been confirmed in a large prospective study.

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4

The number of high-risk factors is related to outcome in stage II colonic cancer patients

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Eur J Surg Oncol. 2011 Nov;37:964-70.

ABSTRACT

Introduction A subgroup of stage II colonic cancer patients are considered to be at high-risk for recurrent/metastatic disease based on 1) tumor obstruction/perforation 2) < 10 lymph nodes 3) T4 lesions and 4) lymphangioinvasion. Their prognosis is regarded as comparable to stage III (T1-4N+M0) colonic cancer and it is therefore strongly advised to treat them with adjuvant chemotherapy. The purpose of this study was *i)* to determine the magnitude of prognostic significance of the conventional high-risk factors and *ii)* to determine whether the number of high-risk factors influences outcome.

Methods We retrospectively analyzed 212 stage II colonic cancer patients undergoing surgery between January 2002 and December 2008. No adjuvant chemotherapy was given. Survival analyses were performed.

Results 154/212(73%) patients were considered to be high-risk patients based on conventional high-risk factors. 58 patients did not meet any high-risk factor, 125 patients met 1 high-risk factor and 29 patients met $\geq$2 high-risk factors. Median follow up was 40 months. Multivariate analysis identified four independent risk factors for recurrent/metastatic disease: age, obstruction, perforation and lymphangioinvasion. The three-year-DFS-rates for the low-risk group, the high-risk group with 1 high-risk-factor and the high-risk group with $\geq$ 2 high-risk-criteria are 90.4%, 87.6% and 75.9% respectively. Patients meeting $\geq$2 conventional high-risk criteria had a significantly worse three-year-disease-free survival ($p < 0.002$*)*.

Conclusions Four independent high-risk factors were identified. The number of high-risk factors does influence outcome. high-risk More attention should be given to the definition and treatment of high-risk stage II patients.

INTRODUCTION

Colonic cancer is the second leading type of cancer in the Netherlands. At the moment, more than 7500 new cases of colonic cancer are diagnosed yearly. Approximately 16% and 38% respectively will have stage I (T1-2N0M0) and II (T3-4N0M0) colonic cancer at time of diagnosis.(1) The number of patients diagnosed with colonic cancer is expected to increase further in the near future due to ageing of the general population.

Usually, patients with curatively resected colonic cancer (CC) without nodal tumor involvement (stage I-II) do not receive adjuvant chemotherapy in the Netherlands. In spite of potentially curative resection, about 10% of the patients with stage I and 15-30% with stage II disease will eventually develop recurrent locoregional disease or distant metastases within 5 years. Therefore, a subgroup of stage I-II colonic cancer patients are currently considered to be at high-risk for recurrent disease and/or metastases based on 1) tumor obstruction OR perforation at presentation 2) less than 10 lymph nodes detected in the surgical specimen 3) T4 lesions and 4) lymphangioinvasion at pathological examination. These patients, especially those having stage II disease, are regarded as comparable to stage III colon cancer and it is therefore, according to guidelines, strongly advised to treat them with adjuvant chemotherapy. (2)

Stage I-II colonic cancer patients without risk factors are generally thought to have no benefit from adjuvant treatment, because individual randomized trials do not show a survival benefit in stage II colonic cancer patients. (2) However, to stress out the importance of further investigation, stage I-II CC patients do suffer from disease recurrence and their overall 5-year survival is only around 70-80%.

Petersen et al proposed a cumulative prognostic index to define different prognostic stage II (T3-4N0M0) subgroups, based on pathological high-risk criteria (peritumoral involvement, venous spread, spread involving the surgical margin and perforation of the tumor). The predictive ability of this index was testes further by Morris et al, showing a 24% absolute difference in 5-year survival between indexed high-risk and low-risk patients ($p<0.001$). (3) Such a prognostic tool might be helpful in selecting the right high-risk patients for adjuvant treatment.

The results of meta-analyses and systematic reviews show at the most a slight disease-free survival benefit of adjuvant chemotherapy in stage II disease. (4) It is because of these results that stage II CC patients do receive adjuvant treatment in Eastern countries and the United States. Thus, there is a (international) need for better delineation of high-risk stage I-II CC patients who should be offered adjuvant treatment.

This study aims to identify the abovementioned and/or additional high-risk factors in stage II colonic cancer patients related to oncological outcome and investigate whether the number of high-risk factors present relates to outcome.

METHODS

Patients

We retrospectively analyzed all colonic cancer patients treated in our large community teaching hospital between 2002 and 2008. A cohort of 236 patients was identified as having stage II (T3-4N0M0) colonic cancer in our hospital. Twenty-four patients received adjuvant chemotherapy and were excluded from this study. A total of 212 stage II colonic cancer patients who were treated by radical surgical resection alone were analyzed.

Methods

We performed surgical, pathological and radiological chart reviews to collect our patient characteristics and treatment data. The following patient characteristics were collected: age, gender, medical history, clinical tumor staging, initial complaints at presentation, emergency presentation with obstruction or perforation, emergency surgery, presence of conventional high-risk factors ((1) tumor obstruction OR perforation at presentation 2) less than 10 lymph nodes detected in the surgical specimen 3) T4 lesions and 4) lymphangioinvasion at pathological examination), number of conventional high-risk factors, type of surgery, size and location of the tumor, operating time, type of anastomosis, postoperative complications, relaparotomy, pathological staging, number of lymph nodes, total hospital stay, intensive care unit admission, recurrent/metastatic disease, survival and death.

T4 status was defined as pathological T4 status. Therefore, patients with obstruction or perforation were not included in the calculation of the number of T4 patients to make is possible to analyze these high-risk factors independently.

Emergency surgery was defined as non-elective surgery for obstruction of perforation.

Preoperative staging consisted of physical examination, laboratory examination, colonoscopy, abdominal ultrasound and/or computed tomography of the abdomen and a X-ray of the chest. In case of laparoscopic surgery patients received Klean prep® bowel preparation. Surgery was performed by a surgeon or a supervised surgical resident. Pathological examination after surgery was performed at our pathological department. Follow up was performed in accordance to national guidelines consisting of CEA every 3 months for the first 2 years, then every six months for 5 years, abdominal ultrasound or CT-scan of the abdomen and chest X-ray every twelve months for 5 years and colonoscopy every 3 years. (2)

Statistical analysis

SPSS 17.0 was used to evaluate our data. Survival analyses for high-risk patients based on obstruction, perforation of lymphangioinvasion were performed by means of the Kaplan Meier survival function.

Disease-free survival (DFS) was defined as the time from operation to the earliest recorded documentation of local/regional or distant recurrence of colon cancer, new 2nd primary colon cancer or death from any cause. A multivariable Cox regression analysis was performed to identify independent high-risk factors for recurrent/metastatic disease in stage II colon cancer patients. P-values <0.05 were considered statistically significant.

RESULTS

We included 212 stage II colonic cancer patients with a mean age of 71 years old. (range 39-95). Median follow up was 40 months ranging from 18 - 108 months. Patient characteristics are outlined in Table 1.

Table 1. Patient characteristics in stage II colonic cancer patients

Patients characteristics (n=212)	n		median
Age (range)		39-95	73
Male gender (%)	110	51.9%	
Tumor location (%)			
– Rightsided	116	54.7%	
– Leftsided	96	45.3%	
Tumor size (range cm)		2-12	5
Perforation (%)	7	3.3%	
Obstruction (%)	25	11.8%	
T4 (%)	2	0.9%	
Number of lymph nodes harvested (range)		1-24	7
< 10 lymph nodes (%)	148	69.8%	
Lymphangioinvasion (%)	6	2.8%	
Follow up (months)		18-108	40
Recurrent disease (%)	7	3.3%	
Metastatic disease (%)	27	12.7%	

Identification of high-risk criteria

The following six factors for recurrent/metastatic disease were identified through univariate analysis: age, length of hospital stay, emergency surgery, obstruction, perforation of the tumor and lymphangioinvasion. The number of retrieved lymph nodes and the presence of a T4 tumor were found not to be statistically significant factors in the univariate analysis.

Multivariate analysis including age, length of hospital stay, emergency surgery, number of lymph nodes, obstruction, perforation, lymphangioinvasion and the presence of a

T4 lesion identified four independent risk factors for recurrent/metastatic disease: 1) age (p=0.016; hazard ratio (HR 1.048), 2) obstruction (p=0.001, HR 3.797), 3) perforation of the tumor (p=0.003; HR 4.906) and 4) lymphangioinvasion (p=0.005; HR 5.643). The often in guidelines mentioned criterion of less than ten lymph nodes detected in the surgical specimen did not reach significance in our multivariate analysis. (Table 2)

Kaplan Meier survival curves for obstruction, perforation and lymphangioinvasion are shown in Figure 1.

Table 2. Univariate and multivariate analysis for recurrent / metastatic disease

	Cox regression analyses for recurrent / metastatic disease					
	Univariate analysis			Multivariate analysis		
Variable	**p value**	**Hazard Ratio**	**95% CI hazard ratio**	**p value**	**Hazard ratio**	**95% CI hazard ratio**
Age	0.016	1.048	1.009-1.089	0.008	1.057	1.014 – 1.101
Length of hospital stay	0.006	1.016	1.004-1.027	0.618	1.002	0.993 – 1.012
Emergency surgery	0.002	3.653	1.624-8.215	0.685	0.698	0.123 – 3.955
Number of harvested lymph nodes	0.527	0.969	0.879-1.068	*		
Less than 10 lymph nodes	0.930	0.981	0.639-1.506	0.849	0.917	0.377 – 2.231
Obstruction	0.001	3.797	1.815-8.721	0.030	5.977	1.186 – 30.13
Perforation	0.003	4.906	1.705-14.011	0.021	4.730	1.263 – 17.713
Lymphangio- invasion	0.005	5.643	1.686-18.894	0.002	7.164	2.038 – 25.175
T4 lesion	0.824	4.511	0-2723026	0.983	0	0.000 – ∞
Length of specimen	0.066	1.016	0.999-1.033	*		
Length of tumor	0.906	1.013	0.819-1.252	*		
Laparoscopy/ laparotomy	0.985	0.994	0.545 – 1.814	*		
Operating time	0.432	1.003	0.996 – 1.010	*		

Significant if $p < 0.05$
Probability of stepwise entry 0.05
Probability of removal 0.10
*Factor not analyzed in multivariate analysis

Survival analysis based on conventional high-risk criteria

After the multivariate analysis we performed a Kaplan Meier survival analysis for the following groups. Group A1 consisted of 58 patients meeting none of the four conventional high-risk factors. Group B1 consisted of 125 patients meeting one of the conventional high-risk factors (<10LNs in 117/125 patients) and group C1 consisted of 29 patients meeting two or more of the conventional high-risk factors.

Three-year disease-free survival (DFS) rates for group A, B and C are 90.4%, 87.6% and 75.9% respectively. Patients in group C1 had a significantly worse three year DFS, with $p = 0.002$. (Figure 2)

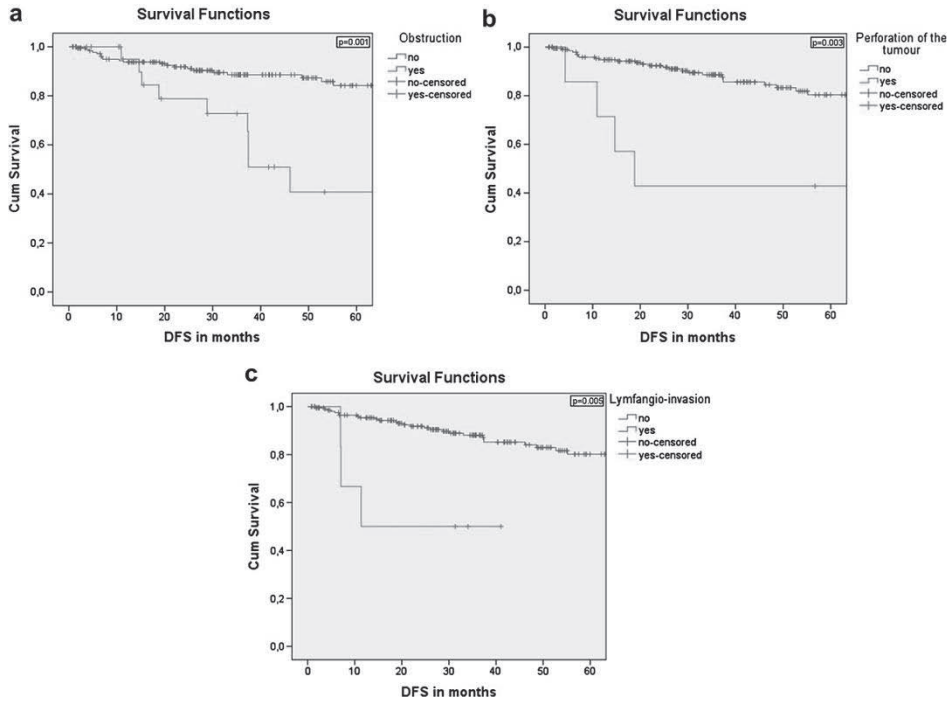

Figure 1: Kaplan Meier survival curves for a) obstruction b) perforation and c) lymphangioinvasion.

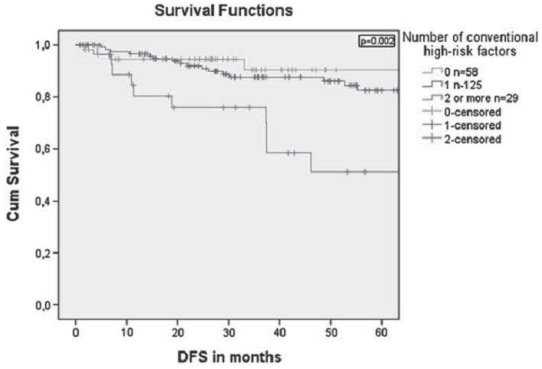

Figure 2: Kaplan Meier survival plot conventional high-risk factors

Survival analysis based on high-risk criteria identified in our study

When performing an additional Kaplan Meier survival analysis using not the conventional risk factors, but the risk factors identified in this series (age, obstruction, perforation, lymphangioinvasion), we identified the following patient groups. Group A2 consisted of 177 patients meeting none of the high-risk factors we identified, group B2 consisted of 32 patients meeting one of the high-risk factors we identified and patient

group C2 consisted of three patients meeting two of the high-risk factors we identified in our multivariate analysis. As expected, even more profound differences in the survival analysis were observed with three-year DFS rates of 90.2% vs.77.6% group A2 and B2 and respectively. (Survival of group C2 not shown, because of the small number of patients in this subgroup (n=3)). Patients in group B2 have a significant worse disease-free survival compared to group A2. (p <0.001) (Figure 3)

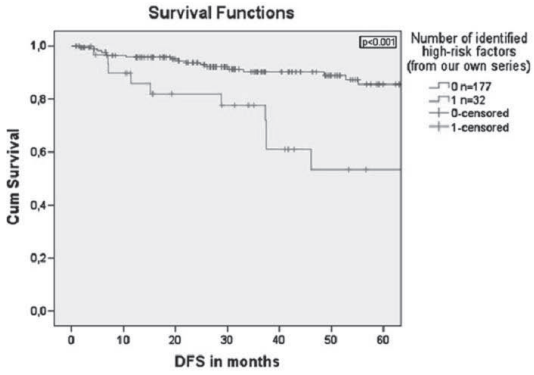

Figure 3: Kaplan Meier survival plot high-risk factors of our series

DISCUSSION

Identification of high-risk patients

In our series of stage II colonic cancer patients, four independent high-risk factors were identified after multivariate analyses, *i.e.* age, obstruction of the tumor, perforation of the tumor and lymphangioinvasion at pathological investigation. Pathological T4 status was not associated with a worse outcome in this study, but this was probably caused by the small number of patients in this subgroup (n=2) and the fact that there were 22 T4 patients in the initial 236 stage II patients identified, however they were excluded from this analysis since they received adjuvant chemotherapy. Patients with obstruction or perforation were not defined as T4 tumors, since that would make it impossible to analyze obstruction, perforation or pathological T4 status as three independent high-risk factors.

No new high-risk factors were identified, although perforation and obstruction were both identified separately as a high-risk factor indicating poor prognosis. All high-risk factors have been observed in other retrospective series, but prospective randomized trials on these "high-risk factors" are lacking (5-7).

Age is a known high-risk factor. However, the hazard ratio in our series analyzed as a continuous factor is only 1.057. Still, younger cancer patients are offered chemotherapy more often than older cancer patients (> 70 years). Many older patients are frail and

therefore not offered adjuvant chemotherapy. However, studies have shown that elderly patients have the same benefit from adjuvant chemotherapy in colonic cancer. So far, no prospective studies, including geriatric assessments, in this vulnerable group of patients have been published (8;9). Currently, multiple randomized trials focusing on chemotherapy schedules in this older patient group are ongoing (10). Hopefully they will provide useful clinical tools for oncologists to distinguish whom to treat and not to treat.

The number of high-risk factors and outcome

The number of high-risk factors is significantly related to outcome. All patients with high-risk factors, but especially those patients with two or more of the conventional high-risk factors (tumor obstruction OR perforation at presentation, 2) less than 10 lymph nodes detected in the surgical specimen, 3) T4 lesions and 4) lymphangioinvasion at pathological examination) have a significantly worse three-year disease-free survival. After survival analysis using the identified high-risk factors from our own data (age, obstruction, perforation, lymphangioinvasion), both perforation of the tumor and obstruction were identified as independent high-risk factors and patients with only 1 high-risk factor had a significantly worse 3-year DFS compared to patients without any high-risk factor.

Nowadays, several DFS definitions are used and DFS is appealing as a surrogate end point for overall survival. Several large randomized trials, including NSABP, X-act and MOSAIC studies have included DFS as a new primary end point and provided DFS definitions in their study reports. These studies can be divided in two groups; those who consider the occurrence of second *non-colorectal* as an event and those who do not. The advantage of including the occurrence of a second non-colorectal cancer is capturing the possible treatment induced secondary malignancies. On the other hand, if cancers are likely to be induced by treatment, the sensitivity of the treatment effect is diminished.

However, since our patients were not treated adjuvantly, we chose to use the DFS definition including local/regional distant recurrence of colon cancer, 2nd primary colon cancer or death from any cause, but not the secondary non-colorectal cancers.

A previous study of Quah identifying risk factors in stage II colonic cancer, also pointed out that the number of high-risk factors was related to outcome. However, they did not use the 'ASCO' high-risk factors, but the three high-risk factors from their own analysis (T4 tumor, preoperative CEA > 5ng/ml, lymphovascular OR perineural invasion). We did not perform a survival analysis on CEA level, since preoperative CEA levels were not known in the majority of our patients. (6)

Our results indicate that certain high-risk factors have more impact on outcome than others and the combination of high-risk factors in the individual patient should be as-

sessed to select and offer the best individual adjuvant treatment schedule, as proposed by Petersen et al. (3)

Lymph node retrieval and survival

The number of lymph nodes detected in the surgical specimen remains a hot topic. We did not show a significant relationship between the number of lymph nodes and prognosis/survival in our series. However, in almost 70% of our patients lymph node retrieval was insufficiently. Because patient group B1 (Figure 2) consisted of 117/125 patients with an insufficient lymph node retrieval as the solely high-risk factor and this factor was not a significant factor in our series, we indeed expected to see that patients in the B2 high-risk group (high-risk factors from our series; lymph node retrieval excluded) in Figure 3 showed a more pronounced negative influence on outcome compared to patients without any high-risk factors.

There seems to be a correlation between the number of lymph nodes found in the surgical specimen and accuracy of staging and prognosis in colonic cancer patients. (11-13)

Recurrence and metastatic disease rates in our study, 12.7% and 3.3% respectively, seem rather low. However, our patient group is a selected group of stage II patients who did not receive chemotherapy. Patients who did receive chemotherapy were excluded and a number of these excluded patients did have recurrent/metastatic disease, so our results probably present an underestimation of the recurrence/metastatic disease rates.

Adjuvant chemotherapy in stage II colonic cancer patients

Currently, patients with stage II colonic cancer are mostly treated by surgery alone. However, patients with high-risk stage II disease, with a prognosis comparable to stage III patients, should be offered chemotherapy in the current (inter)national guidelines. (14;15) Therefore, high-risk stage II patients should be identified properly to select them for adjuvant treatment schedules. However, there is still no international consensus about the definition of high-risk stage II disease colonic cancer patients and the consequences of such a label, because of lacking prospective controlled trials on this subject. In the Netherlands, usually, the ASCO guidelines on high-risk factors are followed. (16)

The ASCO factors are obtained retrospectively from large adjuvant chemotherapy studies in stage II and III colonic cancer. No overall survival benefit of adjuvant chemotherapy was observed in stage II disease in these trials. The results of meta-analyses and systematic reviews of those trials show at the most a slight, but mostly not significant, disease-free survival benefit of adjuvant chemotherapy in stage II disease of 3-6% absolute risk reduction (ARR) in disease-free survival (3.8 – 6% ARR in 5-yr DFS by Figueredo et al, 3% ARR in 5-yr DFS in the IMPACT-B2 study, 4% ARR in 5-yr DFS in the Gill-study. (17;18) The QUASAR study was the first RCT that showed a significant survival improvement in stage II colonic cancer patients after chemotherapy. Relative risk of recurrence

was 0.67 in the chemotherapy group versus 0.91 in the observational group with a p-value <0.001 with an absolute survival five year benefit of 3.6%. Unfortunately, no stage II high-risk factors were indicated. (19)

However, the before mentioned ASCO risk factors were associated with disease recurrence in stage II disease. Therefore, these factors are implemented in (inter)national guidelines. There is no uniform utility of these factors, however, and we were interested in the prognostic role of them in our patient population to better delineate the stage II patient group who could benefit from adjuvant chemotherapy.

CONCLUSIONS

Based on the high-risk factors we identified in this series and the 'ASCO' recommendations, not all recurrences and/or metastases can be explained. Possibly, other high-risk factors can influence or predict outcome in stage II colon cancer patients and further research seems warranted. (20-22) Figueredo et al. concluded in their Cochrane review that the need to further define high-risk features is present and randomization to observational arms remains ethical. Currently, a randomized, prospective study to the relevance of micrometastases with the introduction of the sentinel node procedure and the effect of adjuvant chemotherapy on DFS in stage I-II colonic cancer patients is started in the Netherlands.

High cancer-specific mortality is especially true for stage II colonic cancer. Therefore, it is time for surgeons, pathologists and medical oncologists combined to focus on the selection and treatment of high-risk node-negative colonic cancer patients. (23)

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5

The diagnostic value of one-step nucleic acid amplification (OSNA) for sentinel lymph nodes in colon cancer patients

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Ann Surg Oncol. 2014 Nov;21(12):3924-30.

ABSTRACT

Introduction Lymph node (LN) status in colon cancer is critical for prognosis estimation and treatment allocation. The aim of this study was to compare the performance of one-step nucleic acid amplification (OSNA) through detection of cytokeratin 19 mRNA levels with routine pathological examination (RP) and multilevel fine pathological examination (FP) in sentinel lymph nodes (SLN), detected using the *ex vivo* SLN mapping (SLNM) procedure, in pre-surgically defined non-metastatic colon cancer patients.

Methods In this prospective study, 325 SLNs of 128 patients from the Jeroen Bosch Hospital in 's-Hertogenbosch and the Leiden University Medical Center were investigated by RP (H&E), FP (H&E and Keratin Pan immunohistochemical staining) and OSNA. The SLNs were harvested by the SLNM procedure, using Patent blue or Indocyanine green. SLNs were divided and separate parts were used for RP, FP and the OSNA assay.

Results The diagnostic value of OSNA was 82.1% and 100% for both FP and combined method (OSNA and FP) when compared to RP. An upstaging rate of 20.2% was obtained with the use of OSNA only and 36.4% with the use of FP only. An upstaging rate of 46.5% was obtained by combining the two methods together.

Conclusions OSNA and FP appeared to be promising tools for the detection of lymph node micro-and macrometastases in SLNs after SLNM. The performances of OSNA and FP in this study were superior to RP. Since OSNA allows analysis of the whole lymph node, sampling bias can be avoided. OSNA may therefore improve tumor staging.

INTRODUCTION

In the Netherlands, colorectal cancer (CRC) represents the second most frequent cancer in terms of incidence with over 12,000 newly diagnosed patients and 5,000 deaths each year. For colon cancer, approximately 9,000 new patients are diagnosed each year. (1) Determination of the lymph node (LN) status is an important prognostic factor in CRC, which is critical for staging of these tumors and for allocation to adjuvant therapy when metastases in lymph nodes are found. (2;3) Current guidelines suggest that at least ten LNs in the mesentery of the tumor have to be detected by pathologists. (4) However, the number of resected LNs for accurate tumor staging according to the TNM classification is still debatable, and often decisions for adjuvant treatment allocation are based on the detection of less than ten LNs. (5-7)

Approximately 30% of node-negative CRC patients suffer from recurrent disease within 5 years after surgery, even when ten LNs are detected. (8) It has been suggested that this high recurrence rate is partially caused by nondetection of metastases during histopathological examination. (9-11) The postoperative examination of LNs is based on microscopic examination of one hematoxylin and eosin (H&E)-stained slide. This slide is prepared from the LN section with the largest cutting surface. (12) Therefore, only one part of the LN is examined which might lead to incorrect staging of the tumor as has been shown in earlier studies. (13;14) The role of micrometastases or isolated tumor cells (ITC) in LNs therefore might have an important role in further prognostication.

It has been demonstrated that multilevel sectioning and the use of immunohistochemistry (IHC), defined as fine pathological examination (FP), improves the detection of micro metastases in LNs. (15) Therefore, a novel technique - one-step nucleic acid amplification (OSNA) - using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method to amplify cytokeratin 19 (CK19 mRNA), has been developed. CK19 is an epithelial marker, which is highly suggestive for the presence of metastases when identified in LNs. (16;17) OSNA is already in clinical use for the diagnosis of LN metastases in breast cancer patients. (16-18) In CRC, the use of OSNA is under investigation with promising results. (12;19;20)

Sentinel lymph nodes (SLNs) are the LNs that have the highest potential to harbor metastasis due to the fact that they are directly in communication with the location of the tumor. (21) Different studies showed that ex vivo sentinel lymph node mapping (SLNM) is an easy and feasible technique for ultra-staging CRC patients. This technique was characterized by a high accuracy of 90-100% and negative predictive value of 80-100%. (22;23) A rate of 19-57% upstaging has been observed. (23) Examination of SLNs through the OSNA technique might therefore result in upstaging of patients with otherwise negative LNs. The objective of the current prospective investigation was to compare the performance of OSNA with standard H&E (Routine pathology, RP) and FP

of the SLNs, detected using the ex vivo SLN mapping, in presurgically defined nonmetastatic colon cancer patients.

METHODS

Patients

Patients who were diagnosed with stage I or II colon cancer presurgically, thus not showing any evidence of lymph node involvement or metastasis on radiological examination before surgery, and who underwent surgery between September 2010 and December 2013 at the Jeroen Bosch Hospital and the Leiden University Medical Center (LUMC) were prospectively included in the study. A maximum of three sentinel lymph nodes nodes were harvested from the mesentery of these patients. All other detected LNs were investigated according to local practice (RP). Patients with a history of cancer and patients who received neoadjuvant chemotherapy or other pre-or intraoperative therapy were not eligible for inclusion. In total, 140 patients from two study sites were included, 105 patients at the Jeroen Bosch Hospital and 35 and the Leiden University Medical Center. Twelve patients were excluded from final analysis due to missing pathological data (n=3), missing OSNA data (n=5) or absence of cancer in the resected material (n=4), resulting in a total cohort of 128 colon cancer patients. The medical ethics committee of the participating hospitals approved this prospective study. This study was performed according to the code of conduct for responsible use.

Sentinel lymph node processing

As part of this study, the ex vivo SLN mapping with Patent Blue Dye V (Jeroen Bosch Hospital) or Indocyanine Green (Leiden University Medical Center) was performed within fifteen minutes after the resection, as previously described. (24) Blue-colored or fluorescent SLNs were identified, marked and extracted. If the short axis of a SLN was equal to or larger than 10 mm, the SLN was cut as equally as possible into four blocks, and labeled as “a”, “b”, “c” and “d”. Residual block “c” and block “a” were stored at -80° degrees for up to 6 months until being analyzed by the OSNA Assay. Blocks “b” and “d” were paraffin-embedded and assessed by RP and FP using immunohistochemical (IHC) staining. If the short axis of a SLN was no larger than 10 mm, the SLN was cut as equally as possible into two blocks, “a” and “b”. Block “a” was stored at -80° degrees for up to 6 months until being analyzed by OSNA Assay. Block “b” was paraffin-embedded and assessed by RP and FP using immunohistochemical (IHC) staining (figure 1).

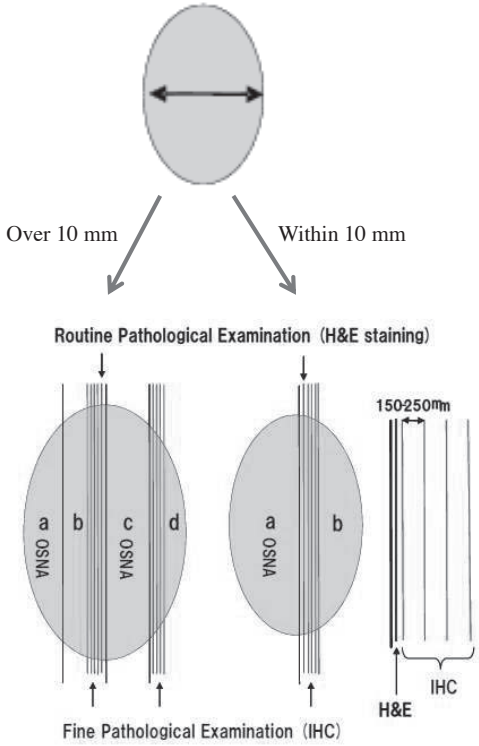

Figure 1. Study Design and Sentinel Lymph Node (SLN) Handling. SLN handling after blue colored or fluorescent SLNs were identified, marked and extracted. If the short axis of a SLN was equal to or larger than 10 mm, the SLN was cut as equally as possible into four blocks, two sections of 3 µm after each 250 µm, and labeled as "a", "b", "c" and "d". Residual block "a" and block "c" were stored at -80° degrees for up to 6 months until being analyzed by the OSNA Assay. Blocks "b" and "d" were paraffin-embedded and assessed by routine pathological examination and fine pathological examination using immunohistochemical (IHC) staining as described in the material and methods section. If the short axis of a SLN was no larger than 10 mm, the SLN was cut as equally as possible into two blocks, "a" and "b". Block "a" was stored at -80° degrees for up to 6 months until being analyzed by OSNA Assay. Block "b" was paraffin-embedded and assessed by fine pathological examination using immunohistochemical (IHC) staining.

Pathological examination

The formalin-fixated SLNs paraffin-embedded blocks were cut into 4 levels with 250 µm between each level. At each level, two sections of 3 µm were taken; one section was H&E-stained using a Sakura Prisma machine; the other section was processed using the IHC method, an anti-Pan-Cytokeratin antibody staining using a Ventana Banchemark XT machine.

For RP level 2 was examined and scored and this level thus served as reference for the performance of OSNA and FP. H&E stained sections on each level served as a reference for the slides cut from the same sections, which were immunohistochemically stained and scored blinded to the results by the pathologist (J.L, R.L. and V.S.)

The non-SLNs and SLNs free from metastasis were judged negative, (sentinel) lymph nodes with at least one observed tumor cell were judged as positive. Each SLN was judged "positive" if cancer cells were observed in any of the sections, and judged "negative" if no cancer cells were observed in all sections.

OSNA

The OSNA method has been previously described in detail. (20;25) Each sample was analyzed by RD-100*i* (Sysmex Corp.) and CK19 mRNA copy number in each sample was determined after validation of each measurement according to the OSNA Assay procedure (manufacturer's instruction). A cutoff value of 250 copies/μL was used in the current investigation, based on a previously described study. (16) A CK19 mRNA copy number of <250 copies/μL was judged negative and a copy number ≥ 250 / μL was judged positive. To avoid observer bias, the OSNA analyses were done in a blinded manner without knowledge of findings from FP.

Comparison between the different techniques

The performances of OSNA or FP on patient base were established by both comparing the results from the OSNA analysis or FP with RP. An upstaging rate of OSNA or FP was determined by calculating the percentage of all patients with a positive OSNA or FP result, but negative RP. This was also performed in pN0 patients by calculating the percentage of patients with a positive OSNA or FP result, but negative routine H&E result in all lymph nodes detected in the resection material, as routinely determined by the pathologist. The false negative rate was calculated by determination of the percentage of patients with a negative OSNA or FP result of the SLNs respectively, but with positive lymph nodes in the resection material (pN+ patient), which was routinely (and standard) assessed with H&E staining by the pathologist (patients in whom the SLNs were "skipped" during metastasis).

RESULTS

In this prospective study, 325 SLNs with RP, 317 SLNs with OSNA and 325 SLNs with FP from 128 colon cancer patients were analyzed. There were 73 men and 55 women, and the median patient age was 71 (±11 SD). The median number of harvested SLNs per patient was 3.0 (±0.7 SD). The mean number of routinely assessed LNs in the resection material was 15.3 (range 4-40). Forty-four patients were diagnosed as pN1 patients and 84 patients as pN0. Patient characteristics are provided in Table 1.

Table 1. Baseline patient and tumor characteristics

Characteristic	No. of patients (%)
Total cohort	128
Sex	
Male	73 (57.0%)
Female	55 (43.0%)
Mean Age (years±SD)	71 (± 11)
Dye	
Patent Blue	97 (75.8%)
Indocyanine green	31 (24.2%)
Median number of detected SLN's (±SD)	3.0 (±0.7)
1 detected SLN	19 (14.8%)
2 detected SLNs	21 (16.4%)
3 detected SLNs	88 (68.8%)
Mean number of routinely assessed LNs (range)	15.3 (4-40)
Tumorlocation	
Right colon	75 (58.6%)
Left colon	15 (11.7%)
Sigmoid	38 (29.7%)
pT-stage	
pT1	6 (4.7%)
pT2	30 (23.4%)
pT3	86 (67.2%)
pT4	6 (4.7%)
pN-stage *	
pN0	84 (65.6%)
pN1	44 (34.4%)
TNM stage	
I	28 (21.9%)
II	56 (43.8%)
III	44 (34.4%)
Differentiation	
Well	8 (6.3%)
Moderate	93 (72.7%)
Poor	18 (14.1%)
Missing	9 (7.0%)

* Lymph nodes routinely assessed in the resection material by a pathologist using H&E staining

First, the performance of OSNA and FP in SLNs were determined by comparing the results of OSNA and FP with the RP of the SLNs. Twenty-three out of 28 patients who were positive in RP were also positive with OSNA, resulting in a diagnostic value of 82.1% (Table 2). An upstaging rate of 20.2% with OSNA was established, as 20 out of 99 patients were positive with OSNA but negative with RP. The diagnostic value of the FP was 100% as 29 out of 29 patients were both positive in the FP and RP (Table 2). An upstaging rate of FP of 36.4% was detected, as 36 out of 99 patients were positive with FP but negative with RP. When both methods were combined, the diagnostic value of OSNA and FP together was 100%. The upstaging rate of the use of both methods, compared to RP, was 46.5% (Table II). This upstaging rate was equally distributed between T-stages (p=0.663). In T1-T2 tumors, 14 of 36 patients (38.9%) were positive with FP or OSNA, but negative with RP. In T3-T4, this was seen for 32 of 92 patients (34.8%).

Table 2. Results of one step nucleic acid amplification (OSNA) and fine pathological examination (FP)* of sentinel lymph nodes (SLN) compared to routine pathological examination (RP) in 128 colon cancer patients

		Routine pathological examination (SLNs)		
		Positive	**Negative**	**Total**
OSNA (SLNs)	**Positive**	23	20	43
	Negative	5	79	84
	Total	28	99	127
FP* (SLNs)	**Positive**	29	36	65
	Negative	0	63	63
	Total	29	99	128
Combined(SLNs)**	**Positive**	29	46	75
	Negative	0	53	53
	Total	29	99	128

SLNs: Sentinel Lymph Nodes

** FP: using anti-Pan-Cytokeratin antibody (LU-5) staining as described in the material and methods section*

*** Results of OSNA and FP of SLNs were combined. A patient was considered positive when in one or both methods a positive SLN was found and negative when with both methods negative SLNs were found.*

When OSNA and the FP of the SLNs were compared, there were 41 discordant cases (31 OSNA negative/FP positive, 10 OSNA positive/FP negative) (Table 3). Only 6 out of 31 OSNA negative/FP positive patients showed a few copy numbers with OSNA, and ranged from 1.4 to 78, which was below the cutoff value of 250 copies/μL. All other discordant cases showed no copy numbers with OSNA. The kappa values were 0.352 for OSNA compared to FP, 0.442 for FP compared to RP and 0.512 for OSNA compared to RP.

Table 3. Results of one step nucleic acid amplification (OSNA) compared to fine pathological examination (FP)* of sentinel lymph nodes (SLN) in 128 colon cancer patients

		Fine pathological examination (SLNs)		
		Positive	**Negative**	**Total**
OSNA (SLNs)	**Positive**	33	10	43
	Negative	31	53	84
	Total	64	63	127

SLNs: Sentinel Lymph Nodes
** FP: using anti-Pan-Cytokeratin antibody (LU-5) staining as described in the material and methods section*

Second, the capability of OSNA and FP to upstage patients with otherwise negative routinely assessed LNs in the resection material (pN0 patients) using H&E staining was investigated. The upstaging rate of OSNA in pN0 patients was 19.0% as 16 out of 84 patients were diagnosed as pN0, while showing a positive SLN with OSNA (Table 4). Sixteen patients were diagnosed as N+ patients while the OSNA result of the SLNs was negative, resulting in a false negative rate of 27.1% with OSNA. With the FP an upstaging rate of 35.7% was achieved. In 30 of 84 pN0 patients, a positive SLN by using FP was detected. The false negative rate of the FP was 12.2% as 9 pN+ patients were negative in the FP. Again, when OSNA and FP were combined, the upstaging rate was even higher with a percentage of 46.4% and a false negative rate of 9.6% (Table 4).

Table 4. Results of one step nucleic acid amplification (OSNA) and fine pathological examination (FP)* of sentinel lymph nodes (SLN) in patients who were with routine pathological examinationof all other lymph nodes (LNs) node positive (pN+) or node negative (pN0)

		Routine pathological examination of LNs***		
		pN+	**pN0**	**Total**
OSNA (SLNs)	**Positive**	27	16	43
	Negative	16	68	84
	Total	43	84	127
FP* (SLNs)	**Positive**	35	30	65
	Negative	9	54	63
	Total	44	84	128
Combined (SLNs)**	**Positive**	36	39	75
	Negative	8	45	53
	Total	44	84	128

SLNs: Sentinel Lymph Nodes
** FP: using anti-Pan-Cytokeratin antibody (LU-5) staining as described in the material and methods section*
*** Results of OSNA and FP of SLNs were combined. A patient was considered positive when in one or both methods a positive SLN was found and negative when with both methods negative SLNs were found.*
**** Lymph nodes routinely assessed in the resection material by a pathologist using H&E staining.*

When separate analyses in the two hospitals were performed, differences in upstaging rate in pN0 patients and false negative rate were seen (Supplementary Table 1). At the Jeroen Bosch Hospital, an upstaging rate of 23.1% with a false negative rate of 23.4% with OSNA was found. The FP showed an upstaging rate of 44.6% with a false negative rate of 8.2%. At the LUMC, OSNA showed an upstaging rate of 5.3% with a 41.7% false negative rate. An upstaging rate of 5.3% with a false negative rate of 30.8% was found by using the FP.

DISCUSSION

It is well known that about 30% of node-negative CRC patients suffer from recurrent disease within 5 years after surgery. (8) One possible explanation for this high recurrence rate is the lack of detection of LNs micrometastases and ITC cells with the current standard of histopathological workup. (26;27) Current guidelines suggest adjuvant chemotherapy for all lymph-node positive colon cancer patients. (28) Optimal tumor staging is therefore crucial for adjuvant chemotherapy allocation and reduction of recurrence.

In this prospective cohort study performed in two Dutch centers on 128 colon cancer patients, OSNA appeared to be a promising tool for the detection of LN micro-and macrometastases in SLNs after the ex vivo SLNM procedure. The performance of OSNA in this study was superior to RP. By using standard conventional pathological examination, which involves examination of solely one H&E stained slide, a certain proportion of micrometastases can remain undetected, possibly leading to sampling bias. OSNA may well be the answer to this problem creating a better detection system for otherwise undetected positive LNs.

In this study, the diagnostic value of OSNA was 82.1% with an upstaging rate of 20.2% comparable with results from Güller et al. (20) However, contrary to Güller et al., in our study we have focused on just SLNs, which were detected by ex vivo SLNM. It is believed that SLNs are the LNs with the highest potential to harbor metastasis because of their close relationship with the tumor. (21) Ex vivo SLNM has proven to be an easy and feasible technique for ultra-staging CRC patients with a high accuracy of 90-100% and negative predictive value of 80-100%. (22;23) A rate of 19-57% upstaging has been observed (23). Therefore, OSNA in combination with ex vivo SLNM may increase detection of positive lymph nodes and may provide a promising staging tool.

Limitations of this study have to be acknowledged. First, the FP of the SLNs with IHC, using an anti-Pan-Cytokeratin antibody, outperformed OSNA in this study. These findings might partly be explained by sampling bias as well. Tumor cells were probably only present in one half of the lymph node, which was analyzed by FP, whereas in the other half, analyzed with OSNA, no tumor cells were present. We also can argue that through

multilevel sectioning of the whole SLN followed by IHC, FP will provide us with the best possible staging method, where ITC can be detected as well. In contrast to FP, OSNA is not intended to detect ITC, because the cutoff value for OSNA positivity (250 copies/μL) will not be reached when only ITC are present. (20) However, for clinical applicability, multi-level sectioning as done in FP is very time-consuming. A major advantage of OSNA is time; 3 to 4 lymph nodes are analyzed in 30-40 minutes (19). In addition, OSNA is a standardized, reproducible method with a good sensitivity and specificity for the detection of positive LNs. (19;20;29) It is noteworthy that the clinical relevance of the detection of ITC, is still unknown and remains under debate. (30-33)

Second, results from our study showed a high false negative rate with OSNA, i.e., the SLNs were negative while the other routinely assessed LNs in the resection material were positive, probably explained by the fact that the SLNs were "skipped" during metastasis. FP showed a lower false negative rate than OSNA, which might be explained by multi-level sectioning with FP, the possibility to detect ITC, and the sampling bias. Therefore, with OSNA more SLNs have possibly shown a negative result and/or more SLNs are "skipped" during metastasis, resulting in a higher false-negative rate.

Nonetheless, OSNA with its great potential as an improved staging method will be able to overcome part of these problems in the future by analyzing the entire LN and increasing its sensitivity to detect ITC.

As shown in the supplementary table, interhospital differences were also noted. Discrepancies on results between the two sites included differences in upstaging rate and differences in false negative rate, i.e., SLNs of pN+ patients, which were negative with OSNA or FP. A higher upstaging rate with a lower false-negative rate was observed at the Jeroen Bosch Hospital, when compared with the LUMC. The discrepancy was most prominent in the FP, because there was a significantly higher positive FP at the Jeroen Bosch Hospital, when compared with the LUMC ($p=0.005$). This might be explained by inter-observer differences in evaluating a positive anti-Pan-Cytokeratin staining. Furthermore, differences in the number of included patients per center, differences in selecting SLNs at each site, and differences in mapping technique could all have contributed to these inter-hospital differences as well. Standardization of the harvesting procedure and staining evaluation could potentially lower this discrepancy rate in the near future.

Difficulties and controversies while using OSNA should be acknowledged as well. First, OSNA is currently only applicable on fresh-frozen material, which requires the immediate presence of a pathologist to harvest the SLNs. Also, more expertise in handling fresh frozen material instead of paraffin-embedded material for tumor staging is essential. Second, the isolation of SLNs from fresh frozen material is time-consuming; however, this will be fully compensated by the speed of the OSNA analysis. Third, because CK19 is an epithelial marker, tissue contamination through dislodged fractions from often large

and friable colon tumors is a possibility when using OSNA for processing SLNs, which should not be underestimated.

CONCLUSIONS

Based on this prospective investigation performed in two hospitals, OSNA proved to be a promising method for the detection of SLN metastases in colon cancer patients after ex vivo SLNM. OSNA appeared to outperform routine pathological examination with H&E-stained slides with an upstaging rate of 20.2%. Because OSNA allows analysis of the whole LN, sampling bias can be avoided. OSNA therefore may improve tumor staging and consequently, through adjuvant chemotherapy allocation, reduction of recurrence. Long-term follow-up of the upstaged patient will further proof the clinical relevance of OSNA and randomized controlled trials are necessary to assess adequately the prognostic relevance of these detected metastases by OSNA.

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Supplementary Table 1. Results of one step nucleic acid amplification (OSNA) and fine pathological examination (FP)* of sentinel lymph nodes (SLN) in patients who were with routine pathological examination (RP) of all other lymph nodes node positive (pN+) or node negative (pN0) – *Separated by hospital*

		Routine pathological examination (all lymph nodes)			
		Jeroen Bosch Hospital		**LUMC****	
		pN+	**pN0**	**pN+**	**pN0**
OSNA (SLNs)	**Positive**	21 (65.6%)	15 (23.1%)	6 (54.5%)	1 (5.3%)
	Negative	11 (34.4%)	50 (76.9%)	5 (45.5%)	18 (94.7%)
	Total	32 (100%)	65 (100%)	11 (100%)	19 (100%)
FP* (SLNs)	**Positive**	27 (84.4%)	29 (44.6%)	8 (66.7%)	1 (5.3%)
	Negative	5 (15.6%)	36 (55.4%)	4 (33.3%)	18 (94.7%)
	Total	32 (100%)	65 (100%)	12 (100%)	19 (100%)

SLNs: Sentinel Lymph Nodes

** FP: using anti-Pan-Cytokeratin antibody (LU-5) staining as described in the material and methods section*

*** LUMC: Leiden University Medical Center*

6

The prognostic value of microsatellite instability, *KRAS, BRAF* and *PIK3CA* mutations in stage II colon cancer patients

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Mol Med. 2015 Dec;17:1-26.

ABSTRACT

Introduction In the era of personalized cancer medicine, identifying mutations within patient tumors plays an important role in defining high-risk stage II colon cancer patients. The prognostic role of *BRAF* V600E mutation, microsatellite instability (MSI) status, *KRAS* mutation and *PIK3CA* mutation in stage II colon cancer patients is not settled.

Methods We retrospectively analyzed 186 patients with stage II colon cancer who underwent an oncological resection but were not treated with adjuvant chemotherapy. *KRAS* mutations, *PIK3CA* mutation, V600E *BRAF* mutation and MSI status were determined. Survival analyses were performed.

Results Mutations were found in the patients with each mutation with each mutation in the following percentages: 23% (MSI), 35% (*KRAS*), 19% (*BRAF*) and 11% (*PIK3CA*). A trend toward worse overall survival (OS) was seen in patients with a MSI (5-year OS 74% versus 82%, adjusted hazard ratio (HR) 1.8, 95% CI 0.6-4.9) and a *KRAS*-mutated tumor (5-year OS 77% vs. 82%, adjusted HR 1.7, 95% CI 0.8-3.5). MSI and *BRAF* mutated tumors tended to correlated with poorer disease-free survival (DFS) (5-year DFS 60% vs. 78%, adjusted HR 1.6, 95% CI 0.5-2.1 and 5-year DFS 57% versus 77%, adjusted HR 1.1, 95% CI 0.4-2.6 respectively).

Conclusions In stage II colon cancer patients not treated with adjuvant chemotherapy, *BRAF* mutation and MSI status both tended to have a negative prognostic effect on disease-free survival. *KRAS* and MSI status also tended to be correlated with worse overall survival.

INTRODUCTION

Despite advances in diagnosis and treatment, a significant proportion of colon cancer patients who undergo resection with curative intent develop disease recurrence. About 15-30% of the patients with stage II (Dukes B) disease develop recurrent locoregional disease or distant metastases within 5 years and their overall 5-year survival is around 70-80%. (1)

In the era of personalized cancer medicine, identifying mutations within patient tumors might play an important role in defining high risk colon cancer patients. (2;3) The role of the *BRAF* mutation in colon cancer is one of recent interest. *BRAF* is a downstream effector molecule of *KRAS*. One particular missense mutation in *BRAF*, *BRAF* V600, accounts for up to 90% of all mutations in human cancers. (4;5) Several studies investigated and confirmed the potential adverse prognostic impact of BRAF mutations but patient categories included in these studies were very heterogeneous. (6-9) As a predictive marker, the *BRAF* status has been studied in metastatic colorectal cancer, where the presence of the *BRAF* mutation was correlated with a lower response rate to cetuximab plus chemotherapy. (10;11)

A probably more well known biomarker is microsatellite instability (MSI), which appears in tumors with deficient mismatch repair (MMR). It is the hallmark of Lynch Syndrome, although it is not solely restricted to hereditary colorectal cancer. Although studies have been equivocal concerning proposed survival benefit, some found that MSI is associated with better prognosis. (12) Recent data support a prognostic role for combined MSI/*BRAF* testing in colorectal cancer. (13;14)

Another potentially promising biomarker is *PIK3CA*. Mutations in this gene have been identified in colorectal cancer (CRC), with most mutations localized in exon 9 and 20. (15)

Among patients who undergo a curative resection for stage I-III colon cancer, *PIK3CA* mutation is associated with shorter cancer-specific survival, but the adverse effect of *PIK3CA* mutation may be potentially limited to patients with *KRAS* wild-type tumors. (16) *KRAS* mutations at the codons 12 and 13 are the most frequent alterations in colon cancer, representing more than 90% of all mutations. (17)

To evaluate the prognostic role of above-mentioned mutations in stage II colon cancer, we aimed to determine the status of the *BRAF* V600E mutation, MSI status, *KRAS* mutation and *PIK3CA* mutation in a well-defined group of stage II colon cancer patients who underwent resection but were not treated with adjuvant chemotherapy. The association of the mutations with disease-free survival and overall survival was assessed.

METHODS

Patients

Patients with stage II (T3N0 or T4N0) colon cancer, diagnosed and surgically treated in one hospital in the southern part of The Netherlands between 2002 and 2008, were included in this study. Patients who received adjuvant chemotherapy were not included.

A tumor was considered right sided when it was located between the cecum and the splenic flexure (C18.0-18.5). The remaining tumors were considered left sided (C18.6-18.9). Demographic and clinical data on the patients were obtained from the medical records of the patients and combined with data from the Netherlands Cancer Registry (NCR) that collects data on all newly diagnosed cancer patients in the Netherlands. Comorbidities are registered according to a slightly modified version of the Charlson Comorbidity index. (18;19)

Patients with insufficient or missing tumor tissue were excluded from analyses. From all patients with sufficient available formalin-fixated paraffin-embedded tumor tissues, DNA was isolated. For this purpose, a tumor area with at least 30% tumor cells from glass slide according to hematoxylin- and eosin (H&E)–stained sections was selected by an experienced pathologist. Subsequently, the selected areas were macrodissected from archival paraffin-embedded tissue after deparaffinization. Subsequently, after proteinase K digestion (1,0 mg/ml TE; overnight 56° C) DNA was extracted using Nucli-SENS® easyMAG® (Biomerieux, Zaltbommel) following manufacturer's instructions. DNA concentration was measured (BioSpec-nano (Shimadzu, 's-Hertogenbosch)) and diluted to 10 ng/µl for subsequent analyses.

KRAS analysis

Mutations in exon 2 (codons 12 and 13) and exon 3 (codon 61) of the *KRAS* gene were detected by PCR high resolution melting (HRM) followed by direct sequencing. Briefly, HRM was performed as previously described (20) using LightScanner Mastermix (Bioke, Leiden, The Netherlands) and LightCycler480 (LC480) Thermal cycler (Roche Diagnostics, Almere, The Netherlands). Positive and equivocal samples in HRM were subjected to Sanger sequencing of the PCR products. Briefly, after the PCR-clean up reaction (Exo-SAP-IT) and purification of the PCR product (MinElute PCR Purification Kit (Qiagen, Venlo, The Netherlands), the sequence reaction was performed using the same primers independently and the Big Dye reagents (Applied Biosystems, Bleiswijk, the Netherlands). Products were separated on the ABI3100 (Applied Biosystems, Bleiswijk, the Netherlands). The sequences were evaluated with the Sequencing Analysis 5.3.1 software.

BRAF analysis

The V600 mutation on the *BRAF* gene was detected by means of a newly developed real-time PCR modified from our previously described V600E assay (4) using the following primers and probes, forward 5' CTA CTG TTT TCC TTT ACT TAC TAC ACC TCA GA 3' and reverse 5' ATC CAG ACA ACT GTT CAA ACT GAT G 3', wild-type probe VIC-5' CTA GCT ACA GTG AAA TC 3' and mutant V600E probe FAM-5' TAG CTA CAG AGA AAT C 3'. In addition, VIC labeled MGB probes to detect V600R (VIC-5' TAG CTA CAA GGA AAT C 3') and V600K (VIC-5' TAG CTA CAA AGA AAT C 3') were included, which also detects the V600D mutation. Furthermore, a BRAF-wildtype (wt) locked nucleic acid (LNA) oligonucleotide was used, which supposedly blocks amplification of the wildtype allele during PCR so that mutant DNA can be efficiently amplified. A PCR product of 136 bp was obtained. The assay showed to have a detection limit of at least 1-5% tumor cells in a given specimen. All PCRs were carried out in 10 ul volume using an ABI7500 Fast realtime cycler (Applied Biosystems, Life Technologies, Bleiswijk, the Netherlands).

PIK3CA analysis

PIK3CA mutations were determined by PCR followed by single nucleotide primer extension assay, as previously described (24) for the hotspots in exon 9, c.1624G>A (p.(E542K)), c.1634A>G (p.(E545G)) and c.1633G>A (p.(E545K)) and in exon 20 the c.3140A>G (p.(H1047R)). Briefly, both exons were amplified by multiplex PCR. After enzymatic purification of the PCR products with EXO SAP IT, the extension reaction was performed using primers published elsewhere (24) and the SNaPshot ready multiplex kit (Applied Biosystems, Life Technologies, Bleiswijk, the Netherlands). Finally, these products were purified and separated by capillary electrophoresis using an ABI 3100 (Applied Biosystems, Bleiswijk, the Netherlands).

MSI analysis

Microsatellite instability was detected using only one marker of the Bethesda panel, i.e. the mononucleotide repeat BAT26, as also previously described (4). This marker was chosen because in the Caucasian race, it detects 99% of the MSI high patients and normal DNA is not necessary. (21;22) Briefly, PCR was performed using the following primers, forward VIC-5´TGA CTA CTT TTG ACT TCA GCC 3´ and reverse 5´ACC CAT TCA ACA TTT TTA ACC C 3´. Subsequently, PCR products were diluted depending on their intensity and denatured using formamide and incubated at 95°C for 3 minutes. Products size were analyzed using the ABI3100 (Applied Biosystems, Bleiswijk, the Netherlands) and GeneMapper 4.0 software package.

Statistics

Differences in demographic and clinical characteristics between patients with various mutations were analysed using Chi-square tests or Fisher's Exact tests where appropriate. Crude 5-year overall and disease-free survival were visualised using Kaplan-Meier curves and tested with the Log-Rank test. Overall survival time was defined as the time from primary colon cancer surgery to death or last follow-up date for patients who were still alive. Disease-free survival time was defined as the time from primary colon cancer surgery to recurrence or death or last follow-up date for patients without recurrence or death. Multivariable Cox regression analyses were used to discriminate independent risk factors for death or recurrence and death for the total study population. Besides microsatellite status, *KRAS*, *BRAF* and *PIK3CA*, models were also adjusted for the variables gender, age, comorbidity, surgery, subsite of the tumor, differentiation grade, number of lymph nodes evaluated, tumor obstruction, tumor perforation and lymphangioinvasion. All variables were included in the models at once.

In the period from January to May 2014, data on diagnosis of recurrences were retrospectively collected from the medical records. Date of death is, in addition to passive follow-up via the hospitals, retrieved through linkage with the Municipal Personal Records Database (GBA). This database contains all death or emigrated persons in the Netherlands since October 1994. Date of death was completed until 31 December 2013.

P values below 0.05 were considered statistically significant. SPSS for Windows (version 16.0, SPSS INc, Chicago, Ill) and SAS/STAT® statistical software (SAS system 9.3, SAS Institute, Cary, NC) were used for all analyses.

RESULTS

The total study population consisted of 211 patients. Twenty-five patients (12%) were excluded owing to insufficient tumor tissue (n=7) and missing tumor tissue in our archive (n=18). Patient and clinicopathological characteristics of the study population are shown in table 1. Of these 211 patients, 43 (23%) had an MSI tumor. *KRAS, BRAF* and *PIK3CA* mutations were found in 35%, 19% and 11% of the patients, respectively.

The relationship between various demographic and clinicopathological features and mutational status can be found in table 2. MSI tumors were significantly associated with female sex (p=0.04), right sided location of the tumor (p<0.0001) and poorly differentiated or undifferentiated tumors (p<0.0001). Furthermore, patients with a MSI tumor less often had a *KRAS* mutation (p=0.001), but more often a *BRAF* mutation (p<0.0001). *KRAS* and *BRAF* mutations were mutually exclusive.

Table 1. Demographic and clinicopathological characteristics of stage II colon cancer patients who underwent resection (n=186)

	N (%)
Gender	
Male	99 (53)
Female	87 (47)
Age	
≤65	52 (28)
>66-75	62 (33)
≥76	72 (39)
Comorbidity	
0	57 (31)
1	50 (27)
≥2	67 (36)
Unknown	12 (6)
Surgery	
Elective	166 (89)
Acute	20 (11)
Subsite	
Left-sided colon	85 (46)
Right-sided colon	101 (54)
Pathological T stage	
3	185 (99)
4	1 (1)
Differentiation grade	
Well/moderate	114 (61)
Poor/undifferentiated	39 (21)
Unknown	33 (18)
Lymph nodes evaluated	
<10	130 (70)
≥10	56 (30)
Tumor obstruction	
No	164 (88)
Yes	22 (12)
Tumor perforation	
No	179 (96)
Yes	7 (4)
Lymphangioinvasion	
No	180 (97)
Yes	6 (3)
Microsatellite status	
MSS	143 (77)
MSI	43 (23)
KRAS	
Wild type	121 (65)
Mutant	65 (35)
BRAF	
Wild type	151 (81)
Mutant	35 (19)
PIK3CA	
Wild type	165 (89)
Mutant	21 (11)

Table 2. Relationship between various demographic and clinicopathological characteristics and mutational status (n=186)

	MSS (n=143) n (%)	MSI (n=43) n (%)	KRAS wt (n=121) n (%)	KRAS mut (n=65) n (%)	BRAF wt (n=151) n (%)	BRAF mut (n=35) n (%)	PIK3CA wt (n=165) n (%)	PIK3CA mut (n=21) n (%)
Gender								
Male	82 (57.3)	17 (39.5)*	70 (57.9)	29 (44.6)	86 (57.0)	13 (37.1)*	93 (56.4)	6 (28.6)*
Female	61 (42.7)	26 (60.5)	51 (42.1)	36 (55.4)	65 (43.0)	22 (62.9)	72 (43.6)	15 (71.4)
Age								
≤65	46 (32.2)	6 (14.0)	34 (28.1)	18 (27.7)	48 (31.8)	4 (11.4)	43 (26.0)	9 (42.9)
>66-75	46 (32.2)	16 (37.2)	42 (34.7)	20 (30.8)	48 (31.8)	14 (40.0)	59 (35.8)	3 (14.2)
≥76	51 (35.6)	21 (48.8)	45 (37.2)	27 (41.5)	55 (36.4)	17 (48.6)	63 (38.2)	9 (42.9)
Comorbidity								
0	46 (32.2)	11 (25.6)	40 (33.1)	17 (26.2)	50 (33.1)	7 (20.0)*	49 (29.7)	8 (38.1)
1	39 (27.3)	11 (25.6)	28 (23.1)	22 (33.8)	43 (28.5)	7 (20.0)	45 (27.3)	5 (23.8)
≥2	48 (33.5)	19 (44.2)	46 (38.0)	21 (32.3)	47 (31.1)	20 (57.1)	62 (37.6)	5 (23.8)
Unknown	10 (7.0)	2 (4.6)	7 (5.8)	5 (7.7)	11 (7.3)	1 (2.9)	9 (5.4)	3 (14.3)
Surgery								
Elective	126 (88.1)	40 (93.0)	112 (92.6)	54 (83.1)*	134 (88.7)	32 (91.4)	149 (90.3)	17 (81.0)
Acute	17 (11.9)	3 (7.0)	9 (7.4)	11 (16.9)	17 (11.3)	3 (8.6)	16 (9.7)	4 (19.0)
Subsite							77 (46.7)	8 (38.1)
Left-sided colon	81 (56.6)	4 (9.3)**	54 (44.6)	31 (47.7)	81 (53.6)	4 (11.4)**		
Right-sided colon	62 (43.4)	39 (90.7)	67 (55.4)	34 (52.3)	70 (46.4)	31 (88.6)	88 (53.3)	13 (61.9)
Pathological T stage								
3	142 (99.3)	43 (100.0)	120 (99.2)	65 (100.0)	151 (100.0)	34 (97.1)	164 (99.4)	21 (100.0)
4	1 (0.7)	0 (0.0)	1 (0.8)	0 (0.0)	0 (0.0)	1 (2.9)	1 (0.6)	0 (0.0)
Differentiation grade								
Well/moderate	98 (68.5)	16 (37.2)**	72 (59.5)	42 (64.6)	100 (66.2)	14 (40.0)*	102 (61.8)	12 (57.1)
Poor/undifferentiated	20 (14.0)	19 (44.2)	29 (24.0)	10 (15.4)	24 (15.9)	15 (42.9)	35 (21.2)	4 (19.1)
Unknown	25 (17.5)	8 (18.6)	20 (16.5)	13 (20.0)	27 (17.9)	6 (17.1)	28 (17.0)	5 (23.8)
Lymph nodes evaluated								
<10	41 (28.7)	15 (34.9)	38 (31.4)	18 (27.7)	47 (31.1)	9 (25.7)	48 (29.1)	8 (38.1)
≥10	102 (71.3)	28 (65.1)	83 (68.6)	47 (72.3)	104 (68.9)	26 (74.3)	117 (70.9)	13 (61.9)
Tumor obstruction								
No	125 (87.4)	39 (90.7)	108 (89.3)	56 (86.2)	133 (88.1)	31 (88.6)	147 (89.1)	17 (81.0)
Yes	18 (12.6)	4 (9.3)	13 (10.7)	9 (13.8)	18 (11.9)	4 (11.4)	18 (10.9)	4 (19.0)
Tumor perforation								
No	139 (97.2)	40 (93.0)	115 (95.0)	64 (98.5)	147 (97.4)	32 (91.4)	159 (96.4)	20 (95.2)
Yes	4 (2.8)	3 (7.0)	6 (5.0)	1 (1.5)	4 (2.6)	3 (8.6)	6 (3.6)	1 (4.8)
Lymphangioinvasion								
No	138 (96.5)	42 (97.7)	118 (97.5)	62 (95.4)	147 (97.4)	33 (94.3)	159 (96.4)	21 (100.0)
Yes	5 (3.5)	1 (2.3)	3 (2.5)	3 (4.6)	4 (2.6)	2 (5.7)	6 (3.6)	0 (0.0)
Microsatellite status								
MSS	N/A***	N/A	84 (69.4)	59 (90.8)*	134 (88.7)	9 (25.7)**	129 (78.2)	14 (66.7)
MSI			37 (30.6)	6 (9.2)	17 (11.3)	26 (74.3)	36 (21.8)	7 (33.3)
KRAS								
Wild type	84 (58.7)	37 (86.0)*	N/A	N/A	86 (57.0)	35 (100)**	108 (65.5)	13 (61.9)
Mutant	59 (41.3)	6 (14.0)			65 (43.0)	0 (0)	57 (34.5)	8 (38.1)
BRAF								
Wild type	134 (93.7)	17 (39.5)**	86 (71.2)	65 (100)**	N/A	N/A	133 (80.6)	18 (85.7)
Mutant	9 (6.3)	26 (60.5)	35 (28.9)	0 (0)			32 (19.4)	3 (14.3)
PIK3CA								
Wild type	129 (90.2)	36 (83.7)	108 (89.3)	57 (87.7)	133 (88.1)	32 (91.4)	N/A	N/A
Mutant	14 (9.8)	7 (16.3)	13 (10.7)	8 (12.3)	18 (11.9)	3 (8.6)		

*p<0.05

**p≤0.0001

***N/A = not applicable

BRAF mutations were associated with female sex (p=0.03), comorbidity (p=0.036), right-sided location of the tumor (p<0.0001) and poorly differentiated or undifferentiated tumors (p=0.002).

PIK3CA mutation was associated with female sex (p=0.016).

Survival

For the total study population, 5-year overall survival was 80% and 5-year disease-free survival 74%. In both univariable and multivariable analysis, higher age, more comorbidities, poorly differentiated or undifferentiated tumors and lymphangioinvasion were significantly associated with poorer overall and disease-free survival (tables 3,4). Although not significant, a trend towards worse overall survival was seen in patients with a MSI tumor (5-year overall survival rate of 74% compared with 82% for patients with a MSS tumor, figure 1A), a *BRAF*-mutated tumor (5-year overall survival rate of 76% compared with 81% for patients with a BRAF wild type tumor, figure 1E) and a *KRAS* mutated tumor (5-year overall survival rate of 77% versus 82% for *KRAS* wild type tumors, figure 1C). As 60% of all patients with a MSI tumor was alive and without recurrence at five years versus 78% of patients with a MSS tumor (figure 1B), MSI correlated with poorer disease-free survival. *BRAF* mutated tumors also correlated with poorer disease-free survival with a 5-years disease-free survival of 57% versus 77% for *BRAF* wildtype (figure 1F). However, the associations between MSI and disease-free survival and between *BRAF* and disease-free survival were no longer significant in multivariable analysis (tables 3,4). Not enough patients with *PIK3CA* mutations were left at the end of follow-up to assess survival for this mutation.

Table 3. Crude 5-year overall survival and hazard ratios[a] for death for the total study population (n=186)

	Crude 5-year survival (%)	Hazard ratio (95% CI)
Gender		
Male	77	1.0 (reference)
Female	84	2.5 (1.2-5.2)
Age		
≤65	98**	1.1 (1.1-1.2)
>66-75	87	
≥76	61	
Comorbidity		
0	96**	1.0 (reference)
1	74	3.4 (1.0-10.9)
≥2	69	4.9 (1.6-15.6)
Surgery		
Elective	82	1.0 (reference)
Acute	^	1.5 (0.2-14.4)
Subsite		
Left-sided colon	78	1.0 (0.5-2.2)
Right-sided colon	82	1.0 (reference)
Differentiation grade		
Well/moderate	86*	1.0 (reference)
Poor/undifferentiated	71	3.9 (1.6-9.3)
Lymph nodes evaluated		
<10	80	1.4 (0.6-3.0)
≥10	81	1.0 (reference)
Tumor obstruction		
No	82	1.0 (reference)
Yes	^	2.1 (0.2-18.4)
Tumor perforation		
No	82	1.0 (reference)
Yes	^	3.4 (0.8-14.4)
Lymphangioinvasion		
No	81*	1.0 (reference)
Yes	^	4.8 (1.2-18.7)
Microsatellite status		
MSS	82	1.0 (reference)
MSI	74	1.8 (0.6-4.9)
KRAS		
Wild type	82	1.0 (reference)
Mutant	77	1.7 (0.8-3.5)
BRAF		
Wild type	81	1.0 (reference)
Mutant	76	0.7 (0.2-2.0)
PIK3CA		
Wild type	80	1.0 (reference)
Mutant	^	0.5 (0.1-1.8)

[a] Adjusted for all variables listed. Included in the analysis but results not shown for comorbidity unknown and differentiation grade unknown.

*p<0.05

**p≤0.0001

^ Number of patients left <10

Table 4. Crude 5-year disease-free survival and hazard ratios[a] for recurrence or death for the total study population (n=186)

	Crude 5-year disease-free survival (%)	Hazard ratio for recurrence/death (95% CI)
Gender		
Male	70	1.0 (reference)
Female	78	2.1 (1.1-4.0)
Age		
≤65	90**	1.1 (1.0-1.1)
>66-75	75	
≥76	60	
Comorbidity		
0	89*	1.0 (reference)
1	64	3.3 (1.3-8.1)
≥2	64	3.3 (1.4-7.9)
Surgery		
Elective	75	1.0 (reference)
Acute	^	1.7 (0.2-14.5)
Subsite		
Left-sided colon	70	1.2 (0.6-2.3)
Right-sided colon	76	1.0 (reference)
Differentiation grade		
Well/moderate	82*	1.0 (reference)
Poor/undifferentiated	52	3.7 (1.8-7.4)
Lymph nodes evaluated		
<10	72	1.1 (0.6-2.2)
≥10	76	1.0 (reference)
Tumor obstruction		
No	75	1.0 (reference)
Yes	62	1.5 (0.2-12.6)
Tumor perforation		
No	75	1.0 (reference)
Yes	^	1.8 (0.5-6.6)
Lymphangioinvasion		
No	75*	1.0 (reference)
Yes	^	6.8 (2.1-21.8)
Microsatellite status		
MSS	78*	1.0 (reference)
MSI	60	1.6 (0.7-3.9)
KRAS		
Wild type	73	1.0 (reference)
Mutant	75	1.1 (0.5-2.1)
BRAF		
Wild type	77*	1.0 (reference)
Mutant	57	1.1 (0.4-2.6)
PIK3CA		
Wild type	72	1.0 (reference)
Mutant	^	0.5 (0.1-1.7)

[a] Adjusted for all variables listed. Included in the analysis but results not shown for comorbidity unknown and differentiation grade unknown.

*p<0.05

**p≤0.0001

^ Number of patients left <10

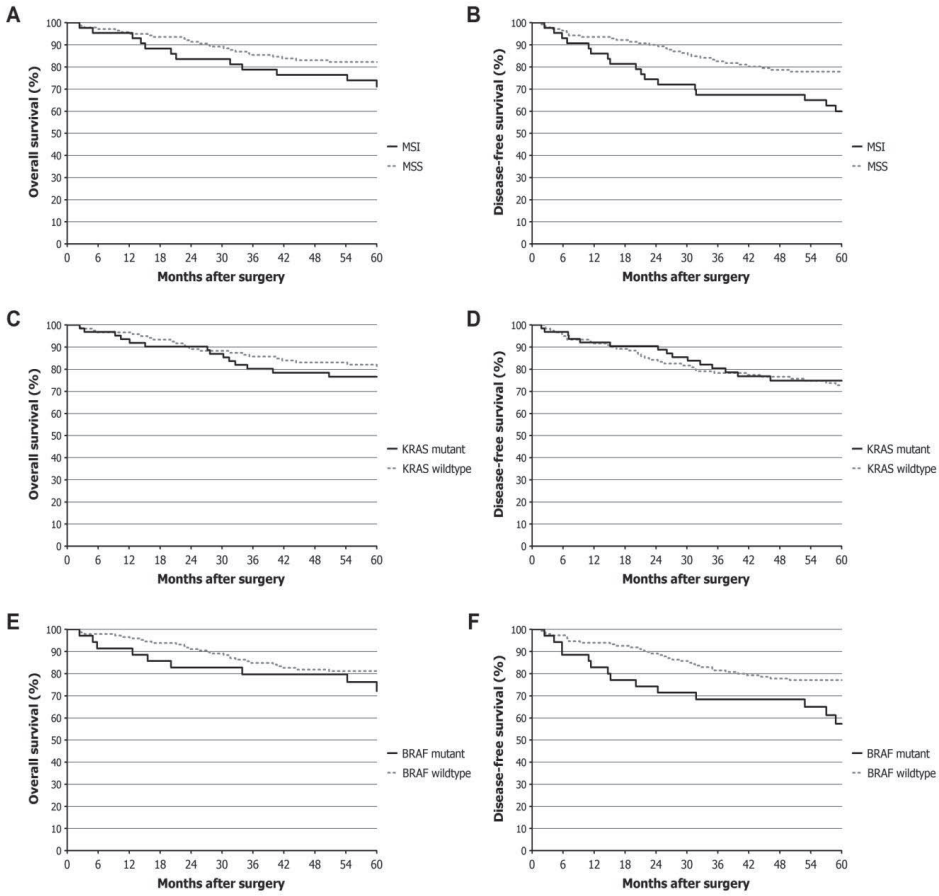

Figure 1. Overall and disease-free survival according to mutational status of microsatellite instability (A-B), KRAS (C-D) and BRAF (E-F) (n=186)

DISCUSSION

In our study we assessed the prognostic value of *BRAF* mutation, *KRAS* mutation, *PIK3CA* mutation and the MSI status with regard to overall and disease-free survival in a well-defined stage II colon cancer cohort of patients who underwent resection but were not treated with adjuvant chemotherapy. *BRAF* mutation and MSI status both tended to have a negative prognostic effect on disease-free survival. *KRAS, BRAF* and *MSI* status also tended to be correlated with worse overall survival.

MSI

MSI positivity was found in 23% of the patients, which is comparable with other subgroup analyses of recent studies reporting 15-25% MSI. (6;8;23;24). Consistent with prior studies (25), MSI status was inversely correlated with the presence of the *KRAS* mutation. The most remarkable finding in our study is the trend towards a negative prognostic effect of an MSI mutation on disease-free survival and overall survival. Although not all studies have verified the association of MSI mutation and improved overall survival, MSI mutation is generally associated with improved overall and disease-free survival. (26;27) On the other hand, like in our study, MSI is associated with poorly differentiated histology which is a known adverse prognostic factor. (27) This gives rise to a paradoxical situation.

Current treatment protocols recommend adjuvant treatment only to stage II patients with high-risk pathological features (for example, T4 stage, bowel perforation or clinical bowel obstruction, inadequate lymph node sampling, (lymph)angioinvasion and poorly differentiated histology). Exceptions are made for MSI positive colon cancer; the most recent Dutch guideline does not recommend adjuvant chemotherapy in high-risk stage II patients with a MSI tumor. Since we only included stage II patients that did not receive chemotherapy, probably a more favourable group of MSS-tumor patients is analyzed and compared with MSI in our study. Therefore, MSI status might have contributed to a relatively poorer survival in our study population. For the MSI determination, we choose the mononucleotide repeat BAT 26 because it discriminates 99% of MSI in the Caucasian population without the requirement of amplified normal DNA, as previously described. (21) The use of only one marker could have diminished the sensitivity of our analysis but not the specificity. (21;22)

BRAF

The presence of the *BRAF* mutation varies more widely between recent studies (6-21%), and was 19% in our cohort. (6;8;23;24) A recent meta-analysis found that the risk of mortality in colorectal cancer patients harbouring the *BRAF*-V600E mutation is more than two times higher than those with wild-type *BRAF*. (28) Although less strongly, our results show a trend towards the *BRAF* mutation having a negative prognostic effect on disease-free and overall survival compared with *BRAF* wild-type tumors.

KRAS

KRAS mutations (codons 12, 13 and 61) were found in 35% of the patients in our study, consistent with other reports. (6;8;24). We found a trend towards worse overall survival for *KRAS* mutated tumors as compared to *KRAS* wild-type tumors. In the prognostic setting, there are conflicts about the role of the *KRAS* mutational status. (6;8;29) A recent large study of more than 1,000 colorectal cancers (stage I through IV) has shown that

KRAS codon 12 mutation is associated with worse prognosis in *BRAF* wildtype colorectal cancers. However, the study is limited by the lack of information on cancer treatment. (30)

PIK3CA

The frequency of *PIK3CA* mutation seems to be dependent on the technique used to evaluate the mutation. (31) We found a *PIK3CA* mutation in 11% of our patients, which is comparable with the literature (10-20%) (31). *PIK3CA*-mutated colorectal tumors have been associated with more proximal location and with a *KRAS* mutation. (31;32) In our study more than 60% of the *PIK3CA* mutated tumors were located in the proximal colon. We found no correlation with *KRAS* mutation. In line with the literature, we did not find a correlation with MSI and *BRAF*. (31;32) *PIK3CA* mutations are more commonly found in exon 9 compared to exon 20. (31) Indeed, 13 of 21 mutations were found in exon 9 in our study. In an earlier report, only a mutation in exon 20 was suggested to be responsible for a worse chance of survival. (33) Because of the small numbers, survival analysis of PIK3CA subgroups in our study was not feasible.

A recent prospective study showed that the total number of lymph nodes harvested is highest for colon cancers with MSI. (34) In this study the nodal harvest is associated with MSI influenced by *BRAF* and *KRAS* genotypes. However, we did not find an association with the number of lymph nodes and the mutational status.

As described above, the relationship between the mutational status and various demographic and clinicopathological variables is comparable with the literature. However, our study population is not completely comparable with those from other studies. Most other reports about the prognostic value of molecular markers in colorectal cancer included more heterogeneous groups of colorectal cancer patients, with patients in different stages. (6;8;23;24) Different studies also evaluated the prognostic value of MSI status, *KRAS* and *BRAF* mutational status in stage II colon cancer patients, but in most of them chemotherapy was given to (a partial cohort) of the patients or information regarding adjuvant therapy was lacking. (6;8;23;24;30)

A new way of sub-staging within stage II colon cancer was suggested by a recent report that defined molecular subtypes by genomic instability. For stage II patients, the numerical difference in chromosomal aberrations between recurrence and no recurrence was statistically significant. Further studies with larger patient samples have to confirm these results. (35)

Cancer care is becoming increasingly dependent on tumor markers to diagnose, anticipate prognosis and select optimal therapy for patients. Although biomarker discovery is thriving, incorporation of biomarkers in clinical practice lags behind. It is imperative that the field of oncology works with a common language and clear standards of evidence

so that the merits of established and emerging biomarkers can be communicated in a clear and unambiguous manner, thereby ensuring that clinicians take full advantage of the current genomic era. (36) Another future direction in (colorectal) cancer research is the host immune response against an invasive tumor process. The recently described 'Immunoscore' classification, demonstrating the prevalence of immune infiltrates, was shown to have a superior prognostic significance in colorectal cancer compared to the classical TNM classification. (37)

Strengths and limitations

To the best of our knowledge we reported the largest study that analyzed MSI, *KRAS, BRAF* and *PIK3CA* mutational status in stage II colon cancer patients who underwent resection but did not receive chemotherapy. Representing 30-40% of all resected colorectal cancers, stage II patients are a very interesting subgroup because clinicians still do not know exactly which of these patients are at high risk of recurrence and therefore may benefit from adjuvant chemotherapy. Furthermore, the percentage of low-stage colon cancers is going to increase because of the screening programmes. (23)

Unfortunately, due to a relative small number of patients, we were not be able to perform adequate subgroup analyses within the different mutations and assess survival for *PIK3CA*.

CONCLUSIONS

The histopathological approach is paramount in colon cancer classification, however, for most patients with stage II disease who are classified as standard risk, there are no additional markers to refine risk assessment. The use of molecular biomarkers in addition to pathological classification will be particularly important in stage II colon cancer, in order to offer the most adequate therapy to each individual patient and avoid unnecessary chemotherapeutic treatment. Our study shows that in stage II patients who have not been treated with chemotherapy, *BRAF* mutation tended to have a negative prognostic effect on survival and also, in contrast to most other reports, MSI tended to be a poor prognosticator. Further studies are needed to verify and further clarify these results.

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7

Impact of anesthetic technique on survival in colon cancer: a review of the literature

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Gastroenterol Rep. 2016 Feb;4(1):30-4.

ABSTRACT

An oncological surgical resection is the mainstay of treatment for potentially curable colon cancer. At the time of surgery, a large fraction of patients do harbour - although not visible - minimal residual disease at the time of surgery. The immunosuppression that accompanies surgery may have an effect on disease recurrence and survival. Regional or neuraxial anesthetic techniques like epidural anesthesia may suppress immune function less than opioid analgesia, by reducing stress response and significantly reducing exposure to opioids. Consistent with this hypothesis, regional anesthetic techniques have been associated with lower recurrence rates in breast cancer and prostate cancer. Results for colon cancer, however, are contradictory. In this review of the literature we describe all studies addressing the association of the use of epidural anesthesia and survival in colon cancer surgery.

INTRODUCTION

An oncological surgical resection is the mainstay of treatment for potentially curable colon cancer. However, even in stage I and II colonic cancer, 10-30% will develop recurrence of disease. It is known that, even with the best surgical technique, surgery for cancer is associated with release of tumor cells. Also it is noteworthy that, at the time of surgery, a large fraction of patients do harbour minimal residual disease, although this may not be visible. (1)

The idea that surgery itself can promote local cancer recurrence and metastasis is not novel and was already described in the 19th century by Velpeau, a French anatomist and surgeon, who noticed that surgical removal of cancer could be associated with the return of the disease and that the operation possibly tended to accelerate tumor growth. (2) Whether this results in recurrence of clinical cancer or metastasis depends largely on the balance between the tumor's ability to spread and the immunosurveillance of the patient. (3)

General anesthesia and surgical stress may suppress immunity by directly affecting the immune system or activating the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. (4) Pre-operative and post-operative opioids may inhibit cellular and humoral immune function in humans, and morphine itself might have a pro-angiogenic effect and promotes tumor growth. (5)

Regional or neuraxial anesthetic techniques may suppress immune function less than opioid analgesia by reducing stress response and significantly reducing exposure to opioids. Consistent with this hypothesis, regional anesthetic techniques have been associated with lower recurrence rates in breast cancer and prostate cancer. (6;7) Results for colon cancer, however, are contradictory. (8) In this review of the literature we describe all studies addressing the association of the use of epidural anesthesia (EA) and survival in colon cancer surgery.

METHODS

Relevant studies were sought in the Pubmed database (starting date January 1990 up to June 2014) using search terms as follows: (i) "regional anesthesia" or "regional anaesthesia" or "regional analgesia" or "anesthetic technique" or "anaesthetic technique", (ii) "recurrence" or "survival" and (iii) "colorectal cancer" or "colon cancer". Also, we searched "related citations" and reference lists to identify other articles. Only full papers published in the English language were included. We did not define a minimum of patients to qualify for inclusion in the analysis.

The following information was gathered from the articles: (i) number of included patients, (ii) design of the study, (iii) age, (iv) type of tumor (colon and/or rectal), (v) tumor stage, (vi) follow up, and (vii) effect of anesthetic technique on overall survival and cancer recurrence.

RESULTS

A total of seven studies were found addressing the impact of EA on survival in colorectal cancer surgery. (8-14) *Table 1* shows the characteristics of each of these.

Prospective studies

Two studies of these seven were prospective. Christopherson *et al*. studied long-term survival after resection of colon cancer as a sub-analysis of a prospective randomized study. This Veterans Affairs Co-operative Study No. 345 was initially designed to compare the short-term effect of general anesthesia with and without epidural anesthesia and analgesia supplementation in patients undergoing abdominal surgery. Randomization was stratified for type of surgery, age and cardiac risk. (9) The second prospective study was the Multicenter Australian Study of Epidural Anesthesia and Analgesia in Major Surgery (the MASTER trial), primarily designed to compare adverse outcomes in high-risk patients managed for major surgery with epidural block or alternative analgesic regimens with general anesthesia in a multicentre randomized trial. (14)

Overall survival

Christopherson *et al*. found a better overall survival in the first 1.5 postoperative years in 177 colon cancer patients for stage I-II patients with a mean age of 69 years old who received EA (HR 0.21 (95% CI 1.40-15.42) p=0.012). Nevertheless, the type of anesthesia did not appear to affect longterm survival. (9) Of 446 patients in the MASTER trial with a mean age of 70-71 years old undergoing major abdominal surgery for different types of cancer, 112 underwent surgery because of stage I-III colon cancer. They did not find that the use of EA (n=58) was associated with improved overall survival. (14)

Disease-free survival

Of the two prospective studies, only the MASTER trial assessed a disease-free survival analysis. EA in this study was not associated with improved disease-free survival.

Retrospective studies

Five retrospective studies were included in this review. Of all reviewed literature, the largest retrospective study was the Surveillance, Epidemiology, and End Results (SEER)-

Table 1. Characteristics of described studies

First author	Year of publication	Study design	No. of patients	Mean age (years)	Cancer type	Stage	Follow up (years)	OS benefit from EA	RFS benefit from EA
Christopherson	2008	Prospective	177 EA: 58 No EA: 92	69	Colon	I-IV	Up to 10 years	Better OS in stage I-II No benefit in stage III-IV	Not assessed
Gottschalk	2010	Retrospective	509 EA: 256 No EA: 253	64	Colon (n=283) Rectal (n=202) 'others' (n=25)	I-IV	Median, 1.8	Not assessed	Better RFS in older patients (≥ 65 years old)
Gupta	2011	Retrospective	655 EA: 562 No EA: 93	73 (colon) 69 (rectal)	Colon (n=360) Rectal (n=295)	I-III	Mean, 2.6	Better OS in rectal cancer	Not assessed
Myles	2011	Prospective	112* EA: 58 No EA: 54	71 (EA) 70 (no EA)	Colon	I-III	Up to 12 years	No benefit	No benefit
Day	2012	Retrospective	424 EA: 107 (251 including spinal) No EA: 173	72 (EA) 70 (PCA) 70 (spinal)	Colon (n=314) Rectal (n=110)	I-III (?) Not clearly described	Median, 3.1 (epidural) 2.3 (PCA) 1.4 (spinal)	No benefit	No benefit
Cummings	2012	Retrospective	42151 EA: 9670 No EA: 32481	≥ 66	Colon (n=33390) Rectal (n=8761)	I-III	Up to 14 years	Better OS	No benefit
Holler	2013	Retrospective	749 EA:442 No EA: 307	Not available	Colon (n=369) Rectal (n=380)	I-IV	Up to 8 years	Better OS (especially in ASA classification 3 to 4)	Not assessed

*As a part of 446 patients undergoing major abdominal surgery for different types of cancer

EA=epidural anesthesia; OS= overall survival; PCA=patient-controlled analgesia; RFS=recurrence-free survival

based study with a large cohort of 42151 patients aged 66 years or older diagnosed with non-metastatic colorectal carcinoma. (8) Holler *et al* studied 749 stage I-IV colorectal cancer patients in their large retrospective analyses. (13) The Swedish study of Gupta *et al.,* with a total of 655 colorectal patients with a mean age of 69 (rectal cancer) and 73 (colon cancer) years old, excluded emergency operations, laparoscopic-assisted resections and stage IV in their analysis. (12) Day *et al.* studied colon- and rectal cancer patients with a mean age of 70 (no epidural) and 72 (epidural) years old. (10) All underwent a laparoscopic resection in this study. Patients received either an epidural (n=107), spinal block (n=144), or a morphine, patient-controlled analgesia (PCA) (n=173) for their primary postoperative analgesia. Gottschalk *et al* analysed stage I-IV patients (n=509) of which 283 with colon cancer, 202 with rectal cancer and 25 'others'. (11)

Overall survival

Four of the retrospective studies assessed overall survival analysis. The large SEER-based study found a significant association between EA and improved overall survival (HR 0.91 (95% CI 0.87-0.94) p<0.001). (8) A significantly better overall survival was also found by Holler *et al*. in 442 patients who received EA (5 year survival rate with EA was 62%, but only 54% without EA; HR 0.73, p< 0.02). (13) The positive impact in this study was the most significant in high risk patients defined as American Association of Anaesthesiologists (ASA) classification 3 - 4 (p= 0.006). (13) The Swedish study found a reduction in all-cause mortality in rectal cancer patients (n=295) who received EA (HR 0.45 (95% CI 0.22-0.90) p=0.025). (12) Day et al. found no overall survival difference in their analysis. (10)

Disease-free survival

In the study of Gottschalk *et al*. during median follow up of 1.8 years, EA was associated with a lower cancer recurrence in 248 patients older than 64 years (p=0.01), but not in younger patients. (11) The SEER-based study adjusted for demographic and clinical covariates and did not find a significant difference in the odds of recurrence between the groups during a mean follow-up of 5 years. (8) Also no recurrence-free survival difference was found in the study of Day *et al*. (10)

DISCUSSION

Because the anti cancer immune response is a primary determinant of cancer progression, it is logical to hypothesize that interventions aimed at reducing exposure to immunosuppressive factors would improve patient outcomes after a potentially curative cancer resection. Although EA is theoretically supposed to be a favourable immune-

modulating intervention, not all studies show consistent beneficial effect from EA in colon cancer patients. Seven studies are included in this review, of which two had a prospective design. Four of the seven studies showed an overall survival benefit in patients receiving EA although, in three of these, the effect was only seen in subgroups (stage I-II in the first one and a half year postoperative, rectal cancer patients and ASA 3 - 4 patients). A cancer recurrence survival benefit of EA was found in one study-in older patients. None of the studies found a negative effect of EA on recurrence-free or overall survival.

Because of the retrospective nature of five of the seven studies, unrecorded factors may have influenced survival: for example, potentially important treatment characteristics like the use of chemotherapy and radiation are missing in all studies except Gupta *et al* (12). Although, in some studies, tumor grade is known (11;13;14), other tumor-specific characteristics that influence prognosis - such as lymphangioinvasion, tumor perforation and microsatellite instability - are unknown.

It is hypothesised that volatile anesthesia and opioids may have a negative effect on the anti-cancer immune system, especially natural killer (NK) cells (5;15;16). EA might reduce the requirement of volatile anesthesia, and obviate the need for opioid administration. None of the studies give detailed information about the used analgetic and anesthetic techniques currently in use.

In two of the seven studies, only colon cancer patients were studied (9;14), while four studies analyzed colorectal cancer patients as one group (8;10;11;13). Only Gupta *et al* made a subanalysis for colon and rectal cancer. (12) As a possible explanation for the better survival for rectal cancer patients with EA in their study, they suggest that rectal cancer may be more susceptible to the protective effect of regional analgesia than colonic cancer patients. No specific pathophysiological mechanism for this hypothesis is given.

NK cells are the major responsible of cytotoxic activity against spontaneously derived tumor cells. Data from literature have shown that both the total and the relative number of circulating NK cells are greater in healthy elderly than in young adult ages. The age-related increase of NK-cell number can be regarded as a compensatory mechanism for the decreased cytolytic activity per cell in elderly subjects. Total NK-cell cytotoxicity is steady, but, the NK-cell cytotoxicity on a 'per cell' basis is impaired (17). Gottschalk *et al* suggested that the favorable benefit from EA on the immune system (and especially the NK cells) might be greater in older subjects, because they only found a recurrence-free survival benefit from EA in patients older than 64 years old (11). Although specific changes of the effect of EA on NK cells in elderly subjects might play a role in different results of the studies, the possible underlying mechanism needs to be further clarified in future studies.

Different surgical techniques may also have influenced the results, especially the laparoscopic versus open approaches. The study of Day *et al* only looked at patients receiving laparoscopic colorectal resections. (10) The reason why no survival advantage was identified with the use of regional analgesia in this study may be due to the laparoscopic approach. Laparoscopy is known to reduce the degree of immunosuppression that occurs during the postoperative period compared with that of an open colorectal resection. (18) If a significant preservation of immune function occurs with laparoscopic colorectal resection, the choice of analgesia used may be less important. On the other hand, a large number of trials comparing laparoscopic and open surgery for colorectal cancer can be identified in the literature. A recent meta-analysis stated that laparoscopic surgery for colon cancer does not differ from open surgery in terms of overall survival. (19) None of the prospective studies in our review stratified for the type of surgery (laparoscopic versus open).

Finally, the effect of EA might not only be anti-tumor, but also favour other mechanisms. Although cancer recurrence will determine survival to a large extent, other putative mechanisms include a reduction in perioperative cardiac-, respiratory- and thromboembolic events, but this effect mainly influences short-term survival. (20) A recent Cochrane review concluded that, compared with general anesthesia, a central neuraxial block may reduce the 0-30-day mortality for patients undergoing surgery with intermediate-to-high cardiac risk. (21)

CONCLUSIONS

This review of seven heterogeneous studies shows that the association between EA and survival for colon and rectal cancer is not clear, as conflicting results are described in literature - although none of the studies showed a negative influence of EA on survival. Randomized prospective, well stratified studies are needed to determine whether the association between EA and (cancer-specific) survival is causative.

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8

Epidural analgesia associated with better survival in colon cancer

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Int J Colorectal Dis. 2015 Aug;30(8):1103-7.

ABSTRACT

Introduction Surgery remains the mainstay of treatment for potentially curable colon cancer. Otherwise, the surgical stress response might increase the likelihood of cancer dissemination during and after cancer surgery. There is growing evidence that the type of anesthesia during cancer surgery plays a role in the metastatic process. Therefore, we assessed if the method of anesthesia is associated with long-term survival after colon cancer surgery.

Methods A retrospective single-center study was conducted including 588 patients who underwent colorectal cancer surgery, TNM stage I-IV, in the Jeroen Bosch Hospital between 1995 and 2003. The Cox proportional hazard model was used for statistical analysis. Adjustments were made for age, sex, comorbidity, TNM stage, chemotherapy, emergency surgery status and year of incidence.

Results Of the 588 primary colon cancer patients with a median age of 70 years, 399 (68%) patients underwent colon surgery with epidural anesthesia, whilst 189 (32%) patients were operated without epidural anesthesia. Five-year survival for patients not receiving epidural analgesia was 42% versus 51% for patients receiving epidural analgesia (P=0.03). This effect remained after adjustment for relevant patient, tumor, and treatment characteristics (Hazard Ratio (HR) 1.30 (95% Confidence Interval (CI) 1,05-1.59), p=0.01). Subgroup analysis in patients of 80 years and older (n=100) showed also a better overall survival after receiving epidural analgesia (HR 1.74 (95% CI 1.11-2.72), p=0.01).

Conclusions Epidural analgesia during colon cancer surgery was associated with a better overall survival. Prospective trials evaluating the effects of locoregional analgesia on colon cancer recurrence are warranted.

INTRODUCTION

Surgery remains the mainstay of treatment for potentially curable colon cancer. On the other hand, the surgical stress response may increase the likelihood of cancer dissemination and metastasis during and after cancer surgery. Furthermore, a large proportion of patients does already have micrometastases at the time of surgery. Whether this results in clinical overt metastases depends largely on the balance between the metastatic potential of the cancer cell and the anti-metastatic immune activity of the patient.(1-3) The possibility that anesthetic drugs can influence this process of cancer recurrence is subject of more recent interest. Based on experimental studies and a few clinical investigations, epidural (or other locoregional) analgesia during cancer surgery has been suggested to reduce the chance of cancer metastasis. (4)

Epidural analgesia or anesthesia has commonly been used for the management of pain after abdominal surgery. The reduction in the neuroendocrine stress by epidural analgesia may prevent immunosuppression induced by surgery and general anesthesia and thereby influence the host's antitumor defense mechanism.(5;6) Recent studies in breast cancer and prostate cancer supported the evidence of reduced cancer recurrence.(7;8)

We conducted a retrospective single-center study using a large cohort of colon cancer patients to evaluate whether the method of anesthesia is associated with long-term survival after colon cancer surgery.

METHODS

Patients

All patients who were operated upon for colon cancer in the period 1995 until December 2003 were selected from the databases of the Jeroen Bosch Hospital in 's-Hertogenbosch and the Eindhoven Cancer Registry (Comprehensive Cancer Centre South), which records and maintains thorough information about all patients diagnosed with cancer in the Southern part of the Netherlands. Patients of whom no information on perioperative epidural use was available and also those who had an unknown status of recurrence after surgery were excluded (n=106). Ultimately, a total of 588 patients were included who underwent surgery for colon cancer, TNM stage I-IV, in the Jeroen Bosch Hospital between January 1995 and December 2003.

Patients were allocated into two groups according to the information in their medical records, i.e. those receiving epidural analgesia perioperatively and those not receiving epidural analgesia, but patient-controlled analgesia as the primary method of analgesia. Furthermore, the following data were collected for each patient: gender, age, comor-

bidity, TNM stage, emergency or elective surgery, chemotherapy and their vital status. Follow-up was assessed and complete until 2012.

Statistical analyses

All data are presented as numbers (percentages). Survival and type of anesthesia was visually depicted using Kaplan-Meier survival curve. The log rank test was used to test differences in univariate survival. The differences in epidural anesthesia use between the different years of incidence, as well as between the elective and emergency procedures were determined by Mann-Whitney tests. The relationship between type of anesthesia (epidural: yes/no) and survival was analyzed using the Cox proportional hazard model adjusted for age, sex, UICC stage, chemotherapy, comorbidity, emergency surgery status and year of incidence. P-values for the differences in patient characteristics and overall mortality risks were obtained by chi-square testing. SPSS software (version 16.0.1, SPSS Inc, Chicago, Ill) was used for statistical analyses. All tests were 2-sided. P-values lower than 0.05 were considered statistically significant.

RESULTS

The total study population comprised 288 males and 300 females with a median age of 70 years. A total of 399 (68%) patients underwent colon surgery under general and epidural anesthesia and 189 (32%) patients were operated upon without epidural anesthesia. Only 58 (9.9%) patients were operated in an emergency setting. Median follow-up was 53 months. Patient and treatment characteristics are shown in *Table 1*.

Epidural analgesia

From 1995 to 2003, an increasing number of patients received perioperatively epidural analgesia ($p < 0.01$). Furthermore, a difference was found in epidural analgesia use for patients electively operated (70%) versus patients who underwent emergency surgery (52%) ($p<0.01$). Mean age in electively operated patients, 69 years, did not differ with those patients who underwent emergency surgery, 67 years old ($p>0.05$).

Survival

Patients receiving epidural analgesia exhibited a better overall survival compared to patients who did not receive epidural analgesia (5 years survival of 51% versus 42%), with a crude hazard ratio (HR) of 1.25 (95% CI 1.02-1.53), $p=0.03$. Figure 1a is the graphical representation of the relationship between epidural anesthesia and mortality in all subjects. After adjustment for age, sex, comorbidity, UICC stage, chemotherapy, emergency surgery status and year of incidence these results did not change (HR 1.30 (95% CI 1.05-

Table 1. Patient and treatment characteristics of patients who did and did not receive epidural analgesia during colon cancer surgery between 1995 and 2003.

	Epidural analgesia n=399	No epidural analgesia n=189	p-value
Sex			0.781
Male	197 (49%)	91 (52%)	
Female	202 (51%)	88 (48%)	
Age (years), median (SD)	70 (12)	71 (13)	0.964
Comorbidity			0.718
0	165 (41%)	73 (42%)	
1	121 (30%)	56 (32%)	
≥ 2	112 (28%)	46 (26%)	
missing	1 (<1%)	14 (7%)	
TNM stage of primary tumor			0.122
I	64 (16%)	27 (14%)	
II	174 (44%)	77 (40%)	
III	92 (23%)	49 (26%)	
IV	69 (17%)	33 (18%)	
Chemotherapy			0.759
Yes	76 (19%)	34 (18%)	
No	323 (81%)	155 (82%)	
Timing of surgery			0.006
Elective surgery	369 (92%)	161 (85%)	
Emergency surgery	30 (8%)	28 (15%)	

Data presented are as number (percentages), unless otherwise stated.
Abbreviation; SD: standard deviation.
P-values were obtained by Mann-Whitney U tests (continuous parameters) and Chi-square test (categorical parameters).

1.59), p=0.01). In Figure 1b the relationship between epidural anesthesia and survival of patient that underwent elective surgery is shown (n=530). Also in this subgroup, patients receiving epidural analgesia exhibited a better overall survival compared to those who did not (5 year survival of 52% versus 42%), with a crude hazard ratio of 1,25 (95% CI 1.00-1.55), p=0.04. Overall mortality risks of patients who did and did not receive epidural analgesia during colon cancer surgery are shown in *Table 2*. Besides, also in patients of 80 years and older (n=100) the use of epidural analgesia was associated with a better overall survival than those who did not receive epidural analgesia, adjusted for all the above mentioned confounders, HR 1.74 (95% CI 1.11-2.72), p=0.01. No survival benefit was found in patients with epidural analgesia who underwent emergency surgery.

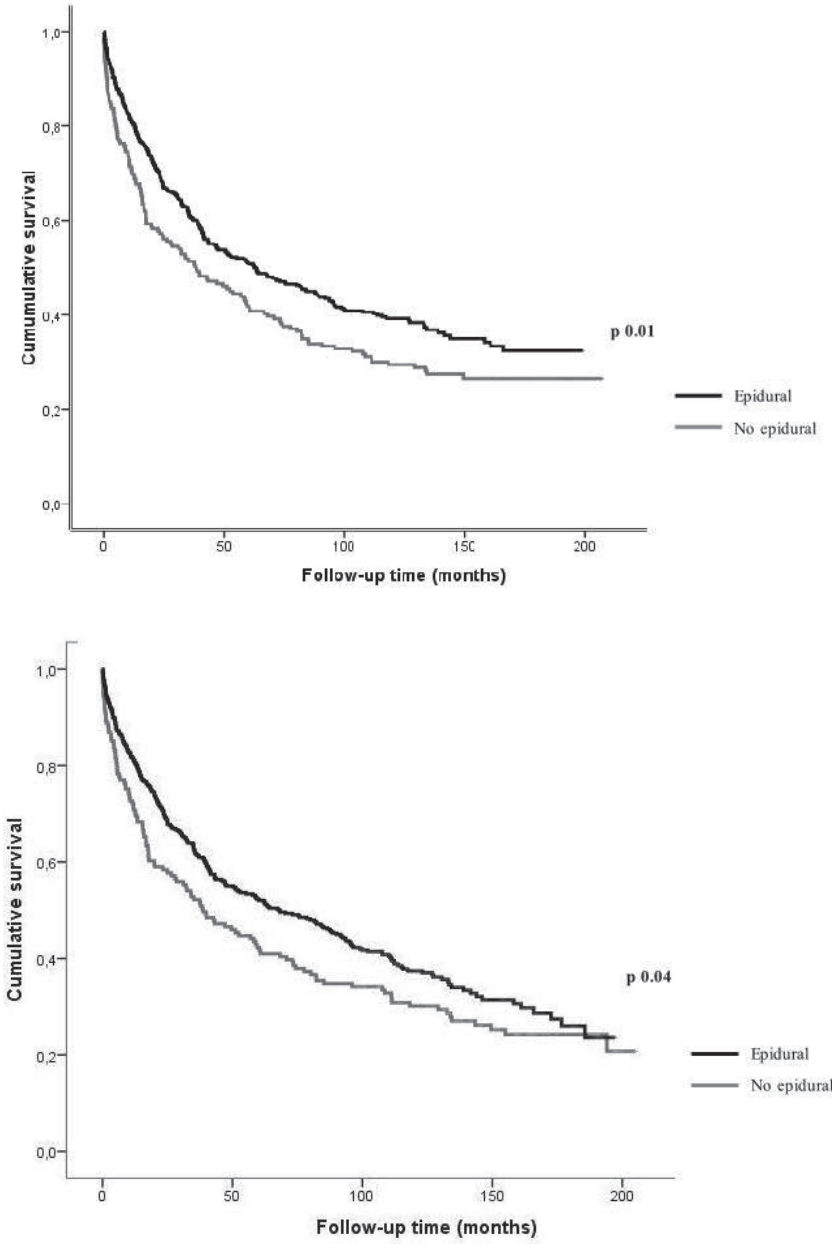

Figure 1 a. Graphical representation of the relationship between epidural analgesia and mortality in all subjects after colon cancer surgery. **b.** Graphical representation of the relationship between epidural analgesia and mortality in all subjects after elective colon cancer surgery.

Table 2. Overall mortality risks of all patients undergoing surgery for colon cancer.

		HR (95% CI)	p
Epidural analgesia	Yes	1 (ref)	
	No	1.30 (1.05-1.59)	0.01
Sex	Male	1 (ref)	
	Female	0.91 (0.75-1.11)	0.39
Age (years), median		1.04 (1.03-1.06)	<0.01
Comorbidity			
0		1 (ref)	<0.01
1		2.10 (1.64-2.70)	
≥ 2		1.78 (1.38-2.29)	
TNM stage of primary tumor			
I		1 (ref)	<0.01
II		1.16 (0.85-1.59)	
III		2.03 (1.42-2.92)	
IV		7.60 (5.30-10.91)	
Chemotherapy	Yes	1 (ref)	
	No	0.95 (0.69-1.29)	0.75
Year of incidence		0.97 (0.93-1.01)	0.21
Timing of surgery	Elective	1 (ref)	
	Emergency	1.69 (1.24-2.29)	<0.01

Hazard ratios were estimated using Cox proportional hazard models.

DISCUSSION

In this retrospective cohort-study comprising 588 patients operated upon for colon cancer, the use of epidural analgesia during colon cancer surgery was associated with a better overall survival.

In recent years, several retrospective observations were done in cancer patients undergoing surgery with or without perioperative locoregional analgesia. Exadactylos *et al* showed a fourfold lower recurrence level in breast cancer patients undergoing breast surgery with paravertebral analgesia. This study can be viewed as hypothesis generating, and as an estimated size effect for future randomized controlled trials. (8) In a retrospective analysis of 225 patients after open radical prostatectomy, substituting epidural analgesia for postoperative opioids, was associated with a lower risk of biochemical cancer recurrence.(7) In a secondary analysis of patients undergoing radical prostatectomy (n=99), no effect of epidural anesthesia on disease-free survival, however, could be found.(7;9) Recent retrospective analysis in 143 patients with an ovarian serous adenocarcinoma showed that the 3- and 5-year survival rates were significantly

better for the 101 patients who underwent surgery with epidural anesthesia and analgesia than patients who had general anesthesia and postoperative intravenous opioid analgesia. (10)

For colorectal cancer, results of earlier studies are conflicting. In a retrospective study of 177 patients, the use of epidural anesthesia was associated with an improved survival in the first 1.5 years, whilst later on, the type of anesthesia did not seem to affect survival. Cause of death in this study might have been different in the early postoperative period, during which epidural anesthesia was associated with a significant increase in survival, from the cause of death in later years, when there was no benefit associated with epidural anesthesia. (11) In another retrospective study, a potential survival benefit was only observed in elderly colorectal cancer patients, aged > 64 years. (12) A recent Swedish study found a reduction of all-cause mortality in patients after rectal cancer surgery but not after colon cancer surgery when comparing epidural analgesia with patient-controlled analgesia. (13) Furthermore, the Multicentre Australian Study of Epidural Anesthesia and Analgesia in Major Surgery (the MASTER trial) (14), a prospective study, primarily designed to compare adverse outcomes in high-risk patients managed for major surgery with epidural block or alternative analgesic regimens with general anesthesia in a multicentre randomized trial, also performed a sub-analysis on overall survival in patients with colon cancer. Of the 446 patients in this trial undergoing major abdominal surgery for different types of cancer, 112 underwent surgery because of stage I-III colon cancer. They did not find that the use of epidural analgesia (n=58) was associated with improved overall survival.

Furthermore, it is worth mentioning that a number of studies using animal models have demonstrated the biological plausibility of an effect of regional anesthesia on long-term outcome after cancer surgery. A key study using a rat model demonstrated that sevoflurane general anesthesia and laparotomy each suppress tumoricidal function in liver mononuclear cells (T-helper cells) and that spinal block attenuates this effect. (15) This study also showed fewer liver metastases in the sevoflurane plus spinal anesthesia group compared with the sevoflurane without spinal group. Another rat study showed that laparotomy conducted during general anesthesia alone increased lung tumor retention up to 17-fold. The addition of spinal block reduced this effect by 70%. The natural killer (NK) cell function in this study was also better preserved by regional anesthesia than by general anesthesia. (16) The same study group assessed that thiopental anesthesia suppresses natural killer cell activity and compromises host resistance to metastatic formation. (17)

The potential ability of regional anesthesia to improve long-term outcome after cancer surgery can be attributed to at least three different mechanisms.(4) First, regional anesthesia attenuates the immunosuppressive effect of surgery. Neuraxial anesthesia can inhibit the neuroendocrine stress response and, in particular, paravertebral analgesia in

humans who did have breast surgery, has also been shown to inhibit this surgical stress response. (18) Secondly, patients who receive regional analgesia have lower opioid requirements, as has been shown in breast cancer surgery. (19) Opioids may themselves inhibit cell-mediated immunity and host anti-tumor defences. (20) Finally, when regional anesthesia is used in addition to general anesthesia, the amount of general anesthetic required during surgery is reduced.(4) Inhaled anesthetics and intravenous opioids may contribute to the process of metastasis by decreasing the activity of NK cells. NK cells are particularly important because they can spontaneously recognize and kill malignant cells, and their suppression is associated with increased rates of metastasis. (21) Low perioperative levels of NK activity are associated with an increased cancer related morbidity and mortality. (22)

Epidural analgesia has commonly been used for the management of postoperative pain after abdominal surgery and is considered fundamental in Enhanced Recovery Protocols, which is up until now considered the standard of care in many hospitals in the Netherlands. However, some authors state that routine epidural anesthesia is unnecessary for laparoscopic- colorectal resection, and, therefore, its ongoing value in the perioperative management of laparoscopic- colorectal surgical patients is still under debate. (23;24)

Potential limitations of our study must be considered. The most important is its retrospective design. Patients were not randomized, so selection bias and the effects of unmeasured confounding variables cannot be excluded. Also, potentially relevant information such as the amount of morphine given and whether analgesia in the epidural group was sufficient, was not available in the records.

The strength of the study is its long-term follow up in a large cohort of colon cancer patients.

CONCLUSIONS

Our study shows that the benefit of epidural analgesia seems to be more than only analgetic. We observed a substantial reduction in long-term death risk when colon cancer surgery was performed with epidural analgesia. The results of the present study suggest that prospective trials evaluating the effects of regional analgesia on recurrence of colon cancer and other malignancies like breast cancer are warranted.

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9

Summary

BACKGROUND

Over the passed decades, significant progress has been made in the treatment of patients with colorectal cancer (CRC) due to advances in surgery, radiotherapy, and chemotherapy. All-stage survival in CRC has increased through improved surgical technique and preoperative care, aggressive management of metastatic disease and advances in (neo-)adjuvant therapies. Furthermore, pre-operative radiotherapy and the introduction of total mesorectal excision technique significantly decreased the local recurrence rate in rectal cancer. (1) Introducing and improving adjuvant chemotherapy regimens in stage III colon cancer has also resulted in better survival. (2) Nowadays, determination of the lymph node status is the most important prognostic factor in CRC, which is critical for staging of these tumors and for allocation to adjuvant therapy when metastases in lymph nodes are found. (3) Despite this, nodal involvement alone is not considered sensitive enough to discriminate between patients with poor or better prognosis.

Unless a curative oncological resection of the tumor, it seems that in a large fraction of patients minimal residual disease (MRD) is present at the time of surgery. These tumor cells escape detection by traditional hematological, pathological and radiological evaluation. (4) MRD is defined by the presence of circulating tumor cells in the blood (CTC), disseminated tumor cells in bone marrow (DTC) or disseminated (isolated) tumor cells (ITC) or micrometastases in lymph nodes not found in conventional staging procedures. (5) Bone marrow appears to be a common homing site for DTCs derived from carcinomas of different organs and also might be a reservoir for DTCs with the capacity to re-enter other distant organs. (6) Since the idea that DTCs as a part of MRD may be responsible for metastasis has arisen, different strategies and applicable technologies have been developed to reliably identify, isolate and analyse assumed DTCs. Based on these technologies and strategies, different studies have been conducted to elucidate the clinical relevance and prognostic significance of MRD. (7;8)

More detailed information about the prognosis of patients with CRC might select high-risk groups that need altered treatment other than the current guidelines. In an attempt to gain insights into prognosis in CRC, this thesis focuses on pathological, molecular and clinical prognostic factors related to recurrence and survival. The first part of this thesis focuses on the clinical impact of disseminated tumor cells in bone marrow, as part of MRD, and aspects of the tumor microenvironment especially tumor-stroma ratio. The second part of the thesis focuses on ultrastaging and risk-stratification in node negative colon cancer by determining the role of potential clinical factors, lymph node mapping and molecular markers. In the final part of this thesis the influence of an external factor during surgery, the impact of anesthesia, on prognosis of colorectal cancer patients is assessed and discussed.

DISSEMINATED TUMOR CELLS IN BONE MARROW AND ASPECTS OF TUMOR MICROENVIRONMENT

Chapter 2 describes the results of the study evaluating whether the presence of DTCs in bone marrow (BM) of patients undergoing surgical resection of colorectal liver metastases is associated with poor clinical outcome. In this study, sixty patients with colorectal liver metastases, scheduled for a curative resection between 2001 and 2007, underwent bone marrow aspiration before surgery. Detection of tumor cells was performed using immunocytochemical staining for cytokeratin (CK-ICC) combined with automated microscopy or indirectly using reverse transcription-polymerase chain reaction (RT-PCR).

DTCs were found in 33% of the patients using CK-ICC and in 20% of the patients using RT-PCR. Patients with negative results for RT-PCR had a significant better disease-free survival and overall survival after resection of their liver metastases. In this study DTCs detected with CK-ICC were not associated with poor clinical outcome. Additional studies are needed to determine the optimal detection method of DTCs. Also further molecular studies are needed to understand the biology of DTC. A more detailed and also functional analysis of the cells found in bone marrow of colorectal cancer patients may help in therapy selection and may give better prognostic information and, as such, can contribute to individual patient management.

In the process of metastasis that requires cancer cells to escape from the primary tumor and survive in the circulation, tumor microenvironment plays an important role. (9;10) The tumor microenvironment is the cellular environment that surrounds tumor cells, including the stroma of the tumor. In recent years, the simple concept that tumor progression depends solely on the intrinsic properties of the cancer cells has recently given way to a more complex paradigm in which tumor progression depends also on the interaction between tumor and host cells. (11) Recent evidence suggests that the tumor-stroma profoundly influences tumor growth, angiogenesis and dissemination. Tumor-stroma is thought to promote tumorigenesis by different mechanisms including remodeling of the extracellular matrix, suppression of immune response and alterations in stromal regulatory pathways affecting the motility and aggressiveness of cancer cells. Assuming these models are correct it can be anticipated that changes in the proportion of stromal compartment in the primary tumor probably reflect progression. (12)

The aim of the study described in **chapter 3** was to determine the clinical importance of DTCs in bone marrow of patients with primary CRC using CK-ICC, from a prospective database with a long-term follow up. DTCs in bone marrow were found in one-fifth of the patients. It seems that, even in early stage, colorectal cancer is a systemic disease in at least a part of the patients. However, the presence of DTCs detected with CK-ICC did not predict a worse clinical outcome in our study, although a trend towards better

disease-free survival was found in node-negative colon cancer patients without DTCs in bone marrow.

Furthermore, we also studied tumor-stroma ratio (TSR) and tested whether this parameter was associated with survival. Moreover, the relationship between TSR and DTCs in bone marrow was studied. The hypothesis that a high amount of stroma in the primary tumor is related to the presence of DTCs was not confirmed by our results. TSR was associated with a worse overall survival (HR 2.16, 95% CI 1.02-4.57, p=0.04) with 5 year survival rates of 84% vs 62%. The TSR is a simple cell based parameter using conventional microscopy without relevant additional costs, which is a strong asset. Different other studies confirmed a significant prognostic effect of TSR not only in CRC, but also in other solid tumors. Considering its simplicity and availability for conventional clinical pathology, TSR may serve as a new prognostic histological characteristic after its prognostic value has been confirmed by large prospective studies.

Interpretation of studies regarding DTCs is complicated by the fact that there are several methods to detect DTCs. CK-ICC is the most frequently used technique and well standardized although prone to inter-observer variability. When ICC is used for DTC detection, the results will be affected by the choice of keratin antibodies, as discrepancies between different antibody mixtures have been reported. Similarly, the choice of mRNA markers, as well as different assays and platforms, affect the performance of RT-PCR based DTC detection. Studies regarding the presence and clinical impact of DTCs in bone marrow of colorectal cancer patients depict a fairly heterogeneous picture. The heterogeneity is due to the non-standardized study designs and variable detection methods which makes a valid comparison difficult; a plea for standardization. (8)

In the future, a more precise molecular characterisation and functional analysis of the detected tumor cells in bone marrow could give more direction to the possible prognostic impact. In the mean time, a pooled analysis of all multi-institutional studies concerning DTCs in bone marrow of colorectal cancer patients could give more solid information about the prognostic impact.

NODE NEGATIVE COLON CANCER

About 15-30% of the patients with stage II colon cancer develop recurrent locoregional disease or distant metastases within 5 years leading to a 5-year survival of around 70-80%. (13) The potential value of adjuvant chemotherapy for patients with stage II colon cancer is controversial. A pooled analysis and meta-analyses have suggested a 2% to 4% improvement in overall survival for patients treated with adjuvant fluorouracil (5-FU)–based therapy compared with observation. (14;15) Current treatment protocols recommend adjuvant treatment only to stage II patients with high-risk features e.g. T4

stage, bowel perforation or clinical bowel obstruction, inadequate lymph node sampling, poorly differentiated histology or lymphangioinvasion.

The study described in **chapter 4** aims to identify the above mentioned and/or additional high-risk factors in stage II colonic cancer patients related to oncological outcome and to investigate whether the number of high risk factors present relates to various outcome measures like recurrent disease and three year disease-free survival.

We identified 212 stage II colonic cancer patients treated only by radical surgical resection, but not with adjuvant chemotherapy in a retrospectively analyzed cohort. The following six significant factors for recurrent disease were identified in the univariate analysis: age, length of hospital stay, emergency surgery, obstruction, perforation of the tumor and lymphangioinvasion. After multivariate analysis, four independent risk factors for recurrent disease were identified: age, obstruction, perforation of the tumor and lymphangioinvasion. The three year disease-free survival for the low risk group, the high risk group with 1 high-risk factor and the high risk group with ≥ 2 high-risk criteria were 90.4%, 87.6% and 75.9% respectively, showing that patients meeting ≥ 2 conventional high risk criteria had a significantly worse three-year disease-free survival. Therefore, in practice, the number of high risk factors should be part in clinical decision making.

SENTINEL LYMPH NODE MAPPING

It has been demonstrated that multilevel sectioning and the use of immunohistochemistry, defined as fine pathological examination, improved the detection of micrometastases in lymph nodes (LNs) in colon cancer. (16) Therefore, a novel technique to detect these micrometastases has been developed which is the one-step nucleic acid amplification (OSNA), using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method to amplify cytokeratin 19 (CK19) mRNA. CK19 is an epithelial marker, which is highly suggestive for the presence of metastases when identified in LNs. OSNA is already in clinical use for the diagnosis of LN metastases in breast cancer patients and has been shown to be very successful. (17) Sentinel lymph nodes (SLNs) are the LNs that have the highest potential to harbour metastasis due to the fact that they are most directly in communication with the location of the tumor. Different studies showed that *ex vivo* SLN mapping is an easy and feasible technique for ultra-staging CRC patients. This technique was characterized by a high accuracy of 90-100%, and a negative predictive value of 80-100%. More importantly, a rate of 19-57% upstaging has been observed and patients that are LN negative after a SLNM procedure have an excellent prognosis. (18-20)

In **chapter 5** we compared the performance of OSNA with standard H&E staining (routine pathology) and fine pathological (FP) examination of the SLNs detected using

the *ex vivo* SLN mapping, in pre-surgically defined non-metastatic colon cancer patients. In this prospective study of 128 patients, we found an upstaging rate of 20.2% with the use of OSNA only and 36.4% with the use of FP only. An upstaging rate of 46.5% was obtained by combining the two methods together. OSNA and FP appeared to be promising tools for the detection of lymph node micro-and macrometastases in SLNs in colon cancer. The performances of OSNA and FP in this study were superior to FP alone. Since OSNA allows analysis of the whole lymph node, sampling bias can be avoided. In future, OSNA may therefore improve tumor staging and consequently, through adjuvant chemotherapy allocation, reduction of recurrence.

MOLECULAR MARKERS

In **chapter 6** the prognostic value of *BRAF* mutation, *KRAS* mutation, *PIK3CA* mutation and the MSI status is assessed with regard to overall and disease-free survival in a well defined stage II colon cancer cohort of 186 patients who underwent resection but was not treated with adjuvant chemotherapy. *BRAF* mutation and MSI both correlated with poorer disease-free survival. A trend to worse overall-survival was found for *KRAS* (Hazard Ratio (HR) 1.7, 95% Confidence Interval (CI). 0.8-3.5), *BRAF* (HR 0.7, 95% CI. 0.2-2.0) and MSI status (HR 1.8, 95% CI. 0.6-4.9). Unfortunately, because of the small numbers and the low minor allele frequency, survival analysis of *PIK3CA* subgroups in our study was not feasible.

The use of molecular biomarkers in addition to pathological classification, will be particularly important in stage II colon cancer, in order to offer the most adequate therapy to each individual patient and avoid unnecessary chemotherapeutic treatment. Our study shows that in stage II patients that have not been treated with chemotherapy, *BRAF* mutation had a negative prognostic effect on survival and also MSI revealed to be a poor prognostic indicator. In contrast to most other reports, the unfavourable prognostic role of MSI in our study is an uncommon finding. A recent meta-analysis concluded that MSI is associated with a favourable prognosis and, also, a significant beneficial effect of 5-fluorouracil (5-FU) therapy was found for microsatellite stable (MSS) tumors. (21;22) Current treatment protocols recommend adjuvant treatment only to stage II patients with high-risk pathological features but exceptions are made for MSI positive colon cancer; the most recent Dutch guideline does not recommend adjuvant chemotherapy in high-risk stage II patients with a MSI tumor because of the favourable prognosis of MSI in colon cancer patients and a lack of benefit from 5-fluorouracil (5-FU) based chemotherapy. Because in our study we only included stage II patients who were not treated with adjuvant chemotherapy, selection bias might have contributed to a

relatively poorer survival of MSI positive colon cancer patients. Therefore further studies are needed to verify and further clarify these results.

IMPACT OF ANESTHESIA ON CANCER SURVIVAL

An oncological surgical resection is the mainstay of treatment for potentially curable colon cancer. However, it is known that even with the best surgical technique, surgery for cancer is associated with release of tumor cells. Furthermore, as shown in chapter 3, it is noteworthy that, at the time of surgery, a large fraction of patients do harbour MRD. (23) A factor that leads MRD into the phase of metastasis formation is the balance between the metastatic potential of the cancer cell and the anti-metastatic immune activity of the patient. (23-25)

The possibility that anesthetic drugs can influence the process of cancer recurrence is subject of more recent interest. Based on experimental studies and a few clinical investigations, epidural (or other locoregional) analgesia during cancer surgery has been suggested to reduce the chance of cancer metastasis. (26)

The potential ability of regional anesthesia to improve long-term outcome after cancer surgery can be attributed to at least three different mechanisms. First, regional anesthesia attenuates the immunosuppressive effect of surgery. Neuraxial anesthesia can inhibit the neuroendocrine stress response and, in particular, paravertebral analgesia in humans who did have breast surgery, has also been shown to inhibit this surgical stress response. Secondly, patients who receive regional analgesia have lower opioid requirements, as has been shown in breast cancer surgery. Opioids may themselves inhibit cell-mediated immunity and host anti-tumor defences. Finally, when regional anesthesia is used in addition to general anesthesia, the amount of general anesthetic required during surgery is reduced. Inhaled anesthetics and intravenous opioids may contribute to the process of metastasis by decreasing the activity of natural killer (NK) cells. NK cells are particularly important since they are able to spontaneously recognize and kill malignant cells, and their suppression is associated with increased rates of metastasis. (27)

The idea that the anesthesiologist can influence long-term outcome after cancer surgery seemed obvious based on the scientific findings of in vitro and animal studies. Although the first clinical reports, in melanoma patients, date back to the 1990s, (28;29) the retrospective analysis by Exadactlylos in 2006 received major attention by showing a fourfold lower recurrence level in breast cancer patients undergoing breast surgery with paravertebral analgesia. (30)

In **chapter 7** we presented an overview of all studies addressing the association of the use of epidural anesthesia (EA) and survival in colon cancer surgery. Four of the

seven studies showed an overall survival benefit in patients receiving EA, although in three of these studies the effect was only seen in subgroups (stage I-II in the first one and a half year postoperatively, rectal cancer patients and ASA 3 to 4 patients). A cancer recurrence survival benefit of EA was demonstrated in one study of older patients. Relevant confounding factors (e.g. age, sex, tumor stage, comorbidities and the use of chemotherapy) were not consequently described in the different studies. In this review of seven heterogeneous studies we showed that the association of EA and survival for colon cancer is not convincing as conflicting results are described, although none of the studies showed a negative influence of EA on survival.

Moreover, we conducted a retrospective single center study using a cohort of 588 colon cancer patients to evaluate whether the method of anesthesia is associated with long-term survival after colon cancer surgery of which the results are described in **chapter 8.** In our study 68% of the patients underwent colon surgery with epidural anesthesia, while 32% patients were operated without epidural anesthesia. Adjusted for relevant patient, tumor, and treatment characteristics we found a significant better five year survival for patients receiving epidural analgesia (HR 1.30, 95% CI. 1.05-1.59). Subgroup analyses of patients that underwent elective colon surgery (n=530) and patients of 80 years and older (n=100) showed also a better overall survival after receiving epidural analgesia. In future randomized prospective well stratified studies are needed to add evidence to the association of EA and (cancer-specific) survival.

CONCLUSION AND FUTURE PERSPECTIVES

Detection of minimal residual disease in bone marrow and lymph nodes

DTCs have the potential to be suitable biomarkers in the era of personalised cancer care. However, due to heterogeneous detection methods and lack of prospective multicenter trials, DTCs have not yet entered into the clinical routine as a valid biomarker. The next step in further examining the prognostic role of DTCs in colorectal cancer patients is the use of pooled multi-institutional individual patient data, which is acknowledged as a reliable method to perform meta-analysis of survival data. Furthermore, the CTC/DTC research field is much more complex than previously assumed. Particularly, the heterogeneous biology of CTC makes it hard to believe that CTCs/DTCs uniformly express a single or a set of biomarkers. (7;8;31) Metastases may be initiated by and may evolve from dormant DTCs from pre-invasive lesions (early DTCs), rather than established primary tumors. These DTCs may generate metastases with different characteristics from those of the primary tumor and may explain the lack of success treating metastasis with therapies based on primary tumor characteristics. (32) Clinical evidence supports that the vast majority of early DTCs seem to be dormant. (33) Persistence in a dormant state

allow these DTCs to remain unscatched after treatment, contributing to late recurrence of disease. (32) Because BM might serve as a reservoir of DTCs from where they may re-circulate, BM aspiration may be used as a 'liquid biopsy', as has been described for CTCs earlier. (34;35)

An important subpopulation of CTCs/DTCs are the cancer stem cells (CSCs). In analogy with their normal counterparts (i.e. stem cells), these cells display a high level of therapy resistance and can effectively repopulate the tumor. A better understanding in the mechanism that make CSCs resistant to therapy and the way that CSCs retain their tumorigenic stem cell capacities is crucial. (36)

Further characterization of CTCs/DTCs and CSCs is pivotal to provide insights into the complex biology of tumor cell spread, with important implications for defining therapeutic targets and eliminating MRD. In addition, we need to identify the most aggressive subset of CTCs/DTCs that are the metastasis-initiating cells. This task can only be accomplished by a combined effort of different research groups. (7;8;31;36)

MRD in lymph nodes can be detected by the OSNA technique as described in this thesis. OSNA proved to be a promising method for the detection of sentinel lymph node metastases in colon cancer patients and it appeared to outperform routine pathological with an upstaging rate of 20.2%. These results were confirmed by a recent European multicentre study. (37)

A meta-analysis concluded that in contrast to micrometastases, disease recurrence was not increased in the presence of isolated tumor cells. (38) Long-term follow up of the upstaged patient will further proof the clinical importance of OSNA and randomized controlled trials are necessary to adequately assess the prognostic relevance of these detected metastases by OSNA.

Tumor microenvironment

Once cancer recurs, survival rates of patients with cancer drastically decline. A better understanding of the mechanisms underlying cancer recurrence is crucial. Tumor microenvironment seems to be a key player in the metastatic process, embodied in the concept that cancer cells do not manifest the disease alone, but rather conscript and corrupt resident and recruited normal cell types to contribute to the metastatic process. (39) TSP as part of the tumor microenvironment is a relatively simple assessment that may be readily incorporated into routine clinical pathology reporting to improve risk stratification following curative CRC resection. Despite recognition of the importance of the TSP in cancer progression, its relationship with other components of the tumor microenvironment has yet to be fully characterised in future studies. (40)

Molecular prognostic factors

The past decade has witnessed spectacular advances in the development of molecular tumor subclassification and its implementation in the clinical setting for solid tumors. This approach is most illustrated in breast cancer. The scientific and clinical community involved in the treatment of breast cancer has embraced molecular classification of this disease and has incorporated it into clinical research, implementing clinical trials that are molecularly based for each subtype. Moreover, the care of patients who have a diagnosis of breast cancer is more heavily based on the specific subtype of tumor than it is for patients with any other kind of cancer. Unfortunately, this kind of advancement has so far not occurred with colorectal cancer. (41) Only a few markers like *BRAF*, *KRAS* and MSI are used in clinical practice. Colorectal cancers consist of a group of heterogeneous disorders with diverse sets of (epi)genetic changes, which accumulate during the carcinogenesis process. In addition, molecular features and behaviour of tumor cells are influenced by host immunity and inflammation, as well as by a totality of exposures from environment and a totality of interactions of various molecules. The future challenge will be to develop and validate combinations of variables with independent contributions to prognosis in multifactorial models. (42)

Regional anesthesia and cancer survival

Our research suggests a potentially important role of regional anesthesia in the prevention of (micro)metastasis. The natural killer (NK) cell, discovered in 1975, (43) seems to be a key player in this mechanism. A recent meta-analysis suggests that epidural anesthesia and/or analgesia might be associated with improved overall survival in patients with operable cancer undergoing surgery (especially in CRC), but it does not support an association between epidural anesthesia and cancer control. Prospective studies are needed to determine whether the association between epidural use and survival is causal. (44) At this moment an encouraging number of prospective randomized controlled trials are ongoing, and it is hoped that their results, when reported, will add evidence to this topic in the near future. And more importantly the specific function of NK cells should also be part of this research.

Other perspectives

Not only molecular biomarkers but also several life-style factors contribute to prognosis in CRC. Prospective observational data suggest that patients who survive colorectal cancer and are physically active have lower rates of cancer recurrence and better survival compared with those who are physically inactive. (45) Furthermore, obesity, an established risk factor for CRC incidence and death, is associated independently with inferior outcome in survivors of CRC. (46) Emerging evidence for adverse effects of smoking on disease-specific and overall survival suggests the potential for promotion

and support of smoking cessation. (47;48) Another recent prospective cohort study support the potential negative prognostic role of sugar sweetened beverage intake in colon cancer progression. (49) Together, all of these are modifiable lifestyle factors; therefore, interventions to modify these prognostic risk factors have the potential to improve patient outcomes. Future life-style interventional studies will offer meaningful recommendations for clinical care and targeted tertiary prevention.

Prognostic factors described in this thesis could be part of a registration database like the nationwide Dutch Surgical Colorectal Audit (DSCA) (50). Future challenge is the development of a multidisciplinary (international) registration that integrates not only the surgical, pathological (including molecular factors) and medical oncological items, but also anesthesiological elements and specific patient characteristics such as life style factors. Using such a robust database, patient-specific risk profiles can be distilled that can lead to a more tailor made approach.

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Samenvatting

ACHTERGROND

De afgelopen decennia is er door vooruitgang in de chirurgie, radiotherapie en chemotherapie veel progressie geboekt in de behandeling van het colorectaal carcinoom (CRC). De prognose van alle stadia CRC zijn verbeterd onder andere door verbeterde operatieve technieken, pre-operatieve voorbereiding, agressieve benadering van metastasen en verbetering in (neo-) adjuvante therapie. Zo hebben pre-operatieve radiotherapie en de totale mesorectale excisie (TME) het percentage lokaal recidief van endeldarmkanker drastisch verlaagd en heeft adjuvante chemotherapie de overleving van stadium III coloncarcinoom doen toenemen.

Anderzijds is adjuvante chemotherapie in stadium II nog controversieel. 30% van deze patiënten krijgt echter binnen 5 jaar een recidief. Het is daarom aannemelijk dat deze patiënten ten tijde van de primaire operatie al occulte metastatische ziekte hadden. De aanwezigheid van positieve lymfklieren zijn de belangrijkste factor die eventuele adjuvante chemotherapie bepaalt. Echter de lymfklierstatus is niet sensitief genoeg om voor iedere patiënt te discrimineren tussen goede en slechte prognose.

Ondanks een curatieve resectie is er bij een deel van de patiënten 'minimal residual disease' (MRD) aanwezig ten tijde van de primaire operatie. MRD is gedefinieerd als circulerende tumorcellen (CTCs) in bloed, gedissemineerde tumorcellen (DTCs) in beenmerg en gedissemineerde/geïsoleerde tumorcellen (ITCs) of micrometastasen in lymfklieren welke niet met conventioneel onderzoek worden gevonden. Omdat DTCs in beenmerg uiteindelijk kunnen resulteren in metastasen, zijn er verschillende methoden ontwikkeld om deze cellen te detecteren, isoleren en analyseren. Verscheidene studies onderzoeken de klinische relevantie van MRD.

Er is duidelijk behoefte aan meer gedetailleerde informatie over het risicoprofiel van een CRC patiënt, zodat hoog-risico patiënten kunnen worden geselecteerd die wellicht baat hebben bij aanvullende therapie. Dit proefschrift beschrijft verschillende pathologische, moleculaire en klinische prognostische factoren in relatie tot recidief en overleving. Het eerste deel gaat over DTCs in beenmerg bij CRC patiënten en aspecten van de micro-omgeving van de tumor, in het bijzonder het tumor-stroma percentage. Het tweede deel gaat over ultrastagering en risico stratificatie van lymfklier negatieve coloncarcinoom patiënten door op zoek te gaan naar potentiële klinische, pathologische en moleculaire hoog-risico factoren. In het laatste deel wordt de invloed van een anesthesie op de prognose van CRC patiënten onderzocht.

GEDISSEMINEERDE TUMORCELLEN IN BEENMERG EN TUMOR MICRO-OMGEVING

Hoofdstuk 2 beschrijft de resultaten van de studie naar de prognostische waarde van DTCs in beenmerg bij patiënten die geopereerd zijn vanwege colorectale levermetastasen. In deze studie werd bij 60 patiënten vlak voor de operatie beenmerg afgenomen. Detectie van DTCs vond plaats op 2 manieren: met immunocytochemische cytokeratine kleuring (CK-ICC) en met reverse transcriptase-polymerase chain reaction (RT-PCR). Met CK-ICC werd bij 33% van de patiënten tumorcellen in het beenmerg gevonden en met RT-PCR bij 20%. Patiënten bij wie met RT-PCR geen tumorcellen in het beenmerg werden gevonden hadden een betere (ziektevrije) overleving na levermetastasectomie. DTC ontdekt met CK-ICC hadden geen associatie met een slechtere overleving. Aanvullende studies zijn nodig om de optimale detectiemethode te vinden van DTC. Ook zal meer (moleculair) onderzoek nodig zijn om het biologisch gedrag van DTCs te begrijpen en om een functionele analyse te doen van deze cellen in het beenmerg.

In het proces waarbij een tumorcel van de primaire tumor in de circulatie komt en overleeft speelt de micro-omgeving van de tumor een belangrijke rol. Deze micro-omgeving bestaat uit een complex aan cellen dat rond de tumor ligt, waaronder het tumor-stroma. Het simpele idee dat het de intrinsieke eigenschappen van de tumorcel zelf zijn die zorgen voor metastasering heeft plaats gemaakt voor een complex concept waarin de interactie tussen de tumorcel en cellen in de omgeving bepalend zijn. In recent onderzoek is aangetoond dat tumor-stroma cellen groei, angiogenese en metastasering stimuleren. Uitgaande van dit concept kunnen veranderingen in het tumor-stroma percentage duiden op progressie van de ziekte.

In **hoofstuk 3** wordt een prospectieve studie met een lange follow-up beschreven waarin is gekeken naar de prognostische waarde van DTCs in beenmerg bij patenten met een primair CRC. Als detectiemethode is CK-ICC gebruikt. Bij 20% van de patiënten CRC patiënten werden tumorcellen in het beenmerg gevonden, hetgeen suggereert dat CRC ook in een begin stadium bij een deel al gedissemineerde ziekte is. Echter, de aanwezigheid van deze cellen was in deze studie niet voorspellend voor een slechte prognose.

Tevens werd in dit hoofdstuk de tumor-stroma ratio (TSR) onderzocht. De hypothese dat een hoog percentage tumor-stroma, geduid als prognostisch ongunstig, geassocieerd zou zijn met DTCs in beenmerg kon niet worden bevestigd. Ons onderzoek toont dat TSR geassocieerd is met een significant slechtere overleving, met een 5-jaarsoverleving van 84% versus 62%. Een belangrijk voordeel van tumor-stroma bepaling is het feit dat het een simpele bepaling is die met conventionele microscopie gedaan kan worden. Verder onderzoek binnen grotere prospectieve cohorten zal het prognostisch effect van tumor-stroma moeten bevestigen.

Een complicerende factor bij het vergelijken van studies naar DTCs zijn de verschillende gebruikte detectiemethoden. Als CK-ICC wordt gebruikt zijn de resultaten ook afhankelijk van de gebruikte CK-antilichamen. Parallel hieraan beïnvloeden de gebruikte mRNA markers de RT-PCR methode. Door de heterogeniteit van de verschillende (kleine) studies is een valide vergelijking lastig, hetgeen een pleidooi is voor standaardisatie. Een moleculaire en functionele analyse van de gedetecteerde cellen kan in de toekomst meer inzicht geven in de prognostische waarde van DTCs. Daarnaast kan een gepoolde analyse van verschillende onderzoeksinstituten ook meer solide informatie verschaffen.

LYMFEKLIER NEGATIEVE COLONCARCINOOM PATIËNTEN

Bij 15-30% van de stadium II coloncarcinoom patiënten komt de ziekte terug binnen 5 jaar, hetgeen resulteert in een 5-jaars overleving van 70-80%. Een recente meta-analyse laat een overlevingswinst zien van 2-4% voor patiënten die zijn behandeld met chemotherapie ten opzichte van patiënten die geen nabehandeling kregen. De huidige richtlijnen adviseren alleen adjuvante chemotherapie bij hoog-risico kenmerken (T4-stadium, perforatie/obstructie, <10 lymfklieren, slechte differentiatie of lymfangio-invasie).

De studie beschreven in **hoofdstuk 4** heeft als doel deze en eventueel andere hoog-risico kenmerken te identificeren in relatie tot ziekte-vrije overleving. Ook is gekeken of het aantal hoog-risico kenmerken van invloed is op de prognose. Het betreft een retrospectief onderzoek van 212 stadium II coloncarcinoom patiënten die alleen geopereerd zijn en geen adjuvante chemotherapie hebben gekregen. In univariate analyse zijn de volgende kenmerken geassocieerd met terugkeer van de ziekte: leeftijd, opnameduur, spoedoperatie, perforatie/obstructie en lymfangio-invasie. Na multivariate analyse werden de volgende risicokenmerken geïdentificeerd: leeftijd, perforatie/obstructie en lymfangio-invasie. De 3-jaars ziektevrije overleving voor de laag risico groep, de hoog risico-groep met 1 hoog-risico kenmerk en de hoogrisico groep met ≥ 2 hoog-risico kenmerken zijn respectievelijk 90.4%, 87.6% en 75.9%, hetgeen laat zien dat patiënten met ≥ 2 hoog-risico kenmerken een significant slechtere overleving hebben. Derhalve zal in de dagelijkse praktijk het aantal risico-kenmerken mee moeten worden gewogen in de besluitvorming.

SCHILDWACHTKLIERPROCEDURE

Het gedetailleerder kijken naar lymfklieren bij coloncarcinoom, door deze in meer coupes te snijden dan gebruikelijk, resulteert in een hogere detectie van micrometastasen en wordt gedefinieerd als 'fine pathology' (FP). Een nieuwe techniek, namelijk 'one-step

nucleic acid amplification' (OSNA), maakt gebruik van de zogenaamde reverse-transcription loop-mediated isothermal amplification (RT-LAMP) methode om cytokeratine 19 (CK19) mRNA te amplificeren. CK19 is een epitheliale marker die zeer suggestief is voor de aanwezigheid van metastasen in lymfklieren. OSNA wordt in de praktijk al succesvol toegepast bij het opsporen van lymfklieruitzaaiingen in borstkanker patiënten.

Het toepassen van beide technieken op alle lymfklieren is zeer arbeidsintensief. De schildwachtklier biedt hier uitkomst. Deze wordt gezien als de eerste lymfklier waar een eventuele metastase naar toe gaat. Onderzoek laat zien dat het ex-vivo opzoeken van de schildwachtklier goed toepasbaar en ook betrouwbaar is bij coloncarcinoom. Een aanzienlijk deel van de patiënten zonder lymfkliermetastasen blijken bij nader onderzoek van de schildwachtklier toch (micro-)metastasen te hebben ('upstaging')

In **hoofdstuk 5** vergeleken we de OSNA techniek en de FP methode met de klassieke methode om de lymfklieren na te kijken. In deze prospectieve studie vindt er bij 20.2% van de patiënten upstaging plaats door OSNA en in 36.4% doorgebruik van FP. De 2 technieken gecombineerd resulteerde in een upstaging van 46.5%. OSNA en FP lijken hiermee veelbelovende methoden te zijn om (micro-)uitzaaiingen te detecteren in lymfklieren.

MOLECULAIRE MARKERS

In **hoofdstuk 6** is de prognostische waarde onderzocht van *BRAF*, *KRAS*, *PIK3CA* en microsatelliet instabiliteit (MSI) bij stadium II coloncarcinoom patiënten die niet adjuvant behandeld zijn met chemotherapie. In dit onderzoek bleek *BRAF* en MSI gecorreleerd te zijn met een slechtere ziektevrije overleving. Een trend naar slechter overleving werd gevonden voor *KRAS*, *BRAF* en MSI.

In het bijzonder bij stadium II coloncarcinoom patiënten zijn moleculaire biomarkers belangrijk als aanvulling op de klassieke pathologisch classificatie, om een patiëntspecifieke afweging te maken in het geven of achterwegen laten van adjuvante chemotherapie. Onze studie laat zien dat bij stadium II coloncarcinoom patiënten die niet behandeld zijn met chemotherapie, *BRAF* en MSI een ongunstig beloop voorspellen. De ongunstige prognostische rol van MSI is, in tegenstelling tot de meeste literatuur, een ongewone bevinding. Een recente meta-analyse concludeert dat MSI een gunstige prognose voorspelt en tevens dat op 5-fluoroacil gebaseerde adjuvante chemotherapie significant effectiever is bij coloncarcinoom zónder MSI. De huidige Nederlandse richtlijn adviseert alleen adjuvant chemotherapie bij hoog-risico patiënten, zoals beschreven in hoofdstuk 4. Adjuvante chemotherapie wordt afgeraden bij MSI positieve patiënten, vanwege het ontbreken van het effect. De resultaten van onze studie kunnen zijn beïnvloedt door bias van de patiëntselectie omdat er alleen stadium II patiënten zijn

geïncludeerd die geen chemotherapie hebben gehad. Verder onderzoek is nodig om deze resultaten te bevestigen en verder te verklaren.

EPIDURALE PIJNSTILLING EN KANKEROVERLEVING

Een oncologische operatie gaat gepaard met het vrijkomen van tumorcellen die in het lichaam achterblijven. Daarnaast is er bij veel patiënten reeds sprake van MRD. Of deze MRD zich ook daadwerkelijk tot metastase ontwikkelt, wordt onder andere bepaalt door de balans tussen het antitumor afweersysteem van de patiënt en de metastatische capaciteit van de tumorcel. Proefdieronderzoek en enkele klinische studies suggereren dat locoregionale anesthesie (ruggeprik) dit proces gunstig kan beïnvloeden.

Drie mechanismen kunnen hieraan ten grondslag liggen. Ten eerste vermindert regionale anesthesie het immuunsuppressieve effect en de neuro-endocriene stress respons van chirurgie. Daarnaast hebben patiënten met regionale pijnstilling minder behoefte aan opiaten, zoals bijvoorbeeld blijkt uit borstkankeronderzoek. Opiaten hebben een remmend effect op het (anti-tumor) afweersysteem. Tenslotte kan door regionale anaesthesie de hoeveelheid anaesthetica worden verminderd. Deze laatste lijken een ongunstig effect te hebben op het anti-tumor afweersysteem en met name de natural killer (NK)-cel. Een verminderde functie van NK-cellen is geassocieerd met toename van metastasen.

Hoofdstuk 7 geeft een overzicht van alle studies die het verband tussen epiduraal anaesthesie en overleving bij coloncarcinoom hebben onderzocht. In totaal zijn er 7 studies onderzocht waarvan er 4 een overlevingsvoordeel vinden bij het gebruik van regionale anaesthesie. Een kankerspecifiek overlevingvoordeel werd in 1 studie gevonden, en dan alleen bij oudere patiënten. De studies zijn van beperkte kwaliteit aangezien relevante factoren zoals leeftijd, geslacht, tumorstadium, co-morbiditeit en behandeling met chemotherapie niet consequent zijn beschreven. De uitkomsten van deze 7 heterogene studies zijn tegenstrijdig, echter geen van de onderzoeken toont een negatief effect op de (kanker)overleving.

In **hoofdstuk 8** worden de resultaten beschreven van een studie bij 588 coloncarcinoom patiënten waarin de relatie tussen het krijgen van een epiduraal en de overleving werd onderzocht. In deze studie kregen 68% van de patiënten een epiduraal. Gecorrigeerd voor relevante patiënt-, tumor- en behandelingskarakteristieken werd een significant betere 5 jaaroverleving (51% versus 42%) gevonden voor patiënten met een epiduraal. Subgroep analyse bij electief geopereerde patiënten (n=530) en patiënten ouder dan 80 jaar (n=100) liet een zelfde overlevingsvoordeel. Prospectief gerandomiseerde studies zullen gedaan moeten worden om de associatie tussen epiduraal anaesthesie en (kanker-specifieke) overleving verder te onderbouwen.

CONCLUSIES EN TOEKOMSTPERSPECTIEVEN

Detectie van MRD in beenmerg en lymfklieren

Gedissemineerde tumorcellen als onderdeel van 'minimal residual disease' (MRD) zijn potentiële klinisch bruikbare biomarkers. De grote variatie in de gebruikte technieken en het ontbreken van grote prospectieve multicenter studies staat op dit moment de vertaling naar de dagelijkse praktijk nog in de weg. Een gepoolde meta-analyse van patiëntendata uit verschillende centra is een logische volgende stap in verder onderzoek naar de prognostische waarde van DTCs. Samenwerking tussen verschillende onderzoeksgroepen zal hiervoor noodzakelijk zijn.

De heterogene biologie van CTCs/DTCs maakt het onderzoek ernaar complex. Het is niet aannemelijk dat CTCs/DTCs allemaal dezelfde kenmerken laten zien. Zo kunnen metastasen ontstaan uit zogenaamde slapende DTCs die voortkomen uit vroege invasieve afwijkingen. Deze vroege DTCs kunnen zorgen voor metastasen met andere kenmerken dan de primaire tumor. Therapie gebaseerd op primaire tumoreigenschappen kan zodanig niet effectief zijn tegen metastasen. Klinisch onderzoek wijst erop dat de meeste vroege DTCs slapende DTCs zijn. Slapende DTCs zijn ongevoelig voor therapie en kunnen dus zorgen voor late metastasering. Omdat het beenmerg dient als een reservoir voor DTCs van waaruit deze de circulatie in gaan, kan een beenmergpunctie worden gezien als een zogenaamde 'liquid biopsy', zoals ook beschreven is voor CTCs.

Een belangrijke subgroep van CTCs/DTCs zijn de kanker stamcellen (CSCs). Deze kenmerken zich door een hoge mate van therapie-resistentie en zijn in staat om de tumor te voorzien van nieuwe tumorcellen. De cruciale vraag waarom CSCs therapieresistent zijn en hun stamceleigenschappen behouden zal in de toekomst beantwoord moeten worden. Verdere karakterisering van bovengenoemde cellen, en met name het herkennen van de meest agressieve subtypes, zal meer inzicht moeten verschaffen in de complexe tumorbiologie van metastasering.

MRD in lymfklieren bij coloncarcinoom kan worden gedetecteerd door de OSNA techniek zoals beschreven in dit proefschrift. Recent europees multicenter onderzoek bevestigd deze resultaten. Door toepassing ervan vindt bij 1 op de 5 patiënten een betere stagering plaats. Een meta-analyse laat zien dat in tegenstelling tot micrometastasen, geïsoleerde tumorcellen in lymfklieren niet geassocieerd is met een slechtere kankervrije overleving. Toekomstig follow-up onderzoek zal de prognostische waarde van de door OSNA techniek vastgestelde lymfkliermetastasen verder moeten bepalen.

Tumor micro-omgeving

Overlevingskansen dalen drastisch op het moment dat kanker terugkeert. Een beter begrip in het mechanisme van kankerrecidief is daarom cruciaal. De tumor micro-omgeving lijkt een belangrijke rol te spelen in het metastaseringsproces. Tumor-stroma

is een onderdeel van deze omgeving en is een eenvoudig meetbare entiteit die in verschillende studies prognostische waarde laat zien. Een relatie met DTCs is door ons niet aangetoond. Toekomstig grotere prospectieve onderzoeken zal de rol van tumorstroma verder moeten bevestigen alsook de relatie ervan met de overige componenten van de tumor micro-omgeving.

Moleculaire prognostische factoren

Steeds meer kunnen tumoren op basis van hun moleculaire eigenschappen worden geclassificeerd. Het implementeren van deze kennis in de dagelijkse klinische praktijk is bij borstkanker het meest ontwikkeld. Bij het colorectaal carcinoom is deze ontwikkeling nog niet zo ver. Slechts enkele moleculaire markers, waaronder *BRAF, KRAS* en MSI worden gebruikt in de klinische praktijk. CRC bestaat uit een heterogene groep moleculaire eigenschappen. Processen als inflammatie en immuniteit alsook omgevingsfactoren beïnvloeden deze eigenschappen. De uitdaging voor toekomstig onderzoek ligt in het ontwikkelen en valideren van combinaties van al deze verschillende variabelen met betrekking tot de prognose.

Regionale anesthesie en kanker overleving

Het in dit proefschrift beschreven onderzoek suggereert een belangrijke rol van regionale anesthesie in de preventie van (micro-)metastasering. De in 1975 ontdekte natural killer (NK-) cel lijkt in dit proces een sleutelrol te vervullen. In een recente meta-analyse is regionale anesthesie geassocieerd met een betere overleving, met name bij CRC, echter de associatie met ziekte-vrije overleving wordt niet gevonden. Op dit moment lopen er verschillende prospectief gerandomiseerde studies die in de toekomst meer helderheid hierover zullen geven. In dit kader zal ook de rol van regionale anesthesie op het immuunsysteem, waaronder de NK-cellen, verder moeten worden onderzocht

Overige perspectieven

Lifestyle factoren hebben ook invloed op de prognose van CRC patiënten. Prospectief observationeel onderzoek laat zien dat lichamelijk actieve patiënten een beter overleving hebben en minder terugkeer van ziekte dan inactieve patiënten. Ook overgewicht en roken hebben een ongunstig effect op de ziektevrije-overleving van CRC patiënten. Een potentieel negatief effect op het ziektebeloop wordt ook toegeschreven aan frisdranken met een hoog suikergehalte. Bovengenoemde lifestyle factoren zijn beïnvloedbaar. Interventies hierin kunnen mogelijk de prognose gunstig beïnvloeden. Lifestyle adviezen uit toekomstig onderzoek kunnen een belangrijke rol spelen in tertiaire preventie.

De in dit proefschrift beschreven prognostische factoren zouden in de toekomst onderdeel kunnen zijn van de DSCA registratie database. Uitdaging is de ontwikkeling van een multidisciplinaire (internationale) registratie met niet alleen chirurgische, patholo-

gische (inclusief moleculaire) en medisch oncologische items, maar ook anesthesiologische elementen en specifieke lifestyle factoren. Op deze wijze kan een patiëntspecifiek risico profiel worden gedistilleerd en daadwerkelijk een meer 'tailor-made' behandeling worden gekozen.

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Dankwoord
Curriculum vitae

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DANKWOORD

Vele patiënten hebben belangeloos meegewerkt aan het tot stand komen van dit onderzoek, waarvoor hen veel dank verschuldigd is.
Aan het begin van mijn opleiding tot chirurg heeft Rob Tollenaar mij de gelegenheid geboden om kennis te maken met "onderzoek doen". Het bleek de belangrijke eerste stap op weg naar dit proefschrift. Gerrit-Jan Liefers, immer enthousiast, kritisch en gezond pragmatisch, is de afgelopen perifere jaren mijn life-line met Leiden gebleven. Gedurende mijn tijd in het Jeroen Bosch Ziekenhuis, welke vormend zijn geweest voor mijn ontwikkeling als oncologisch chirurg, is Koop Bosscha de katalysator voor dit proefschrift geweest.

Het includeren van patiënten en verzamelen van data kost veel tijd en energie. Vele (oud)-collega's, chirurgen (i.o.), pathologen, epidemiologen, laboranten, secretaressen en verpleegkundigen hebben hieraan bijgedragen, waarvoor veel dank. Alle co-auteurs, fantastisch dat jullie wilden meeschrijven, meedenken en meerekenen en mijn collega's uit VieCuri, dank voor de nimmer aflatende interesse in dit onderzoek.
Mark Siemonsma en André Vis, vrienden sinds respectievelijk Tanzania en Leiderdorp, superleuk dat jullie mijn paranimfen willen zijn.

Lieve mam en pap, dank voor alle kansen die ik heb gekregen! Geweldig dat jullie hier samen getuige van kunnen zijn.
Lieve Jo, samen met Sieta heb je mij altijd vol interesse gesteund bij mijn promotie-traject.
Mijn allerliefste boeven, Jelle en Carlijn! Geen dag gaat er voorbij zonder dat ik door jullie een glimlach op mijn gezicht krijg. Op jullie vraag: "Pap, is je boekje nu al klaar?", kan ik eindelijk bevestigend antwoord geven.
Lieve Joor, naast de leukste internist van Nederland ben je bovenal de liefste en sterkste vrouw die ik ken. Zonder jou stond ik hier niet!

CURRICULUM VITAE

Jeroen Vogelaar werd op 18 mei 1975 geboren te Leidschendam. Na het atheneum diploma, behaald in 1993 aan het Oranje Nassau College te Zoetermeer, werd in datzelfde jaar begonnen met de studie Geneeskunde aan de Rijks Universiteit Leiden. Zijn afstudeeronderzoek over bottumoren werd uitgevoerd op de afdeling orthopedie onder leiding van Prof. Dr. A.H.M. Taminiau. Na een klinische stage in Tanzania (Sengerema D.D. Hospital) werd het artsexamen behaald in 2000 waarna hij begon als arts-assistent Heelkunde in het Diaconessenhuis te Leiden. In 2001 werd hij arts-assistent in het Rijnland Ziekenhuis te Leiderdorp (thans Alrijne Zorggroep), alwaar hij in 2003 startte met de opleiding Heelkunde (Dr. S.A. da Costa). Jaar 3 en 4 van de opleiding vond plaats in het Leids Universitair Medisch Centrum (Prof. dr. J.J. Hamming), waarna de opleiding tot chirurg in 2009 werd afgerond in Leiderdorp. Tijdens zijn opleiding werd begonnen met het in dit proefschrift beschreven onderzoek. In Leiderdorp heeft hij tot eind 2009 als chef de clinique gewerkt. In 2010 startte hij met de specialisatie Oncologische Chirurgie in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch (Dr. K. Bosscha) waar het wetenschappelijk onderzoek werd voortgezet. Sinds 2012 is hij werkzaam als chirurg-oncoloog in het VieCuri Medisch Centrum te Venlo. Jeroen Vogelaar en Jorien Willems hebben samen twee kinderen: Jelle (2006) en Carlijn (2009).