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Dissemination and clinical impact of minimal metastatic disease in gastrointestinal cancer

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**Dissemination and clinical impact of
minimal metastatic disease
in gastrointestinal cancer**

Cover: 'Hoe weet het zaadje waar het moet kiemen'.

Afbeelding van een Kankantri (plaat 58) uit *Voyage à Surinam* van Pierre Jacques Benoit. Uitgeverij: Société des Beaux-Arts (De Wasmé et Laurent) Brussel 1839. Bron: Universiteitsbibliotheek Amsterdam (UvA) locatie Bijzondere Collecties, plaatsnummer *OTM KF 62-880*.

Achtergrond: de Kankantri of wilde kapokboom (*Ceiba pentandra*, familie Bombacaceae) is mogelijk de hoogste en breedst vertakte boom van het Surinaamse bos. De boom wordt door boslandcreolen als een heilige boom beschouwd en wordt dan ook zelden gekapt en is vaak een getuige van reeds lang verdwenen bos of een aanwijzing voor secundair ontwikkeld bos. Uit: Encyclopedie van Suriname. Hoofdredactie: Prof. dr. C.F. Bruijning, Prof. dr. J. Voorhoeve. Samensteller: Drs. W. Gordijn. Uitgegeven onder auspiciën van de Stichting Encyclopaedie van Suriname. Uitgeverij: Argus Elsevier Amsterdam Brussel 1977.

Cover design: Fania Doekhie en Klaas van der Ham

Gedicht: coupletten uit 'Wan bon' van Dobru (Robin Ravales) vertaald door Edgar Cairo. Uit de bundel: Spiegel van de Surinaamse poëzie: van de oude liedkunst tot de jongste dichters. Meulenhoff Amsterdam eerste druk, 1995. Samenstelling: Michiel van Kempen, met medewerking van Jan Bongers.

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Dissemination and clinical impact of minimal metastatic disease in gastrointestinal cancer

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Eén boom
zovele bladeren
één boom

Eén rivier
zovele kreken
alle op weg naar één zee

Eén hoofd
zovele gedachten
gedachten om één soort heil

*Aan mijn ouders,
Ajoeb en Helene*

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CHAPTER ONE

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

General introduction

Worldwide in 2007, cancer led to the death of 7.9 million people which comprises around 13% of all deaths.¹ An increase in the number of cancer-related deaths is expected with an estimated 9 million in 2015. The main types of cancer leading to overall cancer mortality are solid tumors arising from lung, stomach, colorectum, liver, esophagus, breast, cervix and prostate.² Screening programmes might result in increased incidence rates of early stage cancer, but treatment of cancers such as colorectal, breast and cervical cancer in an early stage have a high cure rate. In this thesis, we will focus on gastric and colorectal cancer with the emphasis on the latter. We assessed whether cancer patients with minimal metastatic disease at the time of surgery who are at risk for disease recurrence, can be identified by detection of occult tumor cells in lymph nodes or bone marrow or by analyzing the primary tumor for angiogenic and lymphangiogenic markers. Patients groups that were investigated were gastric and colorectal cancer patients who underwent curative resection and had tumor-negative lymph nodes after conventional histopathological examination. In addition, patients with colorectal liver metastases who underwent liver resection or isolated liver perfusion after previous treatment for their primary tumor, were studied for effectivity of treatment.

Gastric cancer

The incidence of gastric cancer, which is one of the most common cancers in the world, varies greatly across different geographic locations being higher in Japan and some parts of South America (> 40 per 100,000) and lower in Western Europe and the United States (< 15 per 100,000).³ Environmental exposure rather than genetic factors play a role in the predisposition to gastric cancer.⁴ Risk factors known for gastric cancer are diets rich in salt, smoked or poorly preserved foods, nitrates and high complex-carbohydrates, diets low in fats, proteins, vegetables and fruits and poor drinking water, smoking, prior gastric surgery, *Helicobacter pylori* infection, gastric atrophy, gastritis and adenomatous polyps.^{4;5}

Ninety-five percent of all gastric cancers are adenocarcinomas of which 8% to 10% have an inherited familial component.⁴ Gastric cancer occasionally develops in families with germline mutations in p53 (Li-Fraumeni syndrome) or BRCA2 and can also develop as part of the hereditary nonpolyposis colon cancer syndrome, as well as part of gastrointestinal polyposis syndromes, including familial adenomatous polyposis and Peutz-Jeghers syndrome.⁴ In 1% to 3% of gastric cancers, germline mutations in the gene encoding the cell adhesion protein E-cadherin leads to an autosomal-dominant predisposition to gastric carcinoma, referred to as hereditary diffuse gastric cancer showing a penetrance of approximately 70%. Therefore, for these people, prophylactic gastrectomy is mandated.⁶ For individuals with high risk factors as mentioned previously, the American Society for Gastrointestinal Endoscopy recommends endoscopic surveillance for every 1 to 2 years.⁴ In Western countries, there are no formal screening programmes for gastric cancer,

in contrast to high-incidence regions such Japan and China, where mass screening is cost-effective and has led to a decrease in mortality rates.

Surgery is the cornerstone in gastric cancer treatment and success depends on the size, location and ability to reach margins free of gross and microscopic disease. Extended D2 lymphadenectomy is the norm in Japan but not in Western countries as large randomized controlled trials have failed to show that the extended D2 lymphadenectomy improves survival when compared with D1 dissection.^{7;8} Since improved survival after postoperative chemoradiotherapy (stage IB or higher)⁹ or perioperative chemotherapy (stage II or higher)¹⁰ has been shown by a limited number of studies, they are administered to patients only within a clinical trial.

Colorectal cancer

In 2007, approximately 1.2 million new cases of colorectal cancer (CRC) and 630,000 deaths due to this disease are expected worldwide.² The incidence of CRC is higher in developed countries, making it the second most common cancer in this part of the world with an estimated 341,000 deaths in 2007.^{2;11}

The risk for developing CRC increases with age as a result of accumulation of (epi) genetic mutations, with more than 90% of new cases being diagnosed in patients older than 50 years. Observational studies show that tobacco avoidance, physical activity, weight control and dietary interventions with increase in fibre, fruits and vegetables and reductions in fat and alcohol can reduce the risk for CRC.^{12;13} Inflammatory bowel diseases, ulcerative colitis and Crohn's colitis, are associated with an increased risk of CRC.¹⁴

Most cases of CRC occur in sporadic forms (88 to 94%) of which about 20% have some component of familial risk (two or more first- or second degree relatives with CRC).¹⁵ Sporadic CRC that occurs in the absence of family history, is usually seen in older patients (60-80 years of age), and generally presents as an isolated colon or rectal lesion. Approximately 5 to 10% of all CRC are hereditary. The main forms are familial adenomatous polyposis (FAP), MUTYH associated polyposis (MAP) and hereditary nonpolyposis colorectal cancer (HNPCC).^{15;16}

Surgery is the treatment of choice in resectable CRC and adjuvant chemotherapy is generally administered to stage III patients with lymph-node involvement, as benefits have been shown.¹⁷⁻¹⁹

Treatment of patients with advanced CRC (approximately 20% of CRC patients at diagnosis²⁰) and distant disease recurrence (almost half of the patients who had previously undergone curative resection²¹) depends on the location of the disease, but surgery is the primary treatment of choice. Surgically treated patients with isolated liver or lung metastases can reach a five-year survival of up to approximately 60%.²²⁻²⁴ Patients are considered for resection of liver metastases depending on the number and location of the lesions, lack of major vascular involvement, sufficient functional residual hepatic tissue and absent or limited extrahepatic disease with the exception of resectable pulmonary metastases.^{25;26}

In addition, multiple studies with multiagent chemotherapy have demonstrated that patients with metastatic disease isolated to the liver, which previously would be considered unresectable, can occasionally be made resectable after administration of chemotherapy.²⁷ Also, improved clinical outcome in metastatic CRC is shown after multiagent systemic chemotherapy²⁸⁻³² with an even better survival after addition of bevacizumab, a monoclonal antibody against vascular endothelial growth factor.³³

Better surgical techniques and advances in preoperative imaging have improved patient outcome after resection of liver metastases over the last two decades. Several prognostic scoring systems have been proposed to optimally benefit from surgical treatment. Nevertheless, numerous patients who have undergone surgical resection of localized liver metastases develop disease recurrence. These patients might benefit from additional systemic treatment as this has been shown to improve progression-free survival over surgery alone.³⁴ Providing only patients at risk for disease recurrence with additional systemic treatment, avoids unnecessary side-effects. Studies are conducted on identifying patients at risk for disease recurrence after resection of liver or lung metastases.

Tumor biology of gastric cancer and colorectal cancer

Both gastric cancer and CRC are multi-pathway and multi-step diseases. They are characterized by considerable genetic heterogeneity. Several molecular markers within the chain of processes leading to cancer have been investigated, the more common of which are shown in *Table 1*.³⁵⁻³⁷

Particular genetic alterations can be clustered that group both gastric cancer and CRC into discrete subtypes. At least two different pathways are seen in gastric cancer leading to the intestinal and diffuse tumor type. The intestinal variant is well differentiated with recognizable gland formation, and usually arises in the setting of a recognizable precancerous condition such as gastric atrophy or intestinal metaplasia.^{5;37} This diploid variant is related to *Helicobacter pylori* infection and nutritional factors and is often located in the corpus and antrum of the stomach. Men are more commonly affected than women, and incidence increases with age. Metastatic spread is generally hematogenous to distant organs.³⁷ The prevalence of the intestinal type is decreasing whereas the prevalence of the diffuse type is rising, particularly in developed countries. The diffuse aneuploid form of gastric cancer is poorly differentiated, lacks gland formation, and is composed of signet ring cells. It is located more frequently in the upper third of the stomach, as well as in the gastroesophageal union and the lower third of the oesophagus. The diffuse variant consists of tiny clusters of small uniform cells, tends to spread submucosally, has less inflammatory infiltration, and metastasizes early. The route of spread is generally by invasion of the stomach wall and through lymphatic invasion. The diffuse form also has an association with blood type A and familial occurrences, suggesting a genetic etiology.

In general, the prognosis is less favorable for patients with diffuse-subtype histology.^{5;37}

There are two major pathways in colorectal carcinogenesis. One is the chromosomal instability (CIN) pathway that evolves through the classical adenoma–carcinoma sequence with usually aneuploid DNA and mutations in the tumor suppressor genes APC and TP53 and the proto-oncogene KRAS.³⁸ The other is a pathway involving defective DNA repair proteins, resulting in microsatellite instability (MSI).¹⁵ These two pathways are not independent and some CRCs show a significant degree of overlap between these two mechanisms.³⁹ Also, other routes, including the transforming growth factor- β /smad pathway, the serrated pathway, and epigenetic pathways, have been reported.⁴⁰ The CIN tumors are more likely to be located in the distal colon and behave more aggressively compared to the MSI subsets. The latter tumors are usually proximally located, are more likely to have diploid DNA and harbor mutations in the mismatch-repair genes. FAP and most sporadic cases originating from adenomas may be considered a paradigm for the CIN pathway, whereas HNPCC more clearly represents the MSI subset.¹⁵

Table 1. Potential molecular markers for primary gastric and colorectal cancer

Mismatch repair gene mutation (MSH2, MSH6, MLH1, PMS1, PMS2)
Chromosomal instability (DNA ploidy) (APC, MCC, DCC)
Allelic imbalances/loss of heterozygosity (18q-)
Epigenetic changes as hypermethylation
Oncogene expression (ras, myc)
Tumor suppressor gene mutations (bcl-2, p21, p27, p53, APC, MCC, DCC)
Proliferation/apoptosis (bcl-2, bax, Ki-67)
Angiogenesis (vascular endothelial growth factor, D2-40, CD31)
Inflammation (cyclooxygenase 2)
Cell adhesion loss (E-cadherin, β -catenin, CD44)
Overexpression or mutation of cell membrane receptors with tyrosine kinase activity (epidermal growth factor receptor, Her2/neu, platelet-derived growth factor receptor, c-met)
Markers of immortalisation (telomerase)

APC, adenomatous polyposis coli; CD, cluster of differentiation; DCC, deleted in colon cancer; DNA, desoxy-ribonucleic acid; MCC, missing in colon cancer; MLH, MutL homolog; MSH, MutS homolog; PMS, postmeiotic segregation.

Prognostic factors

Extensive research has led to a large number of prognostic markers in gastric cancer and CRC. Current practice of prognosis is based on the Tumor-Node-Metastasis (TNM) classification.^{41;42} Under investigation are the presence of occult tumor cells in lymph nodes, blood and bone marrow, the presence of circulating DNA and RNA in blood and expression of molecular markers of the primary tumor.

In *Table 1* several potential molecular markers for primary gastric and colorectal cancer are shown. Although numerous prognostic molecular markers have been reported of which the majority detected by immunohistochemical staining, none has found itself into routine application as yet. In this thesis, only molecular markers related with angiogenesis and lymphangiogenesis will be discussed. In addition, this thesis assesses occult tumor cells in lymph nodes in gastric cancer and CRC, occult tumor cells in sentinel lymph nodes in CRC and disseminated tumor cells in bone marrow from patients with colorectal liver metastases.

Current practice of prognosis in gastric and colorectal cancer

The prognosis of patients with gastric cancer and CRC is related to the degree of penetration of the tumor through the stomach or bowel wall, the nodal status and the presence of distant metastases. These three characteristics form the basis for the TNM staging system which is the most well-established prognostic factors in gastric cancer and CRC and currently the main method for staging.^{41;42} The TNM classification for gastric cancer and CRC is shown in *Table 2 and 3*, respectively. Approximately 40% of gastric cancer patients have stage I (21%) or II (20%) disease at the time of diagnosis.⁴³ One-third have stage III disease, and a quarter has stage IV disease. Five-year survival is approximately 78% for stage IA, 58% for stage IB, 34% for stage II, 20% for stage IIIA, 8% for stage IIIB and 5 to 7% for stage IV.^{43;44}

At diagnosis, approximately 40% of CRC patients have TNM stage I and II disease, 40% have stage III and 20% have stage IV disease.^{20;41} The five-year survival rate decreases from up to 90% for stage I and II CRC to 70% for stage III CRC. Stage IV CRC patients have a worse prognosis showing a five-year survival rate of around 10%.²⁰

Additional prognostic factors in CRC comprise bowel perforation or obstruction, presence of tumor cells in resection margins, number of harvested lymph nodes, vessel invasion, tumor grade, tumor budding, tumor border configuration and serum carcinoembryonic antigen level.⁴⁵⁻⁴⁷ Generally, only the TNM category is being used to decide whether a patient should receive adjuvant therapy although in some clinics bowel perforation or obstruction and vessel invasion are also decisive in administering adjuvant therapy to the patient.

After the introduction in 1987, the TNM system has been revised every few years to allow the incorporation of new evidence. Unfortunately, this staging system does not provide prediction of prognosis for the individual patient, especially for stage II and III CRC patients. According to the last sixth TNM staging manual, stage II CRC is subdivided into IIA (T3N0M0) and IIB (T4N0M0) and stage III is subdivided into IIIA (T1/2N1M0) and IIIB (T3/4N1M0). Worldwide, there is a consensus to administer adjuvant chemotherapy to patients with stage III CRC disease whereas in most countries stage II CRC patients do not receive adjuvant treatment.

Table 2. TNM Classification of gastric cancer^{41;42}

Category	Criteria		
Primary Tumor (T)			
TX	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria		
T1	Tumor invades lamina propria or submucosa		
T2	Tumor invades muscularis propria or subserosa		
T2a	Tumor invades muscularis propria		
T2b	Tumor invades subserosa		
T3	Tumor penetrates serosa (visceral peritoneum) without invasion of adjacent structures		
T4	Tumor invades adjacent structures		
Regional Lymph Nodes (N)			
NX	Regional lymph node(s) cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in 1 to 6 regional lymph nodes		
N2	Metastasis in 7 to 15 regional lymph nodes		
N3	Metastasis in more than 15 regional lymph nodes		
Distant Metastasis (M)			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1	Distant metastasis		
Stage grouping			
Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T1	N1	M0
	T2a/b	N0	M0
II	T1	N2	M0
	T2a/b	N1	M0
	T3	N0	M0
IIIA	T2a/b	N2	M0
	T3	N1	M0
	T4	N0	M0
IIIB	T3	N2	M0
IV	T4	N1-3	M0
	T1-3	N3	M0
	Any T	Any N	M1

However, a study showed stage IIIA colon cancer patients to have a significantly better disease-specific five-year survival (83%) than stage IIB colon cancer (72%) ($P < 0.001$).⁴⁸ This stresses the need for additional prognostic factors to improve individual patient treatment.

Occult tumor cells in lymph nodes

Lymph node metastases are one of the major prognostic factors in solid tumors^{41;42} determining whether patients will receive adjuvant therapy in CRC. However, conventional examination of lymph nodes involves hematoxylin and eosin staining of only one or two 4 to 5 μm sections which is less than 1% of a lymph node of 1 cm in diameter.⁴⁹ Serial sectioning, stepsectioning, immunohistochemical staining, polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) will increase the number of tumor-positive lymph nodes, especially those with relatively small amount of tumor cells, and thus lead to upstaging.^{50;51} Small groups of metastases detected by detailed examination techniques are referred to as occult tumor cells (OTC) which are subdivided into micrometastases (MM) and isolated tumor cells (ITC). MM are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC either as single tumor cells or as clusters of tumor cells of 0.2 mm or less.^{41;42;52} In the last sixth edition of the TNM classification, OTC in lymph nodes were incorporated by stating that ITC do not constitute metastases and should be classified as pN0(i+) whereas the presence of MM should be staged as node positive pN1(mi).⁴¹ This advice was not based on clinical evidence though does make biologic sense and fits with the lack of clinical significance of OTC according to the majority of reported studies in CRC.⁵¹ The majority of studies failed to subdivide OTC into MM and ITC. Up till now, two published CRC studies showed prognostic relevance of MM and none for ITC.^{53;54} Detailed examination of all regional lymph nodes is expensive and time consuming. Since it is of major clinical importance however, sentinel node mapping was introduced.⁵⁵ Sentinel nodes are lymph nodes onto which the tumor directly drains and which therefore have the highest chance of harboring tumor cells (*Figure 1*).^{56;57} By examination of sentinel nodes only, if it reflects the status of the whole regional lymph node basin, OTC detection becomes feasible as the number of lymph nodes to be examined is greatly reduced.

Tumor cells in blood and bone marrow

Tumor cells in blood are referred to as circulating tumor cells (CTC) and tumor cells in bone marrow regard disseminated tumor cells (DTC). Tumor cells in bone marrow and blood can be present in low numbers of approximately one tumor cell per million hematopoietic cells. Therefore, detection methods need to be very sensitive. Enrichment with Ficoll density gradient separation or other enrichment techniques followed by immunocytochemistry, RT-PCR or magnetic cell sorting are reported.^{58;59} In breast cancer, DTC have found to be independent predictors of disease-free and disease-specific survival.⁶⁰⁻⁶²

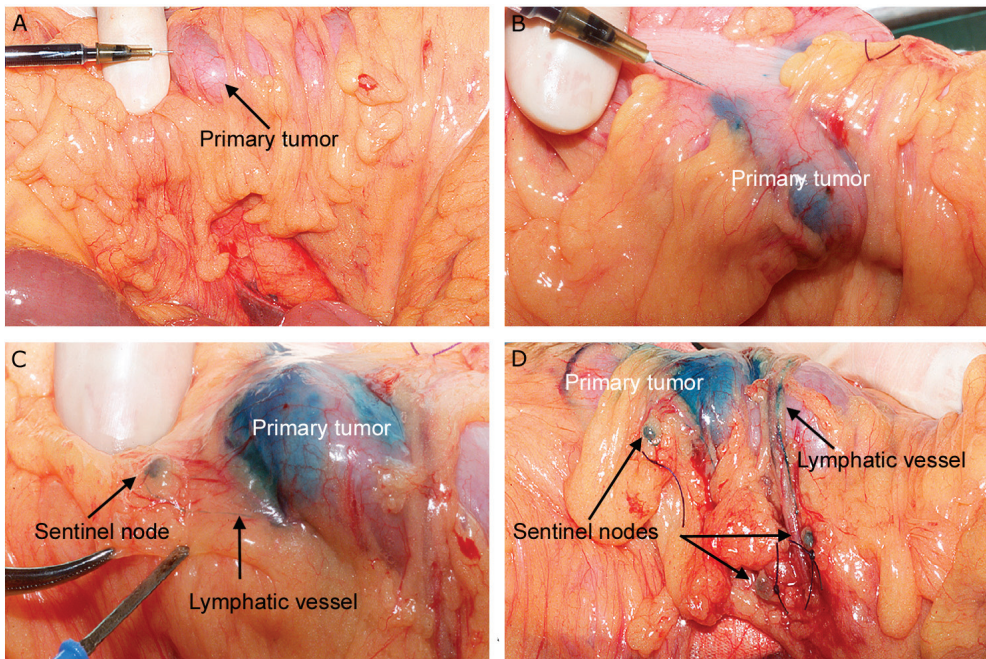


Figure 1. Sentinel node mapping in colorectal cancer. After the colon has been carefully mobilized (A), one-half to one ml isosulphan blue dye was injected subserosally around the tumor (B). Shortly thereafter, a lymphatic channel could be followed to the sentinel node(s) (C). Typically, one to four sentinel nodes are mapped during each procedure. The sentinel nodes were marked with sutures for pathological examination (D).⁹⁶

DTC have also been reported as a marker of systemic treatment response.⁶³ CTC were shown to be independent predictors of overall survival in metastatic breast cancer.^{64;65} As yet, CTC detection cannot replace DTC detection since several breast cancer studies have shown DTC to be superior to CTC detection in prognostic relevance.⁶³

Bone is not a preferential site for metastatic disease in gastric cancer or CRC. Even so, dormant tumor cells detected in bone marrow might represent the metastatic potential of the primary tumor. One study showed that detection of DTC in bone marrow samples could be a marker for assessing the effectiveness of neoadjuvant radiochemotherapy in rectal cancer.⁶⁶ Although, some studies have shown prognostic relevance of CTC and DTC, as yet no clarity has been reached regarding their prognostic significance mostly because of small patient study groups and the lack of standardization in detection methods.⁶⁷⁻⁷⁴

Circulating DNA and RNA in blood

Although higher serum and plasma levels of DNA and RNA have been found in various forms of cancer, their presence has also been seen in autoimmune diseases, postsurgery, stroke, trauma, sepsis and pregnancy rendering it unsuitable for cancer diagnosis.⁷⁵ Nonetheless, decreasing plasma DNA levels

Table 3. TNM Classification of colorectal cancer^{41;42}

Category	Criteria				
Primary Tumor (T)					
TX	Primary tumor cannot be assessed				
T0	No evidence of primary tumor				
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria ^a				
T1	Tumor invades submucosa				
T2	Tumor invades muscularis propria				
T3	Tumor invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues				
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum ^b				
Regional Lymph Nodes (N) ^c					
NX	Regional lymph nodes cannot be assessed				
N0	No regional lymph node metastasis				
N1	Metastasis in 1 to 3 regional lymph nodes				
N2	Metastasis in 4 or more regional lymph nodes				
Distant Metastasis (M)					
MX	Distant metastasis cannot be assessed				
M0	No distant metastasis				
M1	Distant metastasis				
Stage grouping					
Stage	T	N	M	DUKES ^d	MAC ^d
0	Tis	N0	M0		
I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
IIA	T3	N0	M0	B	B2
IIB	T4	N0	M0	B	B3
IIIA	T1-T2	N1	M0	C	C1
IIIB	T3-T4	N1	M0	C	C2/C3
IIIC	Any T	N2	M0	C	C1/C2/C3
IV	Any T	Any N	M1	D	D

^a Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa;

^b Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa: for example, invasion of the sigmoid colon by a carcinoma of the caecum. Tumor that is adherent to other organs or structures macroscopically is classified T4. However, if no tumor is present in the adhesion microscopically, the classification should be pT3. The V and L substaging should be used to identify the presence or absence of vascular or lymphatic invasion;

^c A tumor nodule in the pericorectal adipose tissue of a primary carcinoma without histologic evidence of residual lymph node in the nodule is classified in the pN category as a regional lymph node metastasis if the nodule has the form and smooth contour of a lymph node. If the nodule has an irregular contour, it should be classified in the T category and also coded as V1 (microscopic venous invasion) or as V2 (if it was grossly evident), because there is a strong likelihood that it represents venous invasion;

^d Dukes B is a composite of better (T3N0M0) and worse (T4N0M0) prognostic groups, as is Dukes C (Any TN1M0 and Any TN2M0). MAC is the modified Astler-Coller classification.

have been found in rectal cancer patients with downstaging of disease after chemoradiation whereas constantly high or increasing values were seen in patients not responding to chemoradiation⁷⁶, implying detection of nucleic acids valuable in monitoring response to cytotoxic therapy or disease progression. Mechanisms to explain free nucleic acids in blood might be apoptosis or necrosis of tumor cells at the tumor site, lysis or apoptosis of CTC, or an active release from the tumor or from lymphocytes. A recent study suggests that RNA is protected within a vesicle-like structure implying an active release.⁷⁷ Future studies have to establish a differentiation between free nucleic acids coming from cellular death mechanisms which indicates a good prognosis and those coming from an active release mechanism, probably expressing the activity and proliferative capacity of the tumor representing a risk factor for poor prognosis. Studies regarding detection of circulating nucleic acids in blood show similar variability as studies assessing the prognostic significance of tumor cells in lymph nodes, blood and bone marrow such as a diverse number of potential markers, use of different techniques, studies involving few patients, a lack of consensus on sample type and preparation protocols and false-positive results due to contamination or aspecific background.⁷⁸

Angiogenesis and lymphangiogenesis

Both angiogenesis and lymphangiogenesis are required for growth of the primary tumor and metastases and play a pivotal role in metastatic spread. The absence of a continuous basement membrane makes the lymphatics a primary route for dissemination of many solid tumors.^{79;80} Also, tumor cells experience less stress and mechanical deformation in lymphatic vessels as lymphatic capillaries are five to eight times wider than blood capillaries and have far lower flow velocities.⁷⁹ In tumors, however, blood vessels have shown to be highly abnormal, with immature, disorganized wall structure, detached endothelial cells and an abnormal or missing basement membrane which probably aids in tumor cell metastasis.⁸¹⁻⁸³ These features of lymphatic and blood vessels support the passive intravasation hypothesis suggesting that cells growing in a confined space push against each other, producing stress that can collapse vessels and, potentially force cells to breach fragile vessels (*Figure 2*).⁸³ The opposing theory on active intravasation (*Figure 2*) is based on the finding that highly metastatic cells can change alignment and migrate toward blood vessels with the aid of adhesion mechanisms, whereas less metastatic cells cannot.⁸³ An adhesion molecule that might be involved in this process, is sialyl Lewis X (sLeX), a blood group-related antigen.⁸⁴ In addition to these theories, two models of metastatic spread have been suggested.⁸⁵ In the first model (*Figure 3*), tumor cells disseminate from the primary tumor through lymphatic or blood vessels during the early stage of tumor growth. Disseminated tumor cells proliferate and form solid metastases in lymph nodes, whereas tumor cells that spread to distant sites, through the blood, die or remain dormant. At later stages, tumor cells disseminate from the established lymph node

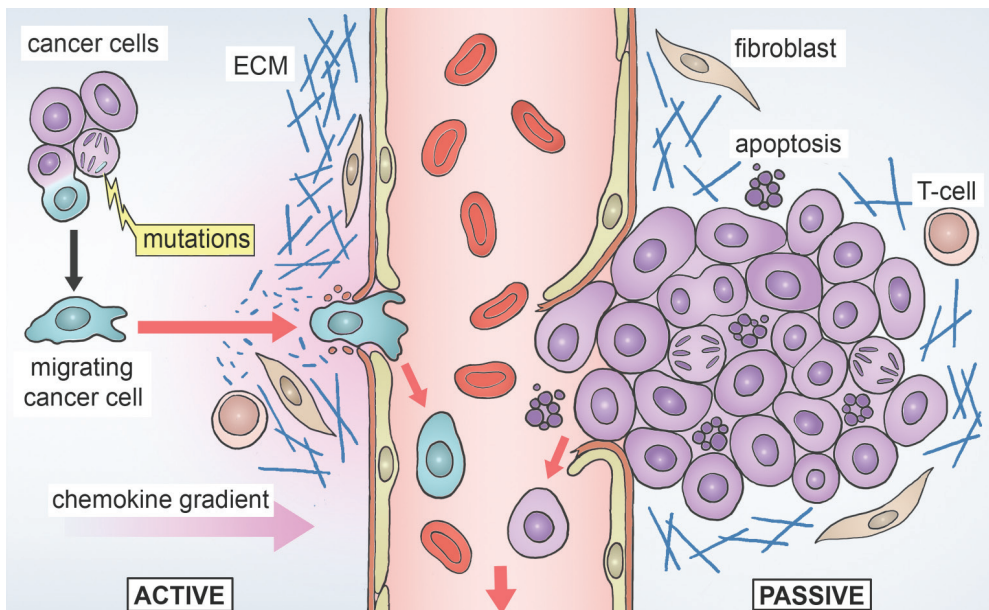


Figure 2. Active and passive mechanisms in initial steps of metastasis. Left: reports that tumor cells accumulate mutations, upregulate migration machinery, and align and migrate up nutrient or chemokine gradients, are in support of active metastasis. Fibroblasts, macrophages, and other stromal cells are also likely to cooperate with cancer cells to actively help with the initial stage of metastasis. **Right:** There is evidence that tumor cells including dead or apoptic cells are shed into the vasculature, which implies a passive mechanism. It is possible that uncontrolled focal growth crushes or impinges upon fragile tumor blood vessels, leading to passive shedding. Similarly, tumor cells may be shed into lymphatic vessels.⁸³ ECM, extracellular matrix.

metastases through the blood circulation to distant sites, where they are able to form solid metastases. As a result, metastasis to other organs is dependent on the presence of lymph node metastases. This correlation has been shown for head and neck cancer.⁸⁶ In the second model (*Figure 3*), cells frequently disseminate, through blood vessels, from the primary tumor to distant sites, where they progress to overt metastases without previous passage through lymph nodes. In patients with breast cancer, this hematogenous dissemination seems to be a very early event in tumor progression.

Blood microvessel density has been assessed by antibodies directed against several endothelial markers, including CD31 (PECAM-1), CD34 and factor VIII (von Willebrand factor) leading to variable results regarding its prognostic value.⁸⁷ In recent years, antibodies specifically directed against lymphatic endothelial cells have been found such as Prox1⁸⁸, LYVE-1⁸⁹, anti-podoplanin⁹⁰ and D2-40.⁹¹ One of the most important growth factors regulating tumor angiogenesis is vascular endothelial growth factor (VEGF) which consists of five ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor and three receptors: VEGFR-1, VEGFR-2 and VEGFR-3.⁹²⁻⁹⁴ VEGF-A is a known ligand for VEGFR-1 and VEGFR-2 which are mainly expressed in blood vascular endothelium. VEGF-C and

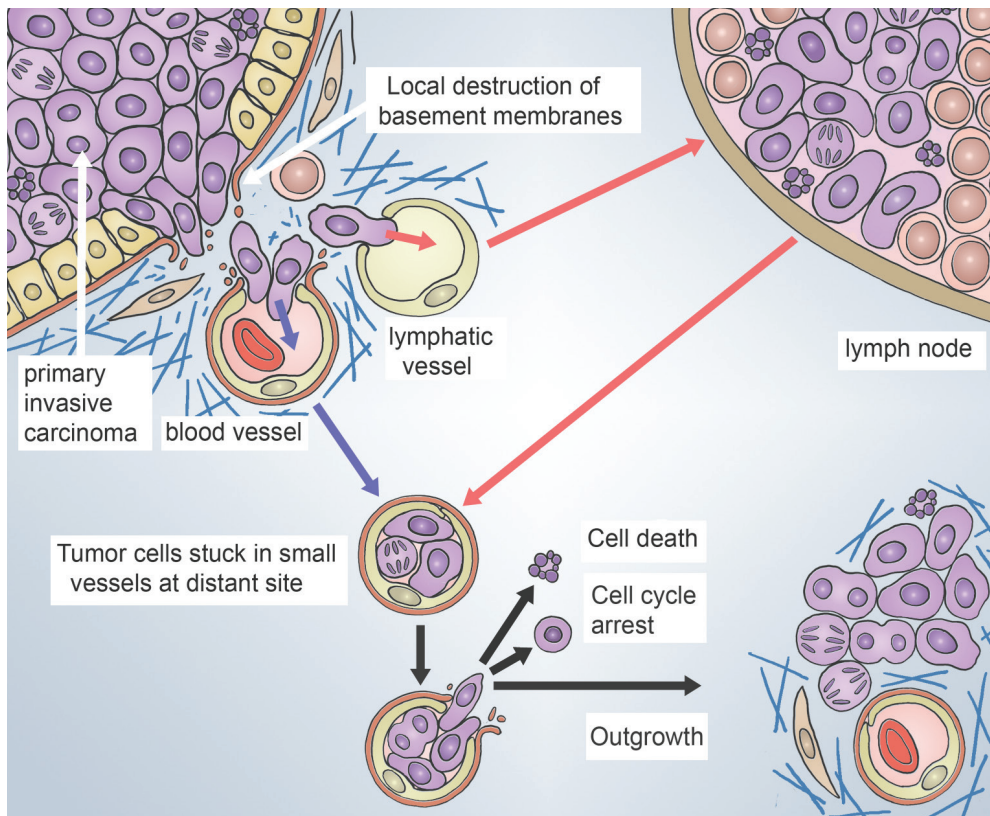


Figure 3. Two models of metastatic spread. Before reaching their final metastatic site, tumor cells must cross several basement membranes: first when progressing from carcinoma in situ to invasive carcinoma, then during intravasation and extravasation (**upper left**) and finally when invading other tissues.⁹⁷ Tumor cell dissemination from the primary site can occur via lymphatic routes (**red arrows**) and by hematogenous routes (**blue arrows**). Secondary hematogenous dissemination also occurs from overt metastases to other distant sites (**black arrows**). In the lymphatic route (**red arrows**), disseminated tumor cells proliferate in the lymph nodes to form solid metastases. At later stages, tumor cells disseminate from the established lymph node metastases to distant sites by entering the thoracic duct and the right lymphatic duct, each draining into its ipsilateral subclavian vein. It is possible that this ability was gained during the progression of these cells in the lymph-node environment. As a result, tumor metastasis is dependent on the presence of lymph-node metastases.⁸⁵ In the hematogenous route (**blue arrows**), tumor cells primarily travel through blood vessels to form distant metastases. Two separate models of metastatic spread is suggested since for instance breast cancer patients can develop metastases at distant organs, whereas the lymph nodes remain tumor free. Again, in head and neck cancer, distant metastases is dependent on the presence of lymph node metastases.⁸⁵ Besides outgrowth into clinically evident metastases, tumor cells that arrested in lymph nodes or that intra- or extravascularly lodged into organ parenchyma for instance bone marrow, can also enter apoptosis or remain dormant either as single cells or as a small group of tumor cells that underwent a proliferative expansion and cannot recruit a vascular bed⁹⁸ (**lower right**).

VEGF-D are ligands for VEGFR-3, mostly expressed in lymphatic endothelium. In this thesis, the clinical relevance of microvessel density detected by CD31 and D2-40 and of VEGF-C, VEGF-D and sLeX tumor expression in CRC was evaluated.

Automated microscopy

Detection of immunochemically stained tumor cells in multiple levels of lymph nodes, in bone marrow and in blood is laborious and time-consuming. The use of automated microscopy by which tumor cells can be detected on basis of different color, can overcome these disadvantages. In addition, automated analysis showed a higher detection rate of ITC in sentinel lymph nodes from breast cancer patients of up to 12.5% compared to conventional microscopy.⁹⁵ In this thesis, automated microscopy has been used to detect tumor cells in lymph nodes and in bone marrow.

Aim and outline of the thesis

After the diagnosis of cancer in a solid organ is established, the main question is whether the tumor is localized or whether it has already disseminated to regional lymph nodes and/or distant organs. Currently, the basis for therapeutic decisions is the anatomically TNM staging system for solid-organ cancers developed by the International Union Against Cancer (UICC)⁴¹ and the American Joint Committee on Cancer (AJCC).⁴² As discussed above, this staging system does not provide accurate prediction of prognosis for the individual patient. Despite improvements in surgical techniques, many deaths from cancer result from the progressive growth of metastases in patients who had been considered curatively treated. In this thesis, we focus on identifying patients at risk for disease recurrence after surgical treatment of gastric cancer, colorectal cancer or colorectal liver metastases.

In **chapter 2** is analyzed whether the presence of OTC in originally considered tumor-negative lymph nodes from curatively resected gastric cancer patients can predict disease recurrence. This is done in a case-control design with a selection of patients from the previously published multicenter Dutch D1-D2 Gastric Cancer trial.⁷ Moreover, the additional value of automated microscopy is evaluated. **Chapter 3** presents an overview of studies regarding the detection methods and clinical relevance of OTC in lymph nodes in CRC. An approach in which immunohistochemical staining and multiple sectioning of lymph nodes in CRC is combined and subjected to novel high-throughput automated imaging is described in **chapter 4**. In addition, the results are compared with RT-PCR data from a previously published study.⁵⁰ **Chapter 5** reports on results of a case-control study with CRC patients assessing whether detailed examination of lymph nodes by multilevel sectioning and immunohistochemical staining can predict disease recurrence.

Sentinel node mapping has been introduced in CRC to improve staging by facilitating OTC assessment in lymph nodes that are most likely to contain tumor cells. In **chapter 6**, studies on the feasibility and reliability of sentinel node mapping in CRC are reviewed with the emphasis on differences in the techniques used.

Although usually no clinically evident bone metastases are developed in CRC, DTC are found in bone marrow and we suspect that the presence of tumor cells

in bone marrow might represent the aggressive nature of the tumor and could therefore be used to predict disease recurrence. This is evaluated in **chapter 7** by assessing whether the presence of DTC in bone marrow from patients who undergo locoregional treatment of colorectal liver metastases is associated with a worse outcome of disease. The presence of DTC in bone marrow is analyzed by using quantitative RT-PCR and immunocytochemistry.

As discussed, it is generally accepted that tumor cells can spread through local invasion and hematogenous or lymphatic routes. To finally reach lymph nodes or other tissue compartments in the body, the tumor cells have to overcome many boundaries with the aid of various proteins. In **chapter 8** several markers related to tumor cell spread through blood and lymphatic vessels are studied for their prognostic significance. Specifically, we examine whether disease recurrence in lymph node-negative CRC patients can be predicted by assessing their primary tumors for the expression of the angiogenic and lymphangiogenic factors sLeX, VEGF-C, and VEGF-D, for blood and lymphatic microvessel density and for the presence of blood and lymphatic vessel invasion.

Finally, the conclusions of above mentioned studies and future perspectives regarding these studies are discussed in **chapter 9**. A summary of this thesis translated in Dutch is presented in **chapter 10**.

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CHAPTER TWO

CLINICAL RELEVANCE OF OCCULT TUMOR CELLS IN LYMPH NODES FROM GASTRIC CANCER PATIENTS

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ABSTRACT

Background: The current method for staging in gastric cancer is not sufficient as even after a complete primary tumor resection, patients with node-negative gastric cancer suffer from disease recurrence. In this study, the relation between disease recurrence and the presence of occult tumor cells (OTC) in lymph nodes from gastric cancer patients was evaluated.

Patients and methods: In a case-control design, lymph nodes from 40 cases (disease recurrence) and 41 controls (no disease recurrence for at least 5 years) with gastric cancer were examined for the presence of OTC, that comprised micrometastases (MM; > 0.2 mm and ≤ 2.0 mm) and isolated tumor cells (ITC; ≤ 0.2 mm). The original hematoxylin and eosin (HE) stained sections of all lymph nodes from cases and controls were previously considered as tumor-negative by a pathologist. Fresh HE stained sections were screened by conventional microscopy. Histological sections stained by immunohistochemistry (IHC) with anticytokeratin antibodies CAM5.2 were screened by conventional and automated microscopy.

Results: Tumor cells were detected in lymph nodes from 40 of 81 (49%) patients. There was no significant difference in the presence of OTC, MM or ITC between the case and control groups ($P = 0.658$; $P = 0.691$; $P = 0.887$, respectively). However, significantly more cases presented with 20% or more OTC-positive lymph nodes ($P = 0.015$). A multivariate logistic regression analysis showed that examination of less than 5 lymph nodes (OR 13.8, 95% CI 1.6-120.6, $P = 0.018$) was the only significant independent risk factor for disease recurrence, especially for locoregional disease recurrence (OR 20.4, 95% CI 2.2-190.8, $P = 0.008$). A similar analysis for distant disease recurrence showed a percentage of 20% or more OTC-positive lymph nodes to be the only significant independent risk factor (OR 15.6, 95% CI 1.6-151.4, $P = 0.018$). The sensitivity of IHC evaluated by microscopy to identify cases with 20% or more OTC-positive lymph nodes increased from 8% for conventional microscopy to 22% for automated microscopy (McNemar's test, $P = 0.063$).

Conclusions: The mere presence of OTC-positive lymph nodes in gastric cancer patients did not predict disease recurrence. However, the number of examined lymph nodes and the percentage of OTC-positive lymph nodes were independent risk factors for locoregional disease recurrence and distant disease recurrence, respectively. Automated microscopy was essential in identifying patients with 20% or more OTC-positive lymph nodes. Therefore a maximum number of lymph nodes should be removed and meticulously examined for OTC to identify high-risk patients. These patients should be considered for additional treatment.

INTRODUCTION

The prognosis of patients with resectable gastric cancer is mainly predicted by tumor involvement of the lymph nodes¹⁻³ and depth of primary tumor invasion into the gastric wall.⁴ The current method for staging, however, is not sufficient as even after a complete primary tumor resection up to 30% of patients with node-negative gastric cancer suffer from disease recurrence.⁵ These recurrences might be explained by inadequate lymph node staging due to missing of occult tumor cells (OTC). OTC comprises of micrometastases (MM) and isolated tumor cells (ITC). MM are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC as single tumor cells or clusters of tumor cells of 0.2 mm or less.⁶⁻⁸ OTC is usually not detected with conventional pathological examination applying the hematoxylin and eosin (HE) staining method. The likelihood of detecting OTC is higher when focused examination techniques such as serial sectioning, immunohistochemistry (IHC) or reverse-transcriptase polymerase chain reaction are used. By detecting OTC, patients at high risk for recurrent disease may be identified. These patients might be considered for postoperative adjuvant therapy.⁹ The aim of this study was to analyze whether the presence of OTC in originally considered tumor-negative lymph nodes from curatively resected gastric cancer patients can predict disease recurrence. Moreover, the additional value of automated microscopy was evaluated.

PATIENTS AND METHODS

Patient source and selection

Between August 1989 and July 1993, a total of 1078 patients were randomized in the multicenter Dutch D1-D2 Gastric Cancer trial. This trial was organized by the Dutch Gastric Cancer Group and compared a D2 (extended) to a D1 (conventional) lymph node dissection. Details of the trial protocol, D1/D2 dissection, the protocol for sampling and evaluation of resection margins and the protocol regarding the handling of the lymph nodes have been reported previously.¹⁰⁻¹³ Large lymph nodes were cut grossly at 2- to 3-mm intervals and totally embedded in paraffin. Follow-up was continued until January 2003 resulting in a median follow-up of 11 years with a range of 9 to 13 years.

Among 1078 patients randomly assigned in the trial, 711 patients had undergone a resection with curative intent with a D1 (n = 380) or D2 (n = 331) lymph node dissection. Of these 711 patients with a curative resection, 304 patients had an R0 resection (i.e. macroscopically and microscopically negative resection margins) and lymph nodes considered tumor-negative by the original pathologist. For the present study, cases and controls were selected from this group.

All patients who suffered from locoregional or distant recurrence (n = 43) were considered cases. Lymph node tissue blocks from 3 cases were not available or not suitable for investigation, leaving 40 cases that were included into the study. Fourteen cases suffered from locoregional disease recurrence, 14 patients

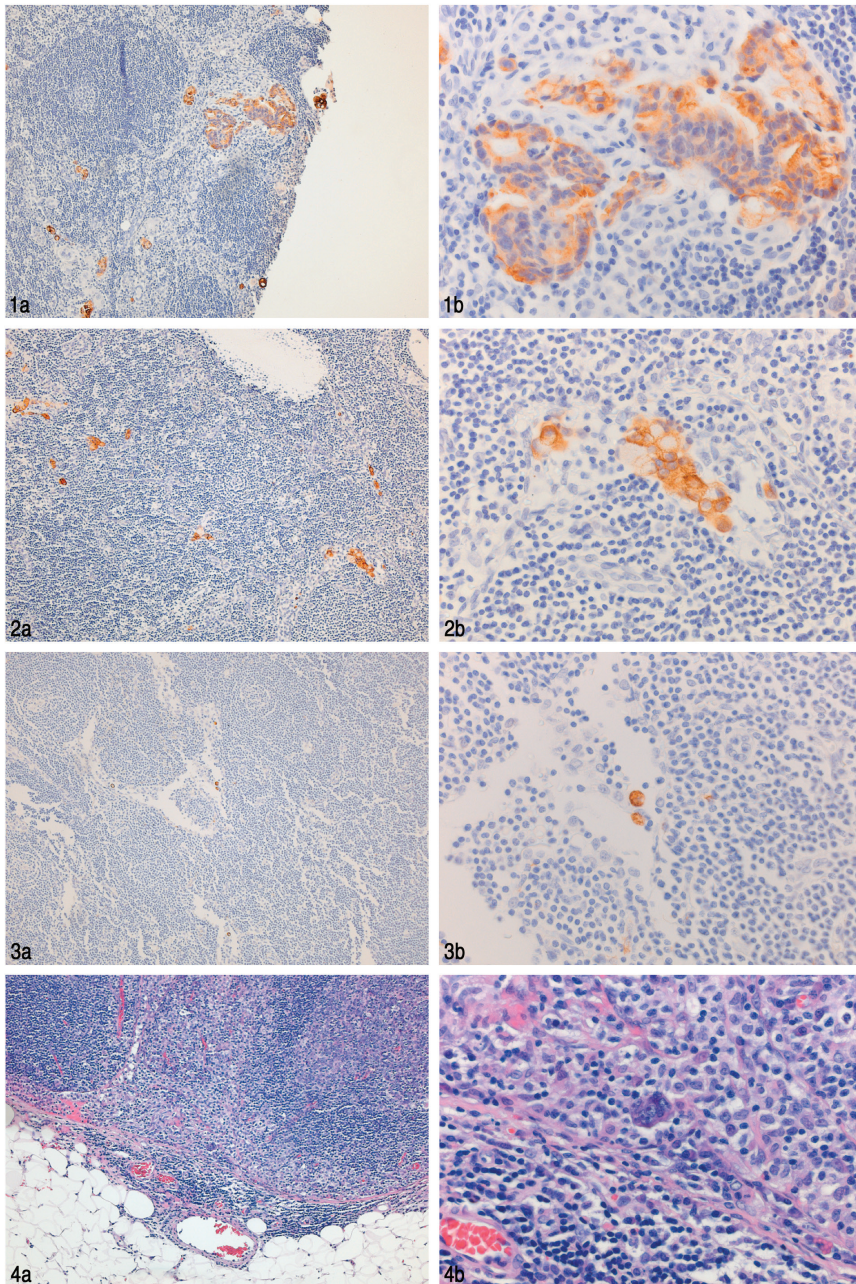


Figure 1-4. Occult tumor cells in lymph nodes from gastric cancer patients. Micrometastases (> 0.2 mm and ≤ 2 mm) at original magnifications of x100 (**1a**) and x400 (**1b**). Isolated tumor cells (tumor cell clusters ≤ 0.2 mm) at original magnifications of x100 (**2a**) and x400 (**2b**). Isolated tumor cells (single cells) at original magnifications of x100 (**3a**) and x400 (**3b**). Isolated tumor cells only seen on the hematoxylin and eosin stained section at original magnifications of x100 (**4a**) and x400 (**4b**).

from locoregional and distant recurrence and 12 patients suffered from distant recurrence. For each case, at least one control was selected from the patient group who did not suffer from disease recurrence for a minimum of 5 years ($n = 261$). Controls ($n = 41$) were matched for depth of tumor invasion and age.

Immunohistochemistry

Three consecutive 4 μm tissue sections were cut from each paraffin block, prepared on aminopropylethoxysilane coated slides, and dried overnight at 37°C. One section was stained with the HE method and two sections were used for IHC. One of these sections was stained with the antibody CAM5.2 (Becton Dickinson, CA), a murine monoclonal antibody specific for cytokeratin 7 (weak) and 8. The other section was used as a negative control stained with the primary antibody omitted. As positive control a known CAM5.2 positive primary tumor was used. As negative control tumor sections were incubated with phosphate-buffered saline (PBS) instead of CAM5.2. The sections were deparaffinized in xylol. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide methanol at room temperature for 20 minutes. Subsequently the sections were rehydrated. After washing in PBS, antigen retrieval treatment was done by incubating the sections in a 0.1% trypsin calciumchloride (0.1%) solution during 20 minutes at 37°C. Hereafter, the slides were rinsed twice in PBS. The primary antibody was applied at a 1:320 dilution in PBS with 1% bovine serum albumine. The sections were incubated overnight at room temperature. The detection of cytokeratin was achieved by incubating the sections for 30 minutes with the biotinylated rabbit-anti-mouse conjugate (Dako, Glostrup, Denmark, 1:200), followed by three PBS washes and incubation for 30 minutes with the streptavidin-biotin-peroxidase conjugate (DAKO, Glostrup, Denmark, 1:100). After three washes in PBS visualization of cytokeratin was achieved by a 10 minutes incubation with 3,3'-diaminobenzidinetetrachloride substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide. Cytokeratin-positive cells stained brown. Counterstaining was done with Mayer's Hematoxylin. For microscopic analysis the sections were dehydrated in graded ethanol followed by xylol and mounted in pertex.

Screening of the slides

The sections stained by the HE and the IHC method (negative control and CAM5.2) were screened by a pathologist using conventional light microscopy at a magnification of 100 times. The sections stained by the IHC method using CAM5.2 were also screened with automated microscopy using the ARIOL system® (Applied Imaging Corporation, San Jose, CA). The ARIOL system consisted of a slidefeeder and a microscope linked to a computer with software for detecting cells with a different color within an evenly stained background. The X, Y and Z coordinates and images of the selected objects were stored for relocation of the slides under the microscope. The slidefeeder could contain 50 slides that were screened within

24 hours. Objects found by this system were reviewed by the operator of the ARIOL system, a technician specialized in cytology. The pathologist reviewed all tumor cells detected by the ARIOL system that were previously missed with conventional microscopy. Nonspecifically stained cells such as macrophages, dendritic cells or white blood cells were excluded. OTC comprised micrometastases (MM) and isolated tumor cells (ITC). MM were defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC as single tumor cells or clusters of tumor cells of 0.2 mm or less. Macrometastases were larger than 2 mm.

Statistical analysis

Statistical analysis was carried out using SPSS software, version 11.5 (SPSS Inc, Chicago, IL). Numerical data are presented as mean $\pm$ standard deviation. The clinicopathologic features of cases and controls were compared either by a chi-square test or a student T-test. Univariate and multivariate odds ratio's (OR), 95% confidence intervals (95% CI) and p-values (P) were calculated by applying logistic regression analysis. Differences between screening results by automated microscopy and conventional microscopy were analyzed by using the McNemar test. A p-value of less than 0.05 was considered an indication of statistical significance. Factors with a (overall) p-value less than 0.10 in the univariate analysis were entered into the multivariate logistic regression model.

RESULTS

Clinical relevance of occult tumor cells

In total, 1396 lymph nodes of 81 patients were studied (median, 15; range, 1 to 92 lymph nodes). The characteristics of these patients are shown in *Table 1*. The percentage of lymph nodes that were found to be tumor-positive and of patients having tumor-positive lymph nodes are shown in *Table 2*. The results with the HE method, IHC evaluated by conventional microscopy and IHC evaluated by automated microscopy are shown separately. Not all lymph nodes were analyzed with all three methods as deducible from the total number of analyzed lymph nodes shown in the table. The combined results, i.e. results from HE staining and IHC staining evaluated both by conventional and automated microscopy, were used for the analysis. The negative control sections did not contain aspecifically stained cells. Tumor cells were detected in lymph nodes from 40 of 81 (49%) patients by screening of HE and IHC sections (*Figure 1-4*). Two cases (5%) and one control (2%) had lymph nodes with macrometastases. These patients were excluded from further analysis leaving a total of 38 cases and 40 controls (*Table 3*). Nineteen cases (50%) and 18 controls (45%) had OTC-positive lymph nodes, which numbers did not differ statistically between both these groups ($P = 0.658$). There was also no significant difference in the presence of MM or ITC between the case and control group (21% vs 18%, $P = 0.691$; 29% vs 28%, $P = 0.887$).

Table 1. Patient characteristics

Characteristics	All patients (n=81)		Cases (n=40)		Controls (n=41)		P ^a
	n	%	n	%	n	%	
Sexe							
Female	32	40	14	35	18	44	NS
Male	49	61	26	65	23	56	
Age (years) ^b	63 ± 11.5		63.9 ± 11.5		62.1 ± 11.5		NS ^c
Stage ^d							
IA	16	20	7	18	9	22	NS
IB	50	62	26	65	24	59	
II	14	17	7	18	7	17	
IIIA	1	1	0	0	1	2	
Location							
C (Upper third)	14	18	7	18	7	17	NS
M (Middle third)	28	35	12	31	16	39	
A (Lower third)	38	48	20	51	18	44	
WHO classification							
Papillary	10	12	5	13	5	12	NS
Tubular	34	42	14	35	20	49	
Mucinous	6	7	3	8	3	7	
Signet ring cell	28	35	17	43	11	27	
Undifferentiated	3	4	1	3	2	5	
Laurén classification							
Intestinal	50	62	21	53	29	71	NS
Diffuse	24	30	16	40	8	20	
Mixed	7	9	3	8	4	10	
Differentiation							
Good	5	6	4	10	1	2	.012
Moderate	33	41	10	25	23	56	
Poor	43	53	26	65	17	42	
Size (cm)							
< 2	6	8	4	10	2	5	NS
2 – 3	34	43	15	39	19	48	
> 3	39	49	20	51	19	48	
Unknown	2	-	1	-	1	-	

^a Chi-square test of cases vs controls, unless mentioned otherwise; ^b Presented as mean ± standard deviation; ^c Student T-test; ^d According to the 5th edition of the TNM classification.¹⁹

- continued

Table 1. Patient characteristics

Characteristics	All patients (n=81)		Cases (n=40)		Controls (n=41)		P ^a
	n	%	n	%	n	%	
Resection type							
D1	54	67	26	65	28	68	NS
D2	27	33	14	35	13	32	
Resection type							
Total	25	31	12	30	13	32	NS
Partial	56	69	28	70	28	68	
Number of lymph nodes							
≥ 5	68	84	28	70	40	98	.001
< 5	13	16	12	30	1	2	

^a Chi-square test of cases vs controls, unless mentioned otherwise.

However, the mean percentage of OTC-positive lymph nodes was significantly higher in the case group compared with the control group (13.4 ± 22.0 vs 5.0 ± 7.0 ; $P = 0.024$). Nine of 38 cases and one of 40 controls were found to have 20% or more OTC-positive lymph nodes (24% vs 3%, $P = 0.015$), indicating that a high number of positive lymph nodes is predictive for disease recurrence. A single variable regression analysis showed that a percentage of 20% or more OTC-positive lymph nodes was a significant predictor for disease recurrence (*Table 3*). In total, three patients had OTC in lymph nodes outside the first level lymph nodes only (i.e. skip metastases). The level of lymph nodes was categorized according to the 4th edition of the TNM classification¹⁴ and the General Rules for the Gastric Cancer Study in Surgery and Pathology by the Japanese Research Society for the Study of Gastric Cancer.¹⁵ Two of these patients were in the case group and one patient was in the control group.

There was a correlation between patients with less than 5 lymph nodes examined and disease recurrence (Chi-square test, *Table 1*; $P = 0.001$). Patient characteristics that in two types of statistical analysis significantly correlated with disease recurrence were tumor differentiation and the number of examined lymph nodes (Chi-square test, *Table 1* and single variable regression analysis, *Table 4*). The difference between the case and control group in having less than 5 lymph nodes examined and in 20% or more OTC-positive lymph nodes was found to be in the D1 dissection group and not in the D2 dissection group (Chi-square; $P < 0.001$ vs $P = 0.586$ and $P = 0.008$ vs $P = 0.793$, respectively). Therefore, patients with less than 5 lymph nodes examined or 20% or more OTC-positive lymph nodes did not have a worse prognosis if they had undergone a D2 dissection.

In a multivariate logistic regression analysis, the variables tumor differentiation,

Table 2. Results from conventional microscopy and automated microscopy.

	Conventional microscopy HE (n=1380)		Conventional microscopy IHC ^a (n=1381)		Automated microscopy IHC ^b (n=1366)		Combined results (n=1396)	
A. Lymph node status	n	%	n	%	n	%	n	%
Presence of tumor cells	43	3.1	70	5.1	108	7.9	119	8.5
Macrometastases	6	0.4	6	0.4	6	0.4	6	0.4
MM	18	1.3	25	1.8	34	2.5	38	2.4
ITC	19	1.4	39	2.8	68	5.0	75	5.4
Negative	1337	96.9	1311	94.9	1258	92.1	1277	91.5
	(n=81)		(n=80)		(n=80)		(n=81)	
B. Patient status	n	%	n	%	n	%	n	%
Presence of tumor cells	19	24	26	33	35	44	40	49
Macrometastases	3	4	3	4	3	4	3	4
MM	8	10	12	15	13	16	15	19
ITC	8	10	11	14	19	24	22	27
Negative	62	77	54	68	45	56	41	51

HE, hematoxylin and eosin staining; IHC, immunohistochemistry; MM, micrometastases; ITC, isolated tumor cells; ^a Seven lymph nodes from three patients were found tumor-positive by HE staining only and scored negative on the IHC slides because these sections did not contain tumor cells anymore;

^b Four lymph nodes from two positive patients were recorded as negative in IHC evaluated by automated microscopy screening because the objects found by the system were considered as doubtful.

number of examined lymph nodes, and percentage of patients with 20% or more OTC-positive lymph nodes were entered. The examination of less than 5 lymph nodes (OR 13.8, 95% CI 1.6-120.6, $P = 0.018$) was the only independent risk factor for disease recurrence. Having a percentage of 20% or more positive lymph nodes was no independent risk factor (OR 9.0, 95% CI 1.0-81.2, $P = 0.051$), although there is a trend of being a risk factor as the P value was close to significance. Distant disease recurrence and locoregional disease recurrence were separately analyzed because of a possible difference in the metastasizing pattern. There was no correlation between the number of examined lymph nodes and distant metastases ($P = 0.374$) but the number of examined lymph nodes did correlate with locoregional relapse ($P = 0.002$). Furthermore, the presence of 20% or more OTC-positive lymph nodes was associated with distant metastases ($P = 0.001$) but not with locoregional recurrence ($P = 0.778$).

The majority of patients (8 of 9) from the case group with 20% or more OTC-positive lymph nodes suffered from distant disease recurrence. A multivariate logistic regression analysis for distant disease recurrence showed the only independent risk factor to be having a percentage of 20% or more positive lymph nodes (OR 15.6, 95% CI 1.6-151.4, $P = 0.018$) and a similar analysis for locoregional disease recurrence showed the examination of less than 5 lymph nodes (OR 20.4, 95% CI

Table 3. Clinical relevance of occult tumor cells in lymph nodes from patients with gastric cancer (patients with macrometastases were excluded from this analysis)

	All patients (n=78)		Cases (n=38)		Controls (n=40)		Univariate analysis			
	n	%	n	%	n	%	P ^a	OR	95% CI	P
Presence of OTC	37	47	19	50	18	45	.658	1.2	(0.5-3.0)	.659
Presence of MM	15	19	8	21	7	18	.691	1.3	(0.4-3.9)	.691
Presence of ITC	22	28	11	29	11	28	.887	1.1	(0.4-2.9)	.887
OTC-positive lymph nodes (mean) ^b	1.3 ± 2.1		1.5 ± 2.3		1.1 ± 1.8		.465 ^c	1.1	(0.9-1.3)	.462
OTC-positive lymph nodes (mean %) ^b	9.1 ± 16.6		13.4 ± 22		5 ± 7		.024 ^c	1.04	(1.0-1.1)	.043
OTC-positive lymph nodes (categorized %)										
0%	41	53	19	50	22	55	.015	1		
> 0% and < 20%	27	35	10	26	17	43		0.5	(0.2-1.3)	.136
≥ 20%	10	13	9	24	1	3		12.1	(1.5-101)	.021
OTC in 2 or more lymph nodes	19	24	11	29	8	20	.357	1.6	(0.6-4.6)	.360
MM in 2 or more lymph nodes	9	12	6	16	3	8	.252	2.3	(0.5-10)	.262

OTC, occult tumor cells; MM, micrometastases; ITC, isolated tumor cells; OR, odds ratio; CI, confidence interval; ^a Chi-square test of cases vs controls, unless mentioned otherwise; ^b Presented as mean ± standard deviation; ^c Student T-test.

2.2-190.8, $P = 0.008$) to be the only independent risk factor.

A sensitivity and specificity calculation for disease recurrence within a period of 5 years for less than 5 examined lymph nodes or having 20% or more OTC-positive lymph nodes revealed a sensitivity of 29% and specificity of 98% ($n = 78$), and a sensitivity of 21% and specificity of 93% ($n = 78$), respectively. The combined condition of less than 5 examined lymph nodes or having 20% or more OTC-positive lymph nodes led to a sensitivity of 45% and a specificity of 95% ($n = 78$). Three of 11 patients from the case group with less than 5 lymph nodes examined had stage IA gastric cancer, 7 had stage IB and 1 had stage II gastric cancer. Two of 9 cases with 20% or more OTC-positive lymph nodes had stage IA gastric cancer, 4 had stage IB and 3 had stage II gastric cancer. All stage IA patients from the case group with less than 5 lymph nodes examined or with 20% or more OTC-positive lymph nodes ($n = 3$) had undergone a D1 resection.

Screening of the slides

IHC staining evaluated by conventional microscopy was compared with HE staining and IHC staining evaluated by automated microscopy was compared with IHC staining evaluated by conventional microscopy. Six lymph nodes from 3 patients containing macrometastases were identified by all 3 screening methods.

Table 4. Single variable regression analysis of disease recurrence (n=78)

Characteristics	Univariate analysis		
	OR	95% CI	P
Sexe			
Female	1		
Male	1.3	(0.5-3.1)	.610
Age (years)	1.0	(1.0-1.1)	.438
Stage ^a			.962 ^b
IA	1		
IB	1.4	(0.4-4.6)	.591
II	1.3	(0.3-5.9)	.705
IIIA	NA		
Location			.732 ^b
C (Upper third)	1		
M (Middle third)	0.6	(0.2-2.3)	.483
A (Lower third)	1.2	(0.3-4.0)	.796
WHO classification			.681 ^b
Papillary	1		
Tubular	0.7	(0.2-2.8)	.602
Mucinous	1.0	(0.1-7.6)	1.000
Signet ring cell	1.5	(0.3-6.3)	.614
Undifferentiated	0.5	(0.0-7.5)	.615
Laurén classification			.178 ^b
Intestinal	1		
Diffuse	2.6	(0.9-7.4)	.067
Mixed	1.1	(0.2-5.2)	.952
Differentiation			.036 ^b
Good	1		
Moderate	0.2	(0.0-1.6)	.121
Poor	0.5	(0.0-5.1)	.551
Size (cm)			.775 ^b
< 2	1		
2 - 3	0.8	(0.3-2.0)	.622
> 3	1.1	(0.5-2.7)	.825

OR, odds ratio; CI, confidence interval; NA, not assessable; ^a According to the 5th edition of the TNM classification¹⁹; ^b Overall P value.

These patients were included in this assessment because by excluding these patients, 3 lymph nodes with MM would also have been excluded. From 1 patient only 1 lymph node was available which was stained by the HE method only.

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Table 4. Single variable regression analysis of disease recurrence (n=78)

Characteristics	Univariate analysis		
	OR	95% CI	P
Resection type			
D1	1		
D2	1.2	(0.5-3.1)	.687
Resection type			
Total	1		
Partial	1.2	(0.5-3.1)	.734
Number of lymph nodes			
≥ 5	1		
< 5	15.9	(1.9-130.4)	.010

OR, odds ratio; CI, confidence interval.

A total of 1365 lymph nodes from 80 patients were examined by both HE staining and IHC evaluated by conventional microscopy.

The number of positive lymph nodes increased from 43 (3%) found by HE staining to 77 (6%) found by IHC evaluated by conventional microscopy. The number of patients with positive lymph nodes increased from 19 (24%) when identified by HE staining to 29 (36%) by IHC evaluated by conventional microscopy. Seven lymph nodes (0.5%) from 3 of the 29 tumor-positive patients were found tumor-positive by HE staining only and scored negative on the IHC slides because these sections did not contain tumor cells anymore (*Figure 4*). IHC slides of 1366 lymph nodes from 80 patients were examined by both conventional microscopy and automated microscopy. When comparing IHC evaluated by conventional microscopy to IHC evaluated by automated microscopy, the number of positive lymph nodes increased from 70 (5%) to 112 (8%) out of 1366 and the number of patients with positive lymph nodes increased from 26 (33%) to 37 (46%). Four positive lymph nodes from 2 of the 37 OTC-positive patients were recorded as negative in IHC evaluated by automated microscopy screening because the objects found by the system were considered as doubtful by the pathologist. The ARIOL system did not miss any tumor cells in lymph nodes. In *Table 5*, the additional value of IHC and automated microscopy in detecting patients with 20% or more positive lymph nodes, is shown. By using IHC evaluated by conventional microscopy 1 patient in the control group was identified with 20% or more positive lymph nodes. However, with IHC combined with automated microscopy, 5 of 8 patients in the case group were additionally identified as having 20% or more positive lymph nodes.

The sensitivity and specificity of the 3 screening methods are displayed in *Table 6*. IHC screened by automated microscopy identified 22% of patients in the case group compared with 8% and 11% identified by IHC screened by conventional

Table 5. The additional value of IHC to HE staining and automated microscopy to conventional screening when considering a patient positive with 20% or more positive lymph nodes (n=77)

		Conventional microscopy IHC	
	Conventional microscopy HE	negative	positive
cases (n=37)	negative	33	0
	positive	1 ^a	3
controls (n=40)	negative	39	1
	positive	0	0

		Automated microscopy IHC	
	Conventional microscopy IHC	negative	positive
cases (n=37)	negative	29	5
	positive	0	3
controls (n=40)	negative	39	0
	positive	0	1

IHC, immunohistochemistry; HE, hematoxylin and eosin staining; ^a Lymph nodes were found tumor-positive by HE staining only and scored negative on the IHC slides because these sections did not contain tumor cells anymore.

microscopy and the HE method, respectively. When comparing the sensitivity of automated microscopy to conventional microscopy, McNemar's test showed a P value of 0.063 suggesting additional value of the former screening method. The specificity of the HE method, IHC screened by conventional microscopy and IHC screened by automated microscopy was 100%, 98% and 98%, respectively.

Original HE slides of 31 of 43 tumor-positive lymph nodes, detected with the HE method, could be retrieved from the involved Pathology Departments. Nine original HE slides with 12 lymph node sections were lost

because material older than 10 years had already been destroyed. Re-evaluation of the original HE slides revealed that in 24 of 31 (77%) positive lymph nodes, tumor cells were also present in these original sections. Six of the 31 positive lymph nodes contained macrometastases and 25 were OTC-positive. Three of 6 lymph nodes with macrometastases in the new HE sections also showed macrometastases in the original sections and 21 of 25 lymph nodes with OTC were on re-observation of the original HE slides also found to be OTC-positive.

Table 6. The sensitivity and specificity of the screening methods in detecting patients with 20% or more positive lymph nodes (n=77)

Screening methods	Sensitivity (%) (n=37)	Specificity (%) (n=40)
HE	11	100
IHC screened by conventional microscopy	8	98
IHC screened by automated microscopy	22 ^a	98

HE, hematoxylin and eosin staining; IHC, immunohistochemistry; ^a McNemar's test comparing automated to conventional microscopy, P = 0.063.

Considering the fact that 24 of 31 (77%) of the positive lymph nodes could already have been identified in the original HE sections, it can be concluded that tumor cells were detected in 7 of 31 (23%) lymph nodes through serial sectioning i.e. additional sections for HE staining.

DISCUSSION

This case-control study showed that simply the presence of OTC, either MM or ITC in lymph nodes from patients with gastric cancer is not correlated with disease recurrence. The examination of less than 5 lymph nodes was the only independent risk factor for disease recurrence. When analyzing the type of disease recurrence separately, the number of examined lymph nodes and the presence of 20% or more OTC-positive lymph nodes were independent risk factors for locoregional disease recurrence and distant disease recurrence, respectively. The number of examined lymph nodes has already been reported as a prognostic factor.¹⁶⁻¹⁸ Current guidelines from the American Joint Committee on Cancer Staging (AJCC)^{7,19} recommend examination of a minimum of 15 lymph nodes to justify the N0 status. However, Klein Kranenbarg *et al.*¹⁶ previously studied patients from the Dutch Gastric Cancer Group Trial (DGCCT) and reported a minimum of 5 examined lymph nodes as a reliable number for staging purposes. Liu *et al.*¹⁷ reported a considerable survival advantage for patients with stage III gastric cancer when more than 15 lymph nodes were removed. Ichikura *et al.*¹⁸ showed that N0 patients with more than 9 lymph nodes examined had a significantly higher survival rate than N0 patients with 5 to 9 lymph nodes examined and patients with N1 or N2 disease¹⁹, tended toward lower survival rates when less than 30 lymph nodes were examined. Therefore, the number of examined lymph nodes is of prognostic significance for patients with N0, N1 and N2 disease. Moreover, our study showed that the number of examined lymph nodes and the presence of 20% or more OTC-positive lymph nodes were independent risk factors for locoregional disease recurrence and distant disease recurrence, respectively. This suggests that locoregional disease recurrence probably is the result of lymph nodes that were not removed. Conversely, when 20% or more of the lymph nodes are OTC-positive, tumor cells may already have travelled to other sites in the body eventually leading to distant metastases. The addition of tumor cell detection in bone marrow²⁰ and peritoneal washes²¹⁻²³ could add to identifying patients at risk for developing disease recurrence.

We have also found in another study²⁴, in which multiple sections of sentinel nodes were examined, that automated microscopy detected more OTC than routine pathology. In our current study, without using automated microscopy, we would have missed 5 of 9 patients in the case group with 20% or more positive lymph nodes. Therefore IHC evaluated by automated microscopy showed additional value in identifying high-risk patients.

Nine²⁵⁻³³ of the 14 previously published studies²⁵⁻³⁸ on the prognostic relevance of

Table 7. Immunohistochemistry studies regarding the clinical relevance of occult tumor cells in lymph nodes

References	Year	Antibody ^a	No of HE-negative patients	Average no of nodes per patient	Upstaging (%)	Impact on prognosis ^b
Siewert <i>et al.</i> ²⁵	1996	AE1/AE3	62	33.8	90 ^c	negative
Maehara <i>et al.</i> ²⁶	1996	CAM5.2	34	12.4	24	negative
Ishida <i>et al.</i> ²⁷	1997	CAM5.2 and or CEA	21 ^d	22.4	67	negative
Kikuchi <i>et al.</i> ³⁴	1999	AE1/AE3	51	27.3	43	none
Cai <i>et al.</i> ²⁸	2000	CAM5.2	69	24.6	25	negative
Bozzetti <i>et al.</i> ³⁵	2000	a pool of 35βH11, 34βE12, CAM5.2, AE1/AE3 and KL1	29	25	28	none
Harrison <i>et al.</i> ²⁹	2000	CAM5.2	25	9.0	36	negative
Fukagawa <i>et al.</i> ³⁶	2001	AE1/AE3	107	41.9	36	none
Nakajo <i>et al.</i> ³⁰	2001	AE1/AE3	67	26.3	15 ^e	negative
Yasuda <i>et al.</i> ³¹	2002	CAM5.2	64	31.9	32	negative
Lee <i>et al.</i> ³²	2002	AE1/AE3	70	23.7	24	negative
Choi <i>et al.</i> ³⁷	2002	anti-CK8	73	25.8	18	none
Ishigami <i>et al.</i> ³³	2003	AE1/AE3	144	23.4	9	negative
Morgagni <i>et al.</i> ^{38, f}	2003	MNF116	300	18	10	None

HE, hematoxylin and eosin staining; CK, cytokeratin; CEA, carcinoembryonic antigen; ^a There are 20 different types of cytokeratins; AE1/AE3 is a mixture of two different clones of monoclonal antibodies (Dako); AE1 recognizes CK10, 13, 14, 15, 16, and 19; AE3 recognizes CK1, 2, 3, 4, 5, 6, 7 and 8; CAM5.2 recognizes CK7 (weak) and 8 (Becton Dickinson); 35βH11 recognizes CK8 (Dako); 34βE12 recognizes CK1, 5, 10 and 14 (Dako); KL1 recognizes CK1, 2, 5, 6, 7, 8, 11, 14, 16, 17 and 18 (Immunotech); anti-CK8 recognizes CK8 (Dako); MNF116 recognizes CK5, 6, 8, 17 and probably 19 (Dako); CEA recognizes carcinoembryonic antigen (IBL, Fujioka, Japan); ^b Impact on prognosis includes impact on overall survival and/or disease-free and/or disease-specific survival based on logrank, univariate and/or multivariate analyses with a P value < 0.05; ^c significant prognostic factor when the threshold of 3 or more tumor cells in more than 10% of the examined lymph nodes was exceeded; ^d Stage II patients; ^e Micrometastases were defined as a cluster of tumor cells with a stromal reaction (15%); no effect on prognosis was shown by individual tumor cells without a change in the stroma (6%); ^f This was the only study examining 3 levels of each lymph node.

OTC in lymph nodes from gastric cancer patients report a negative impact of the presence of OTC in lymph nodes on patient's prognosis (*Table 7*). In the study by Siewert *et al.*²⁵ the percentage of OTC-positive lymph nodes was prognostic relevant. The study by Nakajo *et al.*³⁰ showed a prognostic effect of tumor cells in lymph nodes with stromal reaction but no effect of tumor cells in lymph nodes

without stromal reactions. The other 7 studies reported a prognostic relevance of just the presence of OTC in lymph nodes. Our analysis has important additional value to these 14 studies. First, we used automated microscopy to detect OTC in lymph node sections IHC-stained for tumor cells. Therefore, the chance of finding OTC was highly increased at the level of both marking and detecting tumor cells. Second, our case-control design is more appropriate because the reported number of events i.e. disease recurrences in histologically node-negative patient groups is low.^{28;36;38} This makes it difficult from a statistical point of view, to determine the prognostic role of OTC. A case-control study overcomes this problem. Most studies shown in *Table 7* are retrospective cohort studies with the majority of patients undergoing extended lymphadenectomy. Furthermore, the strength of our study was its original prospective randomised controlled design which ensured identical inclusion and follow-up of cases and controls and identical collection and handling of lymph nodes from cases and controls. In view of the fact that the prognosis of patients with resectable gastric cancer is mainly predicted by the tumor involvement of lymph nodes and the depth of primary tumor invasion into the gastric wall, cases and controls were matched for T stage in the present study. The fourth advantage of our study is the inclusion of almost an equal number of patients with a D1 and D2 dissection type in both the case and control group. We did find the difference between the case and control group in number of examined lymph nodes and percentage of OTC-positive lymph nodes to be in the D1 dissection group and not in the D2 dissection group. Thus, patients with less than 5 lymph nodes examined or 20% or more OTC-positive lymph nodes did not have a worse prognosis if they had undergone a D2 dissection. Klein Kranenbarg *et al.*¹⁶ previously showed that more lymph nodes were examined in the D2 dissection group suggesting a better survival in this group. On the contrary, the DGGCT^{10;11} and other studies^{39;40} reported no difference in survival between patients who underwent a D2 dissection and patients who underwent a D1 dissection, although the DGGCT did show a survival benefit of patients with N2 disease¹⁹ who underwent a D2 dissection.¹¹ The 10-year disease recurrence rate was 97% for N2 patients who underwent a D1 dissection compared with 69% of the patients who underwent a D2 dissection ($P = 0.013$ by the logrank test) suggesting that patients with more than a certain number of positive lymph nodes might benefit from D2 dissection. Our results are in contrast with Petrelli's⁴¹ plea in his editorial to end the debate whether to perform a D1 or D2 dissection. Indeed, the DGGCT showed an increased postoperative morbidity and mortality in patients who underwent a D2 dissection (25% vs 43%; $P < 0.001$ and 4% vs 10%; $P = 0.004$, respectively)¹⁰ and patients showed no significant survival benefit after a follow-up of more than 10 years (30% vs 35%; $P = 0.53$).¹¹ However, morbidity and mortality were mostly caused by performing pancreatectomy and splenectomy. Recent studies show that D2 surgery with organ preservation, performed in specialized centers, is a safe procedure with limited morbidity and mortality.⁴²⁻⁴⁵

Altogether, our results suggest that provided that a decrease of morbidity and mortality can be attained, extended lymphadenectomy might improve patient survival. Another approach may be postoperative chemoradiotherapy for patients with positive lymph nodes who did not undergo a D2 dissection. Macdonald *et al.*^{46;47} showed that chemoradiotherapy after resection significantly improved relapse-free and overall survival among gastric cancer patients who underwent a D0 or a D1 dissection. Postoperative chemoradiotherapy did not show an effect in gastric cancer patients who underwent a D2 dissection. At present, in the United States, gastric cancer patients with completely resected stage IB (T1N1M0, T2a/bN0M0) to stage III are being considered for postoperative chemoradiotherapy based upon data from the INT-0116 trial.^{47;48} All stage IV M0 gastric cancer patients receive postoperative chemoradiotherapy. Considering the results from our study, patients with stage IA gastric cancer, with less than 5 lymph nodes examined or with 20% or more OTC-positive lymph nodes might also be considered for postoperative chemoradiotherapy.

In conclusion, IHC combined with automated microscopy provided additional value in the detection of OTC in lymph nodes. Just the presence of OTC in lymph nodes from gastric cancer patients did not have prognostic value. However, patients with less than 5 lymph nodes examined or 20% or more OTC-positive lymph nodes had a worse prognosis. Therefore a maximum number of lymph nodes should be removed and meticulously examined for OTC to identify high-risk patients. These patients should be considered for additional treatment.

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CHAPTER THREE

PROGNOSTIC RELEVANCE OF OCCULT TUMOR CELLS IN LYMPH NODES IN COLORECTAL CANCER

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ABSTRACT

Background: Presently, in Europe the treatment of node-negative colorectal cancer (CRC) patients consists of surgical resection of the primary tumor without adjuvant systemic therapy. However, up to 30% of these patients will develop disease recurrence. These high-risk patients are possibly identified by occult tumor cell (OTC) assessment in lymph nodes. In this paper, studies on the clinical relevance of OTC in lymph nodes are reviewed.

Methods: A literature search was conducted in the National Library of Medicine by using the keywords colonic, rectal, colorectal, neoplasm, adenocarcinoma, cancer, lymph node, polymerase chain reaction, mRNA, immunohistochemistry, micrometastases and isolated tumor cells. Additional articles were identified by cross-referencing from papers retrieved in the initial search.

Results: The upstaging percentages through OTC assessment and the prognostic relevance of OTC in lymph nodes vary among studies, which is related to differences in techniques used to detect OTC.

Conclusions: We conclude that OTC examination techniques should be standardized to illuminate whether OTC in lymph nodes can reliably identify high-risk node-negative patients.

INTRODUCTION

High-risk node-negative colorectal cancer (CRC) patients may be identifiable through the detection of occult tumor cells (OTC) in lymph nodes. OTC comprise micrometastases (MM) and isolated tumor cells (ITC). MM are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC either as single tumor cells or as clusters of tumor cells of 0.2 mm or less.¹⁻³ OTC are usually not detected with conventional pathological examination, as only one or two 4 to 5 μ m sections of each lymph node are being examined after staining with the hematoxylin and eosin (HE) method. It is calculated that a single 4 μ m section through the center of a lymph node measuring 1 cm in diameter merely samples approximately 0.06 percent of the lymph node⁴ which is presumed to reflect the entire lymph node. Examination techniques focused on detection of OTC include serial sectioning, step sectioning, immunohistochemistry (IHC), polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR). There is a higher chance of detecting OTC with these techniques as a larger part of the lymph node is being examined and because of their higher sensitivity for detecting tumor cells.

The role of OTC detection in CRC is not clear yet although numerous studies on this topic have previously been published. This review deals with the detection methods and clinical relevance of OTC in lymph nodes in CRC. Emphasis is put on differences in examination techniques to detect OTC.

METHODS

A literature search was conducted with PubMed software in the National Library of Medicine, containing articles from 1953 until 2004. The following key words were used in appropriate combinations: colonic or rectal or colorectal neoplasm, adenocarcinoma and cancer, lymph node, polymerase chain reaction, mRNA, immunohistochemistry, micrometastases and isolated tumor cells. Papers with anal cancer in the title were excluded and the language was restricted to English. All hits from this PubMed search were individually checked, and included only if they addressed the subject of this review. Additional articles were identified by cross-referencing from papers retrieved in the initial search. Overall, only articles that included the prognostic relevance of OTC in lymph nodes were included for this overview. There was no limit to the number of patients.

Occult tumor cells in lymph nodes detected with polymerase chain reaction

The PCR assay can be used to detect OTC in lymph nodes by amplification of certain DNA regions that may contain tumor-specific mutations for instance regions in the tumor suppressor genes p53 and K-ras. The K-ras gene can contain mutations at codon 12, 13 or 61 and in the p53 gene mutations cluster in exons 5 to 8.

There are four published studies in which HE-negative lymph nodes were examined with the PCR method and the effect of a positive PCR on the prognosis of the patients was analyzed.⁵⁻⁸ Hayashi *et al.*⁵ screened HE-negative lymph nodes for the presence of mutations in the K-ras or p53 genes. Twenty-seven out of 37 patients with PCR positive lymph nodes suffered from tumor recurrence within 5 years, whereas none of the 34 patients with OTC-negative lymph nodes had a recurrence. Thebo *et al.*⁶ used K-ras mutations at codon 12 and 13 to detect OTC. No recurrences were reported in the OTC-negative group (n = 4) compared to a recurrence rate of 38% in the OTC-positive group (n = 16). Clarke *et al.*⁷ examined lymph nodes by using K-ras mutations at codon 12. No difference was shown in survival between patients with a positive PCR and patients with a negative PCR. Four out of 13 OTC-positive patients died of recurrence within 5 years and one out of four OTC-negative patients also died of recurrence. Belly *et al.*⁸ also used K-ras mutations at codon 12 to detect OTC. Of 14 OTC-positive patients, eight died of disease within 5 years compared to four out of 24 OTC-negative patients.

A disadvantage of the PCR method is the fact that the p53 and K-ras mutations do not occur consistently in CRC. Mutations of the p53 gene and the K-ras gene are present in approximately 70%⁹ and 38%¹⁰ in CRC, respectively. Only when

the primary tumors harbor mutations, these mutations can be utilized to detect OTC in HE-negative lymph nodes. Hayashi *et al.*⁵ could not detect mutations in the p53 or K-ras gene in 41% of 120 primary tumors leading to the exclusion of many patients for OTC detection. An additional problem is the large number of codons or even exons in which mutations can be detected, especially in the p53 gene. This implies that for detection of all the possible mutations many different PCR primers are needed which is not feasible in clinical practice and rather expensive. Concluding, three out of four studies showed clinical relevance of OTC detected with the PCR method. However, technical factors will prevent the PCR from becoming a ubiquitously utilized application for OTC detection.

Occult tumor cells in lymph nodes detected with reverse transcriptase polymerase chain reaction

RT-PCR within the context of OTC assessment in CRC involves amplification of specific messenger RNA present in epithelial cells or malignant cells. A study by Liefers *et al.*¹¹ showed that patients with CEA RT-PCR negative lymph nodes had a significantly better 5-year disease-free survival than patients with positive lymph nodes (91 vs 50%, $P = 0.02$). Five other RT-PCR studies¹²⁻¹⁶ also showed that a positive RT-PCR result had a negative impact on survival. Three of these 6 studies used CEA^{11;12;15}, 2 used CK20^{14;16} and 1 used GCC¹³ as a marker. One of these studies also used CEA as a marker and showed no difference between patients with a positive RT-PCR result and patients with a negative result.¹³ However, with this marker they showed an upstaging percentage of 5 whereas the other three CEA studies showed an upstaging range of 30% to 54%. This suggests a limited sensitivity of this specific protocol what may explain the lack of influence on clinical outcome. The low upstaging percentage of 5% could be due to the use of paraffin-embedded lymph nodes.

During the past years a one-tube one-enzyme quantitative real-time RT-PCR has been developed by several companies. This method involves a closed system and makes use of fluorescence to quantify the PCR product. Real-time RT-PCR is highly sensitive, has a lower risk of contamination and is far less laborious compared to the conventional RT-PCR. Lassmann *et al.*¹⁷ showed no prognostic value of a positive real-time RT-PCR test with the marker CK20. A recent study by Bustin *et al.*¹⁸ neither found any correlation between patient's prognosis and a positive real-time RT-PCR test when using the markers CK20, CEA and GCC at 40 PCR cycles. The higher sensitivity of the real-time RT-PCR is shown in the upstaging percentages with the markers CEA and CK20 of 98% and 75%, respectively. The reason why Lassmann *et al.*¹⁷ found a lower upstaging percentage of 35% with their real-time CK20 RT-PCR might relate to the use of paraffin material. Furthermore, the lower GCC upstaging percentage of 22% in the study by Bustin *et al.*¹⁸ compared to the GCC upstaging percentage of 48% in the study by Cagir *et al.*¹³ could be the result of a higher number of PCR cycles in the latter study i.e.

40 cycles *versus* 70 cycles. A disadvantage of the higher sensitivity of the real-time RT-PCR is the detection of background gene expression in hematopoietic cells or circulating normal epithelial cells, and thus false-positive patients, that might explain the higher percentage of upstaging when compared to the earlier studies. The rate of false-positive patients can be limited by optimizing the number of PCR cycles. Miyake *et al.*¹⁹ reported that under 35 cycles of PCR, lymph nodes from patients with benign diseases did not express CEA and CK20, but, when using 40 cycles, bands for CEA appeared in 7% of normal lymph nodes and bands for CK20 appeared in 20% of normal lymph nodes. These results indicate that there is only a narrow window in which reliable results can be obtained. Similar results were reported earlier by Liefers *et al.*¹¹ who examined HE-negative lymph nodes by using a nested RT-PCR. A very faint band was detected in some negative control samples when 20 or 25 cycles were used in the second PCR round. Therefore, it was decided that 15 cycles should be used in the second PCR round leading to a total of 35 cycles in their nested RT-PCR method.

A pitfall associated with the PCR and RT-PCR method is contamination of lymph nodes by cells from the primary tumor or bowel epithelium leading to false-positive results. Rosenberg *et al.*¹⁴ examined HE-negative lymph nodes with both RT-PCR and IHC. In 13 of 44 RT-PCR positive patients, the positivity was caused by tumor cell contamination located exclusively outside the lymph node capsule. Defining these 13 patients as RT-PCR negative improved the specificity of the RT-PCR assay from 57% to 75%. The overall 5-year survival rates of the IHC-controlled RT-PCR positive group were significantly worse than the negative group ($P < 0.001$), and the differences were greater than with RT-PCR alone ($P < 0.009$). Contamination of lymph nodes by cells from the primary tumor or bowel epithelium can be limited by removing the lymph nodes before incision of the bowel specimen during examination by the pathologist.

From these data it can be concluded that OTC in lymph nodes detected with RT-PCR show prognostic value provided that fresh or frozen lymph nodes, the markers CEA, CK20 and GCC and the optimal number of PCR cycles are used.

Occult tumor cells in lymph nodes detected with immunohistochemistry

Although RT-PCR is potentially more sensitive than IHC, the latter method is commonly available in daily practice and has the advantage of morphological confirmation of detected tumor cells. Conversely, the specificity of IHC seems to be lower.^{15;20} Noura *et al.*¹⁵ studied paraffin-embedded lymph nodes with both CEA RT-PCR and IHC by using the anti-pancytokeratin antibody AE1/AE3 and showed that the former method had prognostic value, whereas the latter did not.

Nine²¹⁻²⁸ out of 28 studies^{15;17;21-41} showed a significantly worse clinical outcome in patients with lymph nodes containing OTC: five^{22;23;25-27} out of eight studies using CAM5.2 (63%)^{22;23;25-27;29-31}, one²¹ out of seven using AE1/AE3 (14%)^{15;21;32-36}, one²⁸ out of two using CEA (50%)^{28;37}, one²⁸ out of two using CK20 (50%)^{17;28}, one²⁴ out

of two using MNF116 (50%)^{24;38}, zero out of two using BerEP4 studies^{28;35}, zero out of one using CC49²¹, zero out of one using anti-CK³⁹, zero out of one using KL-1⁴⁰, and zero out of one using RSP53 antibodies.⁴⁰ One study, using a mixture of antibodies among which AE1/AE3 and CAM5.2, reported no worse prognosis of patients with OTC in lymph nodes.⁴¹ Three^{17;28;30} out of the 28 studies showed a trend towards a worse clinical outcome: one³⁰ out of eight studies using the antibody CAM5.2 (13%), one¹⁷ out of two using CK20 (50%), and one²⁸ of two using BerEP4 (50%) antibodies. The other 15 studies showed no prognostic effect of OTC-positive lymph nodes. Eight²¹⁻²⁸ of 22 studies^{15;17;21-36;38-41} (36%) using antibodies directed against cytokeratin showed a worse prognosis of patients with OTC in lymph nodes compared to one²⁸ of six studies^{21;28;35;37;40} (17%) using antibodies directed against other antigens.

In conclusion, only nine out of 28 IHC studies showed a significantly worse clinical outcome in patients with lymph nodes containing OTC. In particular studies using the antibody CAM5.2, reported OTC-positive lymph nodes to be of clinical relevance.

Factors influencing detection of occult tumor cells with immunohistochemistry

Xu *et al.*⁴² reported undesirable cytokeratin positivity in nonepithelial cells in lymph nodes from breast cancer patients. Cytokeratin positivity was found in reticulum cells and plasma cells with pan-CK and CAM5.2 but not with AE1/AE3^a. Seemingly, antibodies raised primarily against CK8 (CAM5.2 and pan-CK) can detect background CK8 expression in nonepithelial cells, which cannot be revealed by AE1/AE3, though it recognizes a broad spectrum of different cytokeratins. Adversely, use of a broad spectrum antibody such as AE1/AE3, might not be as sensitive as other subset-specific antibodies like CAM5.2 in identifying certain undifferentiated carcinomas. This might explain the higher percentage of CAM5.2 studies showing clinical relevance compared to AE1/AE3 studies as mentioned above. Morphological evaluation is key in distinguishing tumor cells from nonepithelial cells. Others methods that can be used to distinguish tumor cells from plasma cells, dendritic cells, mesothelial cells and macrophages, include double staining with anti-immunoglobulin kappa or lambda light chains, anti-S100, anti-calretinin, and anti-CD68 antibodies respectively. Studies comparing antibodies should lead to the antibody with the highest sensitivity and specificity in recognizing tumor cells in lymph nodes.

Three^{27;28} out of eight studies^{27;28;32;35;41} examining more than one level per lymph node showed prognostic significance of OTC compared to six²¹⁻²⁶ out of 20 studies^{15;17;21-26;29-31;33;34;36-40} examining one level. Two^{22;25} out of six studies^{15;22;25;39;40} examining more than one section per level showed prognostic significance of OTC

^a There are 20 different types of cytokeratins; CAM5.2 recognizes CK7 (weak) and 8; AE1/AE3 is a mixture of two different clones of monoclonal antibodies; AE1 recognizes CK10, 13, 14, 15, 16, and 19; AE3 recognizes CK1, 2, 3, 4, 5, 6, 7 and 8; MNF116 recognizes CK5, 6, 8, 17 and probably 19; anti-CK recognizes CK 8, 18 and 19; KL-1 recognizes CK1, 2, 5, 6, 7, 8, 11, 14, 16, 17 and 18.

compared to seven^{21;23;24;26-28} out of 22 studies^{17;21;23;24;26-38;41} examining one section per level. Adell *et al.*³⁹ reported that 95% of CRC patients with OTC were identified by examining three levels from three lymph nodes with AE1/AE3. McGuckin *et al.*⁴³ examined lymph nodes from breast cancer patients and showed that the majority of lymph node metastases can be detected by examining two levels 300 µm apart. Fisher *et al.*³³ studied the largest CRC patient group thus far and found OTC in lymph nodes in 18.3% of 399 patients but could not confirm any prognostic significance of OTC in lymph nodes. This may be related to the examination of only one level of each lymph node in combination with the use of the antibody AE1/AE3.

In all of the abovementioned papers, OTC were detected through screening by a pathologist using routine light microscopy. This bares the risk of inaccurate screening due to factors such as OTC size, interobserver differences and incomplete section screening. Automated microscopy may facilitate and render IHC more reliable because the aforementioned factors are eliminated.^{44;45}

As yet, an optimum number of two levels has been reported. It can be suggested that in order to assess whether OTC in lymph nodes predicts patient's prognosis, at least two levels of the lymph nodes should be examined using an anti-cytokeratin antibody.

Number and size of lymph nodes

In the studies by Sasaki *et al.*²² and Yasuda *et al.*²⁵, not only the presence but also the number of OTC-positive lymph nodes was considered in relation to decreased survival. The former study showed a significantly higher frequency of OTC-positive nodes in patients with recurrent disease. The latter study reported OTC in four or more lymph nodes to be significantly associated with recurrent disease. Both studies showed a significantly higher frequency of OTC in second tier lymph nodes of patients with poor prognosis.

Overall survival of CRC patients without HE detectable nodal metastases, improves with increasing number of lymph nodes recovered.^{46;47} Cserni *et al.*⁴⁷ studied data from 8574 stage II CRC patients. They could not define a cut-off value for the number of lymph nodes needed to be examined for adequate nodal staging. According to their statistical analysis the risk of death decreased by 2.1% for each negative lymph node. Ruers *et al.*⁴⁸ reported the number of nodes that had to be examined for reliable staging to be T stage dependent. Fifteen nodes for T2 tumors, 10 nodes for T3 tumors and seven nodes for T4 tumors needed to be examined. Current guidelines from the American Joint Committee on Cancer Staging (AJCC) recommend examination of a minimum of 12 lymph nodes for accurate staging.⁴⁹ However, this is not always feasible. By studying 569 CRC specimens, Johnson *et al.*⁵⁰ reported that only 22% of the patients underwent an adequate lymph node harvest according to the current AJCC recommendation. Sometimes only few or no lymph nodes are found, even when surgeons resect a

large part of the perimuscular fatty tissue. Involved factors might be preoperative radiotherapy in rectal carcinoma, leading to decrease in lymph node size⁵¹, or the presence of few or very small lymph nodes.⁵² The former factor was shown in the Total Mesorectal Excision trial where the mean number of examined lymph nodes was 9.7 in the surgery only group and 7.7 in the radiotherapy group ($P < 0.001$).⁵¹ Maurel *et al.*⁵² reported a significantly higher number of examined lymph nodes in patients younger than 75. Fat clearance techniques might facilitate in finding small lymph nodes. Haboubi *et al.*⁵³ showed that by adding fat clearance to conventional lymph node harvesting, the mean number of recovered lymph nodes increased from 6.7 to 58.2 lymph nodes. However, these relatively inexpensive techniques involve the use of xylene and alcohol and, therefore, are considered impractical and unsafe. This is the major reason that fat clearance techniques are not being used worldwide. It should be noted that even very small lymph nodes can contain metastases. Andreola *et al.*⁴¹ reported that 45% of metastatic lymph nodes of 49 stage III patients had a diameter smaller than 5 mm, determining the stage in 15 (31%) of the patients. Haboubi *et al.*³⁰ showed that 86% of lymph nodes with OTC had a diameter smaller than 5 mm. Ruers *et al.*⁴⁸ suggested that a standard of how much cm² of mesocolon should be removed by the surgeon and how many lymph nodes should be retrieved by the pathologist per cm² will contribute to quality control in colon cancer.

Summarizing, the number of OTC-positive lymph nodes should also be considered in relation to decreased survival. Moreover, the problem of defining an optimal number of lymph nodes and finding small lymph nodes might be tackled by standardization of how much cm² of mesocolon should be removed by the surgeon.

Size and location of occult tumor cells in lymph nodes

Using the actual size of OTC instead of dividing them into categories such as ITC and MM might answer the question whether the size of metastases matters.³³ As yet it is not clear whether MM and ITC have the same influence on the patient's clinical outcome. It is possible that in immunocompetent individuals, OTC are destroyed by the immune system before growing into large metastases or that not all OTC are viable and therefore do not have the capacity to proliferate. Determining the viability of OTC could shed some light on the metastasis potential of these tumor cells e.g. by determining the number of apoptotic cells *versus* vital cells.

Furthermore, it may be important to consider the intra-nodal location of OTC in the analysis. Tumor cells in lymph nodes can be located in subcapsular sinuses, also referred to as peripheral sinuses, paracortical sinuses or medullar structures. They can also be located intrafollicularly or show a diffuse distribution. It has been shown that tumor cells are usually present in the subcapsular sinuses^{29;32;37;54;55} but the impact of the location of OTC on patient's prognosis has not been clearly addressed yet. In summary, when considering OTC in lymph nodes in CRC, it might be necessary to include the size and intra-nodal location of OTC in the analysis.

CONCLUSIONS

Most of the studies indicate a clinical significance of detecting OTC in lymph nodes of CRC patients. Conditions and techniques, however, vary considerably among the different studies. Therefore, OTC examination should be standardized. We recommend comparing studies using RT-PCR and IHC to establish optimal conditions to reliably identify high-risk patients that are, using the current techniques, considered as lymph node-tumor negative patients.

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CHAPTER FOUR

AUTOMATED ANALYSIS OF MULTIPLE SECTIONS FOR THE DETECTION OF OCCULT CELLS IN LYMPH NODES

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ABSTRACT

Background: At present, reverse transcription polymerase chain reaction (RT-PCR) against carcinoembryonic antigen mRNA is one of the few research tools for the detection of occult cells in histopathologically assessed negative lymph nodes from patients with colorectal cancer. The aim of this study was to investigate the suitability of supervised low-resolution image analysis of immunohistochemically stained sections as alternative.

Patients and methods: Multiple sections ($n = 50$) of regional lymph nodes from patients with colorectal cancer were immunohistochemically stained and analyzed by applying low-resolution image analysis (flatbed scanning) for semiautomated detection of cytokeratin (CK)-positive stained cells. The sensitivity of this approach was demonstrated for 20 patients with stage II colorectal cancer and compared with RT-PCR regarding the detection of clinically assessed recurrence of disease within 10 years.

Results: CK+ cells were detected in all of the patients ($n = 6$; 100%) with recurrence, compared with five patients (83%) found positive by carcinoembryonic antigen RT-PCR. From patients ($n = 14$) who did not develop a recurrence, eight (57%) had positive lymph nodes. In all patients with recurrence, we visually identified at least one group of CK+ cells (>2 cells).

Conclusions: Automated image analysis is a promising tool for the detection of occult cells in histopathologically negative nodes. It is potentially more sensitive but less specific for detecting recurrence of disease than conventional histopathology or RT-PCR and is particularly useful for the evaluation of sentinel nodes. Furthermore, it opens new ways for basic research of occult cells based on molecular profiling after laser-microdissection.

INTRODUCTION

The presence of lymph node metastasis is one of the most important prognostic factors and therapeutic selectors for many types of cancer. Despite the prognostic value of lymph node status as assessed by conventional histopathology, a significant percentage of patients with node-negative colorectal carcinoma (diagnosed as lymph node negative by conventional examination) develop recurrence of disease.¹⁻³ Reasons for such exceptions are the biology of the tumor but also the limited sensitivity of conventional histopathology to detect rarely occurring occult cells in lymph nodes. For practical reasons only a few hematoxylin and eosin (HE) stained sections are examined, which limits the sensitivity of the technique to detect occult tumor cells.

Recently, alternative approaches to detect occult cells in lymph nodes have been described. Liefers *et al.*¹ examined lymph nodes from a group of 26 patients with stage II colorectal cancer, originally reported as negative by histopathology, using RT-PCR against carcinoembryonic antigen (CEA) messenger RNA. In their retrospective study, 14 of 26 patients were reported as positive for CEA. The 5-year recurrence-free survival for the CEA positive patients was 50% and for CEA negative patients, 91%. Others found similar results.^{2;3}

A different approach to increase sensitivity is by immunohistochemistry (IHC). This method relies on the analysis of IHC stained sections of lymph nodes for the detection of immunostained cells. It has been reported for patients with breast carcinoma that the application of IHC in combination with the analysis of multiple sections results in the detection of up to 35% more positive nodes as compared to conventional histopathology.⁴⁻¹² The clinical value however of this observation needs to be confirmed.

The sensitivity of the detection of occult cells has been shown to increase with the number of sections per lymph node examined up till a certain number of sections,¹³ but the practical applicability of multiple sectioning is limited by the labor intensive nature of preparing a large amount of IHC stained sections. For the analysis of sentinel nodes, however, multiple sectioning is highly recommended by the Association of Directors of Anatomic and Surgical Pathology (ADASP) and considered practically feasible.⁷

In this article, we describe an approach in which IHC staining and multiple sectioning is combined and subjected to novel high-throughput automated imaging. This imaging system uses a high performance flatbed scanner (FBSc), which is able to digitize, in one A3 format, hundreds of cytological or histological specimens. Digitally acquired images of the IHC stained tissue are then automatically analyzed for the presence of positive-stained occult cells. Storage of cell coordinates allows for direct morphological evaluation using conventional microscopy.

In this study, we have compared visual examination of cytokeratin-immunostained serial sections (as gold standard) with automated analysis and with the RT-PCR data from the referred Liefers article¹ with the emphasis on the sensitivity of the method to detect patients with recurrence of disease within a period of 10 years after a diagnosis of colorectal cancer tumor-node-metastasis (TNM) stage II.

PATIENTS AND METHODS

Patients

From 20 patients with TNM stage II colorectal cancer, lymph nodes were obtained consecutively from curative resections performed at the Department of Surgery of the Leiden University Medical Center (LUMC) between January 1990 and February 1992. From this material, originally studied using RT-PCR by Liefers *et al.*¹, 119 of 246 lymph nodes were still available. Twenty-one blocks showed poor material not suited for analysis (fat tissue, degenerated material, no histological material remaining).

Preoperative and perioperative examinations of the patients showed no evidence of metastatic disease. Follow-up was carried out in accordance with the department's protocol (Department of Surgery, LUMC) and was based on periodic evaluations of the patient.¹ The follow-up of the patients was at least 10 years and was updated by checking the patient files as of 1 February, 2002.

Sectioning and immunohistochemical staining

In the original study, one-half of the node was fixed in formalin and was embedded in paraffin for routine histopathological examination.¹ The other half of the resected node was used for RNA isolation for the analysis of CEA-specific mRNA using RT-PCR. For the present study, all of the available lymph nodes were analyzed for those patients who were originally PCR-positive for CEA. From the PCR-negative group for each patient, six lymph nodes were chosen randomly to match the average number of nodes in the positive group. From this material, serial sections (10 sections of 5 μ m in series at each level) were cut with intervals of 200 μ m until no material was left in the paraffin block. Sections were stained for cytokeratin (CK) using the antibody AE1/AE3.¹⁴ The sections were hydrated and subjected to sodium citrate (0.01 M, pH 6.0 at 100°C) for 10 min before incubation with the mixture of primary biotinylated monoclonal anti-cytokeratin antibodies AE1/AE3 (Dako, Glostrup, Denmark). Immunostaining was based on the avidin-biotin-peroxidase technique using 3,3'-diaminobenzidine (DAB) as the endpoint product; all of the sections were counterstained with hematoxylin.

Method of analysis of the slides

All of the IHC slides were first manually examined in a very thorough way. The results served as the gold standard for the automated analysis. A node was called positive when at least one IHC positive cell was found (excluding white blood cells, macrophages e.g. known for nonspecific staining) confirmed by a second person (a pathologist). Subsequently sections were recorded using the flatbed scanner. Automated analysis was performed on all of the recorded nodes. The location of all manually detected CK+ cells was marked on printouts of the recorded images and compared with the automated analysis.

Automated analysis

The system consists of a flatbed Agfa XY-15 scanner interfaced to a 933 MHz Power Mac G4 computer via a SCSI-2 interface. The optical resolution was 5000 dpi in both directions corresponding to a pixel distance of 5 μ m image acquisition. A special mold has been constructed to scan a maximum of 45 microscopic slides automatically (*Figure 1*). Digitization was performed using the ColorExact software package from Agfa. In addition to the system software, we designed dedicated image analysis software to analyze the bed-scan for the presence of microscopic slides within the mold and the lymph node sections on each slide.

The analysis of the lymph node sections was divided into the following steps:

the selection of a lymph node, the detection of candidate occult cells, and the measurement of cell features (area, shape, peak intensity, and averaged probe color). On the basis of these features, eventual falsely selected objects (i.e. other than CK+ cells) were recognized as such. The analysis of a lymph node section resulted in a gallery of images of the candidate occult CK+ cells found and an overview image of the lymph node with position markers where the events were found (*Figure 2*). On the basis of the gallery, the majority of falsely selected objects was easily recognized visually. When needed, the stored cell coordinates were used to relocate events by automated microscopy for visual interpretation at high spatial resolution (*Figure 3*). The total time necessary to automatically process a lymph node, which involved 80 slides with 5 sections per slide, was 81 minutes, of which 1 minute was required to make a full bed-scan at lower resolution and to determine the coordinates of the image crops for the scanning of the individual sections.

For the optimization of the selection algorithm of the automated analysis program, a positive node was analyzed. Parameters were set on the detection of groups and single CK+ cells. Recorded images were automatically analyzed, and the results were compared with those obtained by conventional microscopy. Using optimized selection criteria for automated analysis, we detected 34 (94%) of 36 visually recognized cells by automated analysis. These algorithms were used for the present study.

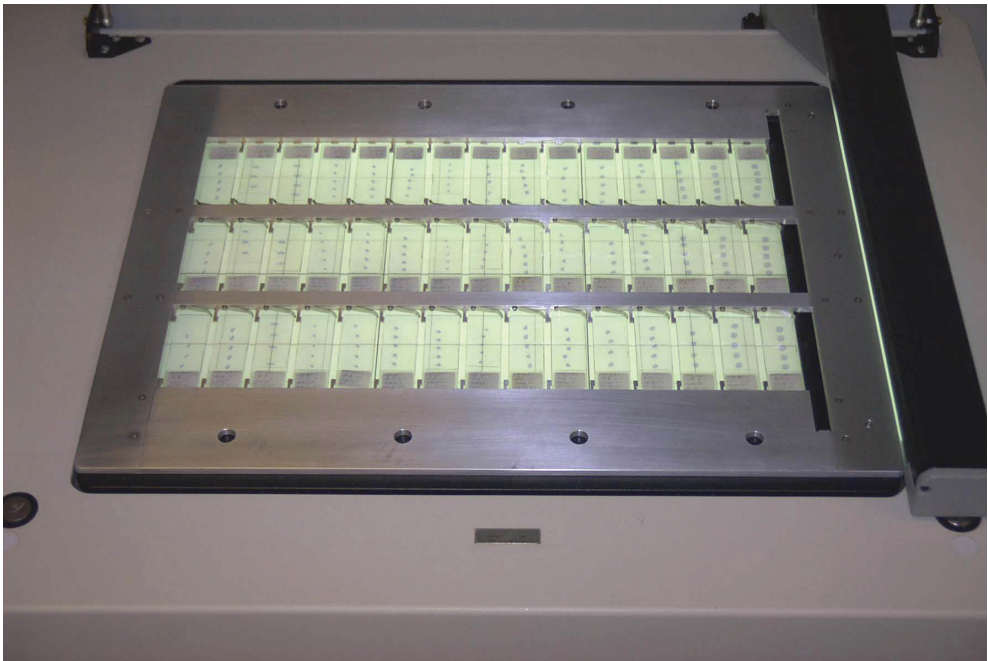


Figure 1. A3 size mould to hold $15 \times 3 = 45$ slides on the AGFA XY15 scanner. Each slide may contain several tissue sections.

Statistical analysis

We have reanalyzed the set of patients previously investigated by Liefers *et al.*¹ because of the availability of the material and the long-term follow-up (10 years). Obtained results using our method (IHC-automated analysis) were compared with the updated data of the RT-PCR study on the same set of patients. Carefully performed manual examination of the same slides served as the gold standard for this comparison. First, the automated analysis was compared with the manual evaluation. The data were described comparing patients with a recurrence of disease *versus* nonrecurrence, regarding IHC-automated analysis (*Table 1*) and RT-PCR (*Table 2*). Then, IHC was compared to RT-PCR by calculating the sensitivity and specificity (*Table 3*). Finally, we calculated the optimal distance for the sectioning of the paraffin material to detect all of the patients with a recurrence of disease (*Figure 4*).

RESULTS

IHC-automated analysis compared to conventional microscopy

A total of 119 lymph nodes (from 20 patients) were available; 33 were found positive and 65, negative, and 21 were not analyzed (see "Patients and Methods"). Per lymph node on average, 49 (range, 8-81) histological sections were analyzed. Comparing automated analysis with visual analysis using conventional microscopy revealed that, from a total of 33 visually evaluated positive nodes, two nodes were missed using automated analysis. Both nodes contained only one cell resulting in a sensitivity of 94% to detect a positive node. However, both patients had three more nodes in which occult cells were detected. Therefore, no positive patients were missed by automated analysis. One patient was missed with visual analysis but was found positive on visual verification of the candidate cells after automated analysis. This case illustrates the imperfectness of conventional screening compared with an automated performance of the image analysis procedure. *Table 4* presents the results of the lymph node analysis and the clinical outcome of the 20 patients.

IHC-automated analysis compared with RT-PCR

All of the patients ($n = 6$) who had developed a recurrence of disease were detected by IHC-automated analysis (*Table 1*). From 43 analyzed nodes in this patient group, 17 (40%) were found positive for CK-stained cells with on average eight cells per positive node. In all of these patients, groups of cells (number cells ≥ 2) were visually recognized.

From all other patients ($n = 14$) who did not develop recurrence of disease, 8 (57%) were found positive. In 16 (21%) of 77 nodes analyzed in this group, CK+ cells were found. Only five patients (36%) had cells located in groups. One patient (7 CK+ groups) died within 1 month after operation because of a gastric hemorrhage. One patient died from a cause other than disease, and three patients showed no evidence of disease 10 years after surgery.

Table 1. Number of automatically detected cytokeratin-positive (CK+) cells and groups in lymph nodes of patients with and without recurrence of disease

Disease status	Recurrence (n=6)	Non-recurrence (n=14)
Positive patients	6 (100%)	8 (57%)
Positive lymph nodes	17/43 (40%)	16/77 (21%)
Patients with CK+ cells	6 (100%)	8 (57%)
No of CK+ cells	81	40
Patients with CK+ groups ^a	6 (100%)	5 (36%)
No of CK+ groups ^a	31	12

^a "groups" is defined as ≥ 2 CK+ cells.

In three patients, no groups of cells were detected.

Using the RT-PCR method, we detected five (83%) patients with recurrence of disease. Forty-five nodes were analyzed in this group, of which 12 (27%) were found positive for CEA expression. Three patients (21%) with no recurrence of disease had positive lymph nodes (*Table 2*).

Table 2. Results of RT-PCR for CEA expression (same patients as shown in Table 4)^a

Disease status	Recurrence (n=6)	Non-recurrence (n=14)
Positive patients	5 (83%)	3 (21%)
Positive lymph nodes	12/45 (27%)	5/109 (5%)

CEA, carcinoembryonic antigen;^a Data from Liefers *et al.*¹

Sensitivity and specificity

Realizing that a relatively low number of patient samples has been investigated, we, nevertheless, calculated the sensitivity and specificity of both methods.

The sensitivity of IHC-automated analysis to detect CK+ nodes in patients with recurrence of disease was 100%; the specificity was 43%. The sensitivity and specificity of the RT-PCR method on the same set of patients studied by Liefers *et al.*¹ are, respectively, 83% and 79% (*Table 3*).

Table 3. Calculation of the sensitivity and specificity of IHC-automated analysis and RT-PCR with respect to the detection of patients with recurrence of disease

	Sensitivity	Specificity
IHC-automated analysis <i>cut-off level: 1 CK+ cell</i>	100%	43%
RT-PCR	83%	79%

Sampling distance of paraffin blocks

By reanalyzing the data, we calculated the effect of the distance of sectioning of the paraffin material with respect to successful detection of recurrence of disease. *Figure 4* shows the effect of varying the distance between sections with respect to

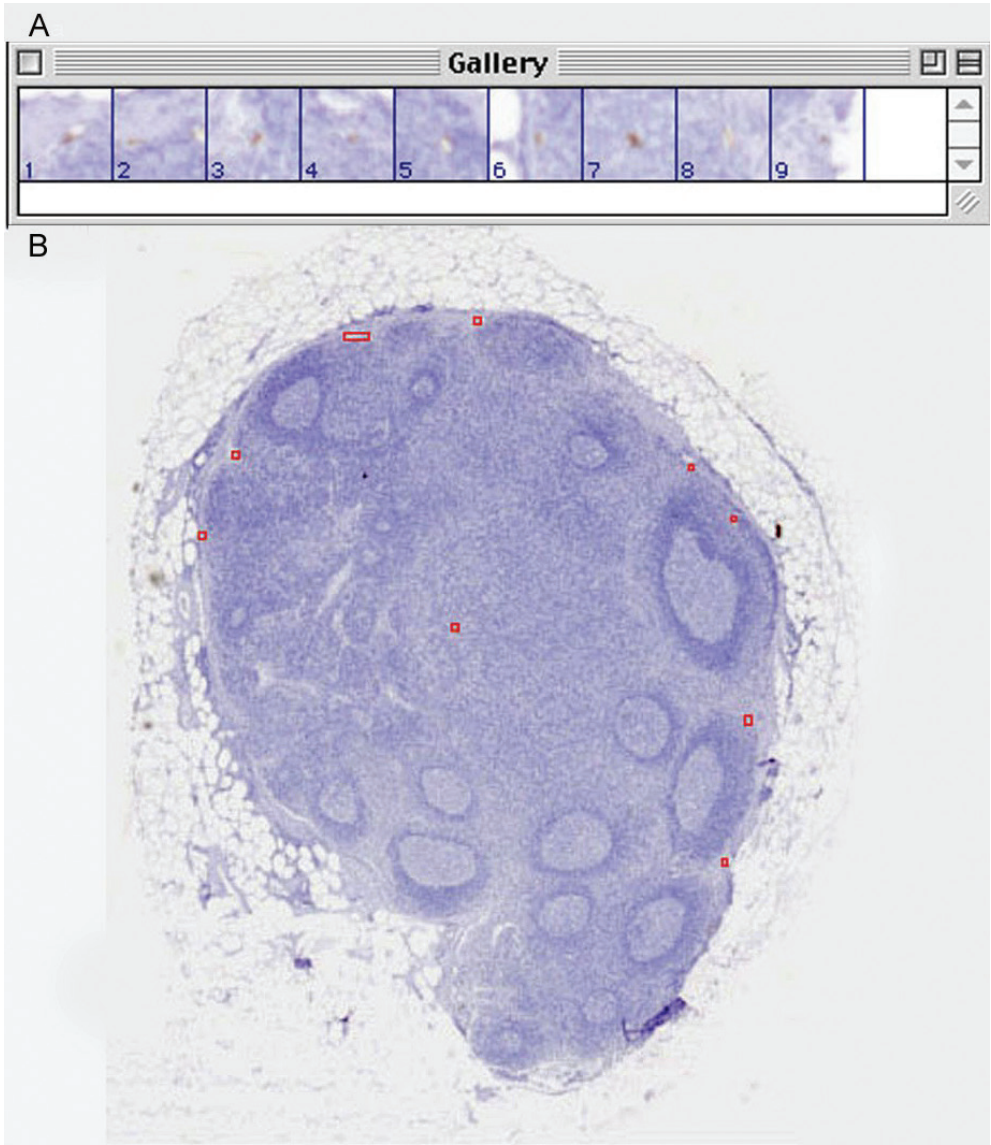


Figure 2. A. Gallery with images of candidate occult cells detected in a lymph node section of a patient with colorectal carcinoma. Selection of candidate cells is based on immunoperoxidase (**brown**) staining for cytokeratin. Counterstaining is performed with hematoxylin (**blue**). **B.** Overview of the lymph node with markers displaying the location of the candidate cells.

the detection of positive lymph nodes (“positive” was defined here as containing at least one CK+ cell). Additionally, the percentage of lymph nodes is shown in which at least one group of cells (≥ 2 CK+ cells) was found. The sensitivity of both the visual and automated detection decreases from 100% and 93% to, respectively, 98% and 85% when one-half of the number of sections is analyzed.

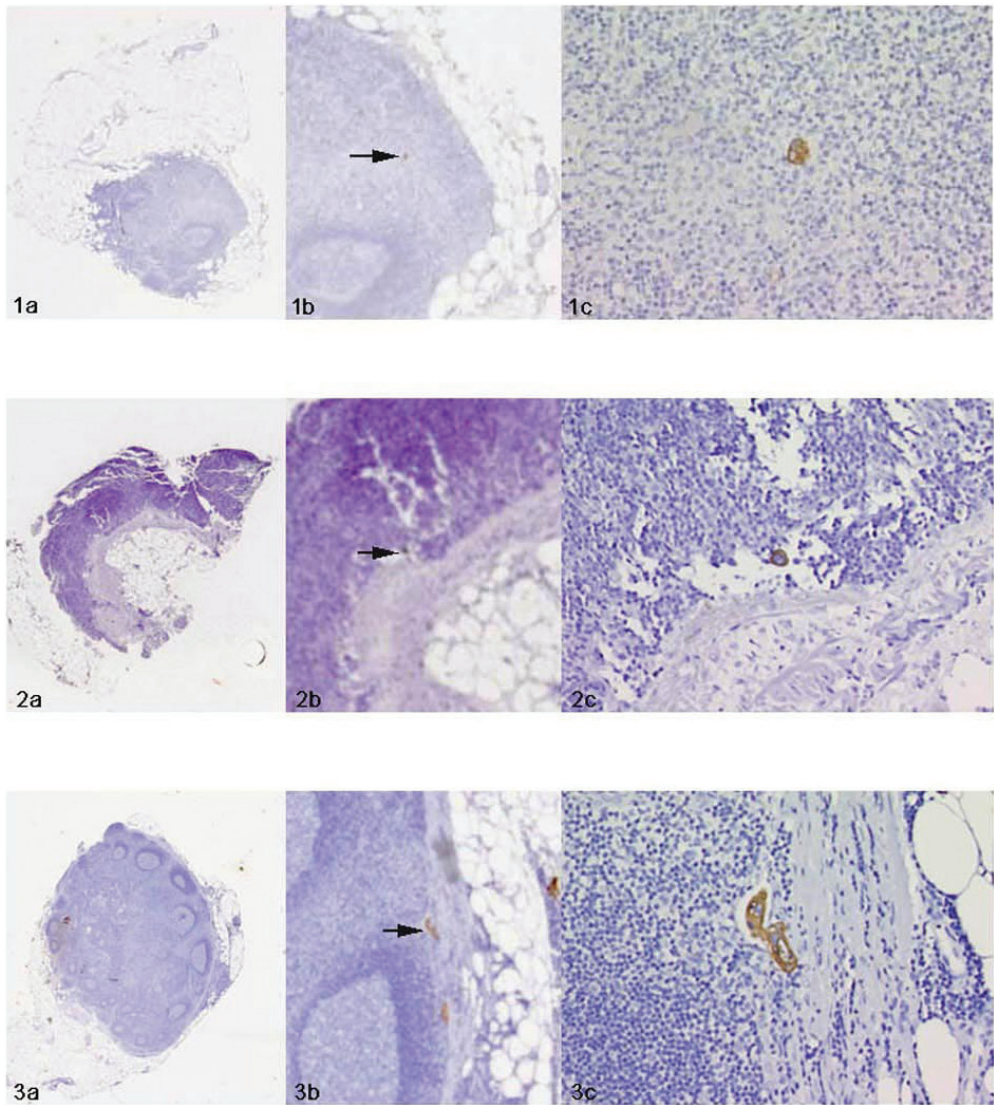


Figure 3. A. Examples of images recorded by the flatbed scanner at 5600 dpi. Sections of lymph nodes from patients with colorectal carcinoma. Cells are positively stained for cytokeratin and counterstained using hematoxylin. **B.** Zoom function of recorded image; arrow marks single cytokeratin-positive cells (**1b**, **2b**) or a small group of cytokeratin-positive cells (**3b**). **C.** Conventional microscope image of the same cells. Recorded with color CCD camera using x16 objective.

One CK+ lymph node containing one group (2 cells) was missed when only one in every two sections was analyzed. When the detection of at least one CK+ group of cells was used as the criterion, sensitivity decreased from 100% to 90% and from 93% to 73% for automated and visual inspection, respectively.

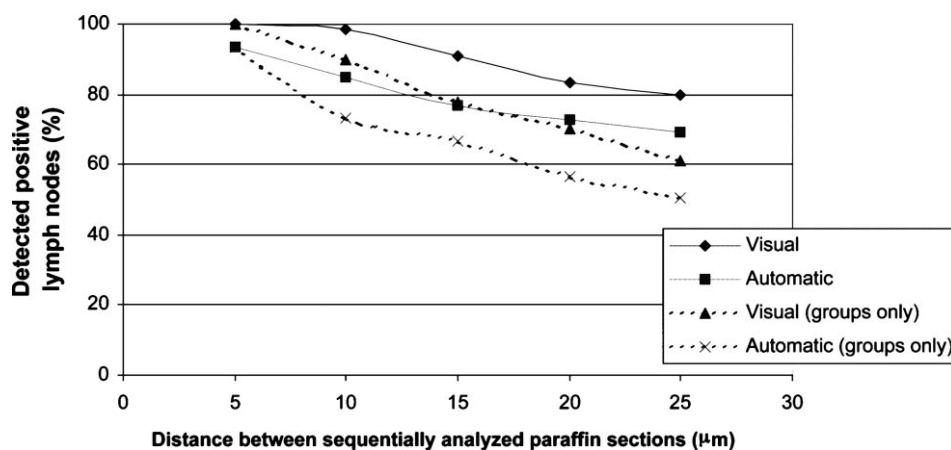


Figure 4. Percentage of detected lymph nodes as function of the distance between sequential sections for both visual and automated detection based either on cytokeratin-positive cells or on positive groups of cells only. A lymph node was classified as positive when at least one verified cytokeratin-positive cell (or a group: ≥ 2 cells) was detected.

DISCUSSION

This study shows that visual examination of multiple IHC stained sections for the presence of occult tumor cells can be automated by low-resolution image analysis. As such, this method may serve as a useful alternative for RT-PCR, particularly when large numbers of sections are analyzed (at least 50 per node for this study). In all of the patients ($n = 6$) with recurrence of disease, IHC positive cells were detected using this method, demonstrating its feasibility for this application. Compared with the RT-PCR study previously performed by Liefers *et al.*¹, automated analysis of IHC stained sections appeared to be more sensitive in detecting patients with recurrence of disease (100% vs 83%). We also found more positive nodes in this group (40% vs 27%). However, in 57% of the patients who did not develop a recurrence of disease, positive cells were also found, compared with 21% for the RT-PCR method. When the cut-off level was increased from one cell to two CK+ cells, the specificity increased from 43% to 57%. This makes automated analysis more sensitive but less specific than the referred RT-PCR method and, therefore, requires additional analysis of the detected positive cells.

The number of analyzed patient samples, however, is considered too small to conclude that the differences are significant and meaningful. Discrepancies with the RT-PCR results may be attributable to unavoidable sampling errors. Yasuda *et al.*¹⁵ analyzed six serial sections using IHC and detected micrometastases in 92% of patients with recurrence but also found a high percentage of positive patients in the nonrecurrent group (70%). When more specific markers than pan-cytokeratin are available as published by Izbicki *et al.*¹⁶ for esophageal cancer (BerEp4), the high rate of false positive findings can possibly be reduced, thereby increasing the specificity of the current assay.

It is estimated that routine HE analysis only has a 1% chance of identifying a focus of cancer cells less than three cells in diameter.¹⁷ This level of sensitivity implies the finding that about 25% of patients with colorectal cancers who are node negative by routine HE examination may develop distant metastases.¹⁻⁴ The histopathological criteria for occult metastases are far from clear. Most of the studies identifying occult tumor cells have been performed in breast cancer. For instance, Turner *et al.*¹⁸ and Kell *et al.*¹⁹ report that, for patients with breast cancer with minimal axillary involvement, the presence of efferent vascular invasion or nodal hilar tissue invasion and the location of a micrometastasis in sinusoidal rather than parenchymal tissue may indicate a less favorable prognosis. Others have suggested the inclusion of the size of the metastasis, groups *versus* single cells, and the microanatomical location of occult cells as prognostic features.²⁰ However, evaluation of the potential value of these parameters has not been systematically pursued.

Table 4. Detection of micrometastasis and outcomes of patients with stage II colorectal cancer

Patient no	Lymph nodes		Outcome	
	Examined	Positive	Vital status	Disease status
1	4	3	Dead	Recurrence ^a
2	10	1	Dead	Recurrence
3	2	1	Dead	Recurrence
4	3	2	Dead	Recurrence
5	17	4	Dead	Recurrence
6	7	6	Dead	Recurrence
7	3	0	Dead	Other ^b
8	6	0	Dead	Other
9	5	0	Dead	Other
10	4	2	Dead	Other
11	6	3	Dead	Other
12	6	0	Dead	Other
13	5	0	Alive	NED ^c
14	10	4	Alive	NED
15	6	0	Alive	NED
16	4	1	Alive	NED
17	10	2	Alive	NED
18	4	2	Alive	NED
19	6	1	Alive	NED
20	1	0	Alive	NED

^a Recurrence death from local or distant recurrent disease; ^b Other death from a cause other than cancer; ^c NED, no evidence of disease.

Although RT-PCR methods have been investigated to improve sensitivity, specific markers still do not exist for many tumors. RT-PCR is proven to be very sensitive and able to detect 1 in 10 million cells but is often prone to false positive classification because of contamination, and illegitimate low-level expression of marker transcripts in normal lymph nodes has also been reported.²¹ Furthermore, PCR has the restriction that detected events cannot be morphologically evaluated and confirmed. Microscope-based analysis has the advantage of enabling morphological analysis of the detected occult cells by the pathologist. The use of monoclonal antibodies to further characterize the detected cells (either by bright-field or fluorescence microscopy) may be useful.²² It is evident that the number of positive nodes will increase on analysis of more sections, and may strongly increase.²³ Automated analysis as described here may be further improved by connection of the scanner to an off-line automated microscope for rapid relocation of the detected events and by fine tuning of the cell classification algorithm with respect to the accuracy. The analysis is rather time consuming, and speed can be increased but is, when focusing on sentinel nodes, not a prerequisite. For use in a clinical setting, the sectioning and staining of all resected lymph nodes is too labor intensive. However, for the analysis of sentinel nodes, which most of the time involves only one to three lymph nodes, serial sectioning is highly recommended by the ADASP and is practically feasible.⁷

The clinical significance of immunohistochemically detected tumor cells present in excised lymph nodes in case of colorectal cancer remains unclear. In a recent multi-institutional study of 736 patients with breast cancer, the presence of immunohistochemically detected occult cells in axillary lymph node metastases was found to be significant and, in case of postmenopausal women, was an independent predictor of overall survival. For colorectal cancer, this reaffirms the need for larger studies with longer follow-up.⁴

The relatively large data set (4569 sections of 119 nodes of 20 patients) of the presented study allowed to examine the effects of the sectioning density on the detection of CK+ cells in a particular lymph node. It appeared that the number of CK+ cells decreased inversely proportional to the sampling distance (data not shown). This relation suggests that the CK+ cells are more or less randomly distributed throughout the node. Because the presence of CK+ cells was rather low in a number of CK+ lymph nodes, a large proportion of the lymph node has to be analyzed to classify the node as positive. As can be seen in *Figure 4*, one CK+ lymph node containing only two CK+ cells (group) was missed (even visually), when only one in every two sections was analyzed. When the detection efficiency of lymph nodes with at least one CK+ group is considered, one would expect that detection of groups, being larger than single cells, would be less dependent on the sectioning distance. *Figure 4* shows the opposite, however. This can be explained by the low frequency of groups present in most of the lymph nodes when compared with the number of single CK+ cells and by the fact that most of those groups consisted of only 2 to 3 cells.

To better understand the biology of metastasis, research is needed to further characterize the detected cells, which may ultimately lead to an increase in specificity and diagnostic accuracy. Such information can be obtained by physical isolation of these cells by laser microdissection followed by single-cell RT-PCR and analysis of gene composition.²⁴

Such research has recently been published by Klein *et al.*²⁵, who found a different genetic make-up for single cells *versus* groups of cells. The clinical importance of these findings, however, is not yet known.

This information can be used to produce specific markers for diagnostic assays that may ultimately allow the identification of biologically important populations of cells that can be directly linked to clinical outcome.

The present study was performed on a well-documented selection of colorectal cancer patients and served as a model. It is obvious that a similar approach is also indicated for breast cancer or for melanomas in which lymph node involvement and, particularly, the role of the sentinel node is an important focus of research.^{8;9;26-31}

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CHAPTER FIVE

DETAILED EXAMINATION OF LYMPH NODES IMPROVES PROGNOSTICATION IN COLORECTAL CANCER

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ABSTRACT

Background: Up to 30 percent of stage II patients with curatively resected colorectal cancer (CRC) will develop disease recurrence. We evaluated whether examination of lymph nodes by multilevel sectioning and immunohistochemical staining can improve prognostication.

Patients and methods: Lymph nodes ($n = 780$) from 36 CRC patients who had developed disease recurrence (cases) and 72 patients who showed no recurrence of disease for at least 5 years (controls) were analyzed. Sections of four levels at 200 μm interval were immunohistochemically stained for cytokeratin expression. The first level was analyzed by conventional and automated microscopy and the three following levels were analyzed by automated microscopy for the presence of tumor cells.

Results: Overall, cases showed more micrometastases (three patients) than controls (one patient). Analysis of a second level led to the additional detection of one patient with micrometastases (case) and one patient with macrometastasis (case). Examining more levels only led to additional isolated tumor cells which were equally divided between cases and controls. Likewise, automated microscopy resulted only in detection of additional isolated tumor cells when compared to conventional microscopy. In multivariate analysis, micrometastases (OR 26.3, 95% CI 1.9-364.8, $P = 0.015$), T4 stage (OR 4.8, 95% CI 1.4-16.7, $P = 0.013$) and number of lymph nodes (OR 0.9, 95% CI 0.8-1.0, $P = 0.028$) were independent predictors for disease recurrence.

Conclusions: Lymph node analysis of two levels and immunohistochemical staining add to the detection of macrometastases and micrometastases in CRC. Micrometastases were found to be an independent predictor of disease recurrence. Isolated tumor cells were of no prognostic significance.

INTRODUCTION

The presence of lymph node metastases is one of the most important prognostic factors in colorectal cancer (CRC) for which adjuvant systemic chemotherapy is generally recommended.¹⁻⁴ Patients with curatively resected stage I and II CRC without nodal tumor involvement do not receive adjuvant systemic therapy since only small improvements in survival have been shown.⁴⁻⁶ However, ten to 30 percent of these node-negative patients will develop locoregional recurrence or distant metastases.^{1;7;8} Adjuvant systemic treatment of all node-negative CRC patients is not recommended by the ASCO (American Society of Clinical Oncology) as it would lead to overtreatment and unnecessary complications.⁹

Identification of node-negative CRC patients with a high risk of disease recurrence may lead to a more appropriate selection for adjuvant treatment.

Conventional histopathology has a limited sensitivity to detect occult tumor cells in lymph nodes, described as micrometastases (> 0.2 mm and ≤ 2 mm) and isolated tumor cells (≤ 0.2 mm or single tumor cells).¹⁰⁻¹² Alternative approaches to detect occult tumor cells in lymph nodes have been reported such as: polymerase chain reaction¹³, reverse transcriptase polymerase chain reaction (RT-PCR)¹⁴, multilevel sectioning¹⁵, immunohistochemical staining¹⁶ and automated microscopy.¹⁷ In a study published by Liefers *et al.*¹⁴ was shown that stage II patients with carcinoembryonic (CEA) RT-PCR negative lymph nodes have a significantly better five-year disease-related survival than patients with CEA RT-PCR positive lymph nodes (91 vs 50%, $P = 0.02$). These results were confirmed by several other groups.¹⁸⁻²² The detection of immunohistochemically stained tumor cells has the advantage of visual examination of the detected cells using microscopy. Multilevel sectioning and immunohistochemistry have been shown to increase the detection rate of lymph node metastases in CRC patients.¹³ Since screening of multiple immunohistochemically stained sections is time consuming and difficult to reproduce, automated microscopy has been implemented.^{23;24}

In this case-control study, we evaluated whether detailed examination of lymph nodes for the presence of occult tumor cells by multilevel sectioning and immunohistochemical staining can improve prognostication in CRC.

PATIENTS AND METHODS

Patient selection

Between January 1981 and December 2001, 1044 patients underwent surgery for a primary CRC at the Leiden University Medical Center. For the present study, a selection was made for patients with negative lymph nodes (N0) and no metastases (M0) at the time of surgery ($n = 506$). Patients who were operated on their first CRC in another hospital, or who were diagnosed with another invasive malignancy before or within five years after the date of diagnosis of the primary colorectal carcinoma, and patients who developed a local recurrence were excluded for the present study. The latter group was excluded to rule out the factor of inadequate surgery. Cases ($n = 40$) were defined as patients who had regional or distant recurrent disease at least three months after but within five years after the date of diagnosis of primary CRC. Regional metastases were considered intra-abdominal or intrapelvic metastases in lymph nodes or in connective tissue. Thirty-six cases could be included in this study because lymph nodes from four cases could not be retrieved from the archive. Controls ($n = 189$) were patients who did not develop locoregional or distant disease within five years after diagnosis of primary CRC. For each case two controls were matched for Tumor-Node-Metastasis (TNM) stage, date of incidence and date of birth, leading to a total number of 72 controls. The median follow-up of the case group was 2.5 years (range 5.3 months – 6.3 years)

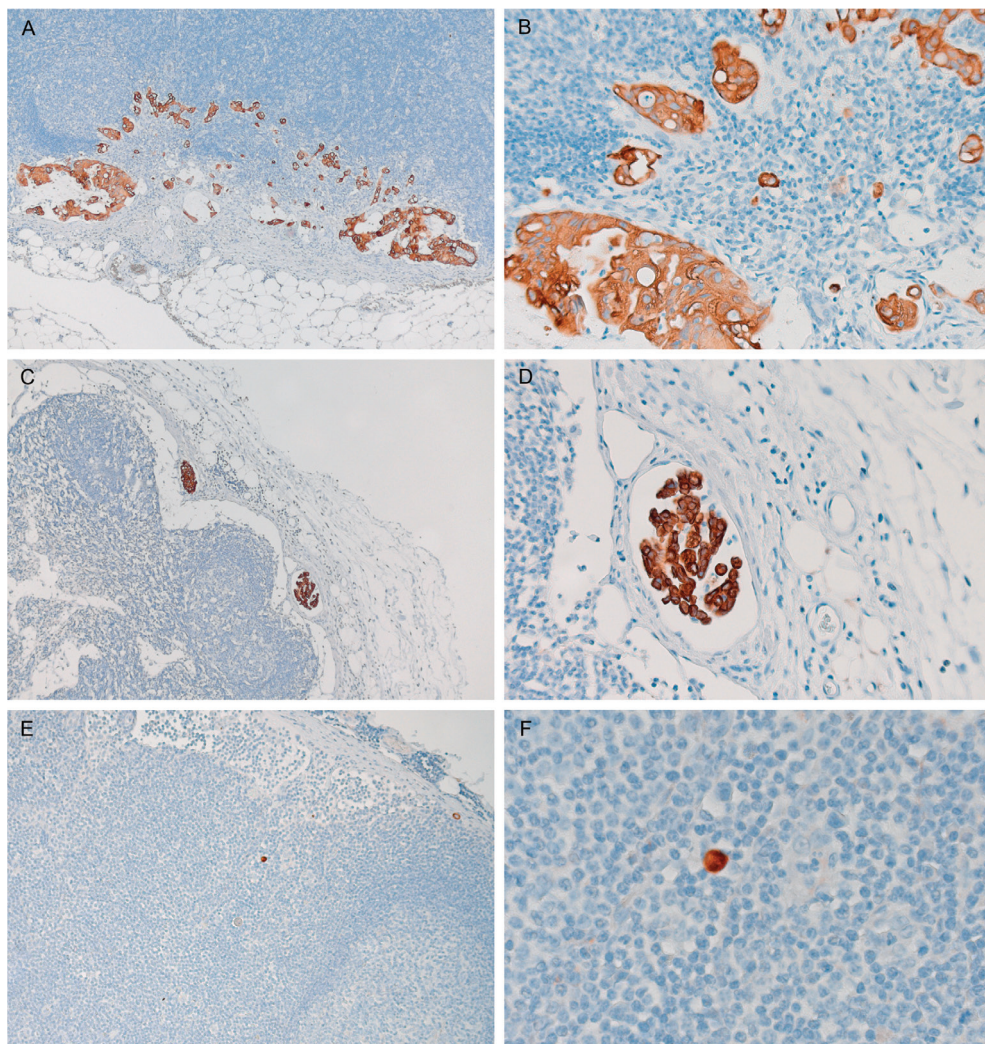


Figure 1. Lymph node sections stained for cytokeratin by AE1/AE3 antibodies resulting in brown cells and counterstained using hematoxylin and eosin. Micrometastases (> 0.2 mm and ≤ 2 mm) at original magnification of x125 (A) and x250 (B); Isolated tumor cells (tumor cell clusters ≤ 0.2 mm) at original magnification of x125 (C) and x500 (D); Isolated tumor cells (single cells) at original magnification of x250 (E) and x1000 (F).

and the median follow-up of the control patient group was 10.8 years (range 5.1 – 21.4 years). Out of 19 patients with rectal carcinoma, two patients had received preoperative radiotherapy and three patients had received postoperative radiotherapy. None of the patients had received adjuvant chemotherapy.

Tissue specimens

After resection the lymph nodes were fixed in formalin, processed through

graded ethanol, and embedded in paraffin as part of a routine procedure for histopathological investigation. From 108 patients, a total of 780 lymph nodes was harvested (median 6; range 1 to 26 lymph nodes). The lymph nodes were embedded in a total of 225 paraffin blocks.

Immunohistochemistry

For detection of tumor cells, a cocktail of murine monoclonal antibodies AE1 and AE3 (Dako, Denmark), was used. AE1 recognizes cytokeratin 10, 13, 14, 15, 16, and 19 and AE3 recognizes cytokeratin 1, 2, 3, 4, 5, 6, 7 and 8. Four micron sections were cut at four levels of each paraffin block with intervals of 200 μ m. The sections were situated on aminopropylethoxysilane coated slides and dried overnight at 37°C. The sections were subsequently deparaffinized in xylene, rehydrated and blocked for endogenous peroxidase in 0.3% hydrogen peroxide/methanol at room temperature for 20 minutes. After washing in phosphate buffered saline (PBS), antigen retrieval treatment was performed by incubating the sections in a 0.01 M sodium citrate solution (pH 6.0) for 10 minutes at 100°C. Then slides were rinsed twice in PBS and the primary antibody AE1/AE3 was applied at a 1:200 dilution in PBS with 1% bovine serum albumine, respectively. The sections were incubated overnight at room temperature, washed with PBS and incubated for 30 minutes with Envision- horseradish peroxidase (Dako, Denmark). After three PBS washes and one rinse in 0.05 M Tris/HCl (pH 7.6), visualization of cytokeratin was achieved by incubation for 10 minutes with 3,3'-diaminobenzidinetetrachloride substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide. Sections were counterstained with Mayer's Hematoxylin and dehydrated in graded ethanol followed by xylene and mounted in glycerol. Cytokeratin-positive cells showed a brown staining of the cytoplasm.

Additionally, we intended to analyze the 200 μ m tissue of paraffin-embedded lymph nodes between the sections used for immunohistochemical staining by using RT-PCR. However, this was not feasible since yield and quality of extracted RNA was insufficient (data not shown).

Analysis of the slides

All sections were analyzed using the ARIOL system® (Applied Imaging a Genetix company). The features of the ARIOL system® have been published previously.²⁵ Sections of the first level were also analyzed by a pathologist (AML) using conventional microscopy at a total magnification of 100 times. Selected candidate tumor cells were verified by the operator and also visually confirmed by an independent pathologist (HM). Nonspecifically stained cells such as hematopoietic cells were visually recognized and excluded from the analysis. Macrometastases were defined as groups of cells larger than 2 mm. Deposits of tumor cells of 2 mm or less but larger than 0.2 mm were considered as micrometastases and single tumor cells or clusters of tumor cells of 0.2 mm or less were classified as isolated tumor cells.¹⁰⁻¹²

Statistical analysis

Statistical analysis was carried out using SPSS software, version 12.0.1 (SPSS Inc, Chicago, IL). Numerical data are presented as mean $\pm$ standard deviation or median and range in case of skewness. The clinicopathologic features of cases and controls were compared either by a Chi-square or Fisher's exact for categorical variables or by a Mann-Whitney or student T-test for numerical variables. Differences between screening results by automated microscopy and conventional microscopy were analyzed by using the McNemar's test. Univariate and multivariate odds ratio's (OR), 95% confidence intervals (95% CI) and p-values (P) were calculated by applying logistic regression analysis. A p-value of less than 0.05 was considered an indication of statistical significance. Variables with a p-value lower than 0.10 in the univariate analysis were entered in the multivariate analysis. Since the number of examined lymph nodes has been shown to be prognostically relevant in several studies²⁶, this variable was also entered in the multivariate analysis.

RESULTS

Clinicopathological features

Clinicopathologic characteristics of the patients are shown in *Table 1*. No significant differences in sex, T stage, tumor size, tumor location, tumor differentiation, mucinous tumors, serum CEA level and harvested lymph nodes (Mann-Whitney test, median 6 (range 1 to 18) vs median 6 (range 1 to 26), $P = 0.096$) were seen between the case and control group. If T2 and T3 stage were combined, more cases than controls were staged as T4 than T2/3 (Fisher's exact test, 8 of 36 (22%) vs 6 of 72 (8%), $P = 0.066$). Except for T stage (T4 vs T2/T3: OR 3.1, 95% CI 1.0-9.9, $P = 0.050$), the single variable regression analysis for disease recurrence of clinicopathological parameters (*Table 1*) did not show any other significant risk factors. The single variable regression analysis for the number of lymph nodes showed the following: OR 0.9, 95% CI 0.9-1.0, $P = 0.132$.

Multilevel sectioning, immunohistochemical staining and automated microscopy

Analysis of the first lymph node level identified micrometastases in three (3%) patients (two cases and one control) and isolated tumor cells in 39 (37%) patients (13 cases and 26 controls) (*Table 2*). One micrometastasis found in an immunohistochemically stained section was also present on the original hematoxylin and eosin (HE) stained slide but was not recognized by the pathologist. Macrometastases in three (3%) patients (two cases and one control) that had not been recognized as lymph node metastases on the original HE stained slides were also seen. They had been described as vascular invasion or tumor deposits without lymphoid tissue in the pathology reports. On the immunohistochemically stained slides of all levels, lymphoid tissue was present around the tumor cells. These macrometastases were excluded from further analysis.

All macrometastases and micrometastases were detected by both conventional and automated microscopy. Automated microscopy led to the detection of additional isolated tumor cells (McNemar's test, 39 vs 10 patients, respectively, $P < 0.001$)

Table 1. Patient and primary tumor characteristics (n=108)

Characteristics	Cases (n=36)		Controls (n=72)		P ^a
	n	%	n	%	
Sex					
Female	16	44	37	51	.496
Male	20	56	35	49	
Age (years) ^b	67 ± 12		67 ± 12		.911 ^c
TNM stage ^d					
I	2	6	4	6	1.000 ^e
II	34	94	68	94	
T stage ^f					
T2	2	6	4	6	.126
T3	26	72	62	86	
T4	8	22	6	8	
Tumor size (cm) (n=104) ^{b, g}	4.9 ± 1.9 (n=34)		5.2 ± 2.1 (n=70)		.539 ^c
Tumor location					
Colon (coecum – sigmoid)	30	83	59	82	.858
Rectum (rectosigmoid – rectum)	6	17	13	18	
Differentiation					
Good	9	25	18	25	.912
Moderate	22	61	46	64	
Poor	5	14	8	11	
Mucinous ^h					
No	32	89	66	92	.728 ^e
Yes	4	11	6	8	
Preoperative serum CEA level (n=43) ^j					
< 6 µg/l	5	56	25	74	.417 ^e
≥ 6 µg/l	4	44	9	26	
Number of examined lymph nodes ^b	6.1 ± 4.5		7.8 ± 5.7		.096 ^k

CEA, carcinoembryonic antigen; ^a Chi-square test of cases vs controls, unless mentioned otherwise; ^b Presented as mean ± standard deviation; ^c Student T-test; ^d According to the 6th edition of the TNM classification¹¹; ^e Fisher's exact test; ^f If T2 and T3 stage were combined, the P value was 0.043; this comparison was therefore used in the logistic regression; ^g Tumor size could not be found in pathology reports from four patients; ^h A tumor was considered mucinous when more than 50% of its volume existed of mucinous component; ^j Serum CEA had been determined in only 43 of 108 patients as it was not a standard procedure; ^k Mann-Whitney test.

(Table 3). All but one isolated tumor cell found by conventional microscopy was also detected by automated microscopy. The missed isolated tumor cell was overlooked during visual inspection of ARIOL system results by the operator.

Analysis of a second level, resulted in additional detection of one patient with macrometastasis (a case), one patient with micrometastases (a case) and nine patients with isolated tumor cells (two cases and seven controls) (Table 2). These patients had no tumor cells detected in the first level nor in the original HE stained slide.

When analyzing a third level, eleven patients (three cases and eight controls) were additionally identified with isolated tumor cells and assessment of a fourth level, identified two patients (two controls) with isolated tumor cells. This led to a total number of 61 patients with isolated tumor cells (Table 2). No additional macrometastases or micrometastases were found when analyzing a third and fourth level. In Figure 1 examples of micrometastases (1A, B) and isolated tumor cells (1C-F) are shown.

Concluding, after analysis of two lymph node levels, macrometastases were observed in one patient (one case) and micrometastases in four patients (three cases and one control). Analysis of two additional lymph node levels solely identified the presence of isolated tumor cells.

Table 2. Detection of tumor cells in paraffin-embedded lymph nodes from colorectal cancer patients (n=105) after multilevel sectioning^a and immunohistochemical staining combined with automated microscopy^b

	One level		Two levels		Three levels		Four levels	
A. Lymph node status (n=772)	n	%	n	%	n	%	n	%
Macrometastases	-	-	1	0.1	1	0.1	1	0.1
Micrometastases	6	0.8	8	1.0	8	1.0	8	1.0
Isolated tumor cells	99	12.8	126	16.3	153	19.8	172	22.3
B. Patient status (n=105)	n	%	n	%	n	%	n	%
Macrometastases	-	-	1	1.0	1	1.0	1	1.0
Micrometastases	3	2.9	4	3.8	4	3.8	4	3.8
Isolated tumor cells	39	37.1	48	45.7	59	56.2	61	58.1

^a Four micron sections were cut at four levels of each paraffin block with intervals of 200 µm; ^b Patients with macrometastases in the first lymph node level that had previously not been recognized as such, were excluded.

Prognostic significance of lymph node metastases

More patients with micrometastases were seen in the case group than in the control group (Fisher's exact test, three of 34 (9%) vs one of 71 (1%), $P = 0.099$). There was no difference in the presence of lymph nodes harboring isolated tumor cells, between the case and control group (Chi-square test, 18 of 34 (53%) vs 43 of 71 (61%), $P = 0.459$) (Table 4). Separate analysis of patients with colon

Table 3. Detection of tumor cells in lymph nodes from colorectal cancer patients after immunohistochemical staining of the *first lymph node level*: conventional microscopy *versus* automated microscopy^a

	Conventional microscopy		Automated microscopy		Combined microscopy	
A. Lymph node status (n=772)	n	%	n	%	n	%
Macrometastases	-	-	-	-	-	-
Micrometastases	6	0.8	6	0.8	6	0.8
Isolated tumor cells	15	1.9	99	12.8	100 ^b	13.0
B. Patient status (n=105)	n	%	n	%	n	%
Macrometastases	-	-	-	-	-	-
Micrometastases	3	2.9	3	2.9	3	2.9
Isolated tumor cells	10	9.5	39	37.1	39	37.1

^a Patients with macrometastases in the first lymph node level that had previously not been recognized as such, were excluded; ^b One lymph node with isolated tumor cells was missed by ARIOL automated microscopy.

carcinoma, rectal carcinoma, right-sided or left-sided carcinoma, did not result in significant difference in the presence of isolated tumor cells or percentage of lymph nodes harboring isolated tumor cells for the case and control group. Neither did excluding patients with T4 tumors or disregarding single tumor cells as isolated tumor cells.

From the clinicopathological variables, the T stage and the number of examined lymph nodes were entered in the multivariate logistic regression analysis with the presence of micrometastases. These results showed that the presence of micrometastases (OR 26.3, 95% CI 1.9-364.8, $P = 0.015$), a T4 stage (OR 4.8, 95% CI 1.4-16.7, $P = 0.013$) and the number of harvested lymph nodes (OR 0.9, 95% CI 0.8-1.0, $P = 0.028$) were independent predictors for disease recurrence. When including the patient with macrometastasis detected after analysis of a second level, the OR changed in favour of the presence of macrometastases or micrometastases in lymph nodes, showing the presence of macrometastases or micrometastases (OR 34.5, 95% CI 2.7-440, $P = 0.006$), a T4 stage (OR 2.9, 95% CI 0.4-23.5, $P = 0.040$) and the number of examined lymph nodes (OR 0.9, 95% CI 0.8-1.0, $P = 0.025$) to be independent predictors for disease recurrence.

Relation between lymph node metastases and clinicopathological features

There was no correlation observed for the presence of micrometastases in lymph nodes and patient's gender, tumor location, tumor size, T-stage, TNM stage and serum CEA level (all P values > 0.05). In rectal carcinoma fewer lymph nodes were harvested than in colon carcinoma (Mann-Whitney test, median 3, range 1 to 13 vs median 7, range 1 to 26, $P = 0.037$). Also patients with less than 12 harvested lymph nodes were significantly older than patients with 12 or more harvested lymph nodes (Student T-test, 69 ± 10 vs 61 ± 15 , $P = 0.008$). No difference was seen in the number of lymph nodes harvested for the different T stages.

Table 4. Clinical relevance of occult tumor cells in lymph nodes from patients with colorectal cancer (n=105)^a

Lymph node status	Cases (n=34)		Controls (n=71)		P ^b
	n	%	n	%	
Tumor-positive	22	65	44	62	.786
Macrometastases	1	3	0	0	.324 ^c
Micrometastases	3	9	1	1	.099 ^c
Isolated tumor cells	18	53	43	61	.459

Cases were defined as patients who had regional or distant disease recurrence within five years after the date of diagnosis of primary colorectal cancer; Controls were patients who did not develop locoregional or distant disease within five years after diagnosis of primary colorectal cancer; ^a Patients with macrometastases in the first lymph node level that had previously not been recognized as such, were excluded; ^b Chi-square test of cases vs controls, unless mentioned otherwise; ^c Fisher's exact test.

DISCUSSION

In this study, we analyzed paraffin-embedded lymph nodes from patients with CRC by using multilevel sectioning combined with immunohistochemical staining and demonstrated that the presence of micrometastases was an independent predictor for disease recurrence. Analysis of a second lymph node level led to detection of additional prognostically relevant macrometastases and micrometastases. Examination of more levels and the use of automated microscopy led to detection of additional isolated tumor cells which were prognostically not relevant.

We performed this study because the prognostic significance of occult tumor cells in lymph nodes in CRC is still a matter of debate. A meta-analysis showed that immunohistochemical detection of occult tumor cells combined with serial or multilevel sectioning led to upstaging of 32% of previously considered node-negative patients but the presence of occult tumor cells did not lead to a statistically significant adverse clinical outcome.²⁷ The meta-analysis reported an upstaging of 37% by RT-PCR and in contrast to immunohistochemistry studies, most RT-PCR studies found the presence of occult tumor cells in lymph nodes to predict a worse clinical outcome (overall three-year survival of 78% vs 97%, $P < 0.001$).^{13;14;18-22;27} However, although several alternative techniques are evaluated for improving RNA yield^{28;29}, optimal results with RT-PCR are mainly achieved when using fresh frozen tissue.^{30;31} This is more laborious and lacks morphological assessment. Therefore, serial or multilevel sectioning combined with immunohistochemical staining is preferred since it can be reliably used on paraffin-embedded tissue and the morphology of stained cells can be examined.

The fact that most immunohistochemistry studies show no difference in clinical outcome between patients with lymph nodes containing occult tumor cells and patients with tumor-negative lymph nodes, we ascribe to the lack of making a distinction between micrometastases and isolated tumor cells.^{13;32-39} The last sixth TNM edition^{11;12} recommends classifying occult tumor cells in lymph nodes into micrometastases and isolated tumor cells.

According to this TNM edition, isolated tumor cells in lymph nodes which are less than 0.2 mm in diameter, are insignificant and should be classified as pN0(i+). Lymph-node deposits with a diameter between 0.2 mm and 2 mm should be classified as micrometastases (mi) and staged as node positive pN1(mi). Even so, this recommendation published in 2002, was not based on evidence but more on logical reasoning. In our study isolated tumor cells were found of no clinical relevance and do confirm the guidelines of the TNM classification system. Only two other research groups^{31;40} have previously published study results regarding CRC patients in which they differentiated between micrometastases and isolated tumor cells, emphasizing the importance of our study. Messerini *et al.*⁴⁰ examined lymph nodes from 395 stage IIA CRC patients by immunohistochemical staining of 12 serial sections with antibodies directed against cytokeratin 20. Micrometastases were detected in lymph nodes from 9.9% of the patients and isolated tumor cells were seen in 28.4% of patients. Similar to our findings, they did not find prognostic relevance of isolated tumor cells and showed a lower survival rate in patients with micrometastases compared to patients with isolated tumor cell-positive and tumor-negative lymph nodes. We suggest that the lack of prognostic significance of isolated tumor cells might be explained by assuming that these cells are shed from the primary tumor and transported through lymphatic vessels to the lymph nodes, but do not have the potential of independent outgrowth, and therefore, do not form established metastases. The second research group³¹ who made a distinction between micrometastases and isolated tumor cells in CRC, examined two lymph node levels at a 200 µm interval with antibodies AE1/AE3. They detected micrometastases in seven of 234 patients (3%) which corresponds to the detection rate in our patient group. This group did not evaluate the prognostic relevance of micrometastases in CRC leaving only two studies by Messerini *et al.*⁴⁰ and our study who did.

Although we choose for a case-control design since this was more cost-effective than a retrospective cohort study, our study has advantages such as reliable follow-up of the patients by our Department of Oncological Documentation and the matching of two controls for each case. A limitation of our study which is known when evaluating archival material for research purposes, was the infeasibility to select for patients with at least 12 lymph nodes examined for accurate staging as recommended by the American Joint Committee on Cancer Staging (AJCC)¹² and the International Union against Cancer (UICC-TNM).¹¹ Twenty percent of our patient group underwent an adequate lymph node harvest of at least 12 lymph nodes. Other studies examining large archival material also demonstrated this limitation. Only 22% of 569 CRC specimens studied by Johnson *et al.*⁴¹ and 37% of 116,995 CRC patients in a study by Baxter *et al.*⁴² received adequate evaluation of 12 or more lymph nodes. Factors that have been reported to affect the number of lymph nodes retrieved in CRC specimens were the effect of different pathology assistants, older age, rectal cancer and the T stage.⁴²⁻⁴⁴

We also saw less patients with a harvest of at least 12 lymph nodes in rectal carcinoma than in colon carcinoma and patients with at least 12 lymph nodes harvested were significantly younger than patients with less than 12 lymph nodes harvested. Even so, evidence exists that the number of harvested lymph nodes has increased during the last two decades. In our group, an RT-PCR study regarding detection of occult tumor cells in lymph nodes from 26 CRC patients who were included from January 1990 and February 1992 has been previously published.¹⁴ In this study, 246 lymph nodes were freshly isolated leading to an average of 9.5 compared to an average of 7.2 in our present study in which lymph nodes were isolated after fixation in formalin. Experience in our pathology laboratory has shown that more lymph nodes are harvested when isolated freshly than when harvested after formalin fixation (data not published). Also, in a prospective sentinel lymph node study in CRC patients³¹ between 1996 and 2001, more lymph nodes were harvesting in the study group (average 14) than in the control group (average 10), suggesting that more lymph nodes are harvesting within a trial design. Fat clearance techniques increases the number of harvested lymph nodes, especially small lymph nodes less than 5 mm.^{45;46} However, these methods are time consuming, expensive and impractical as they involve noxious volatile agents.

Nevertheless, in our study the number of examined lymph nodes was included in the multivariate analysis and in line with the literature, we found that a lower number of examined lymph nodes was an independent risk factor²⁶ for disease recurrence together with T4 stage and the presence of micrometastases. Since detailed analysis of lymph nodes using serial or multilevel sectioning and immunohistochemistry is costly and time- and labor-consuming, we should take into account restriction of lymph node sampling or ultrastaging of sentinel lymph node(s) which have the highest risk for harboring metastases. In a recent study by Pusztaszeri *et al.*⁴⁴, the value of sampling lymph nodes located at distance sidelong CRC specimens was assessed. Mesocolic and perirectal fat were divided into two fractions: close to (< 5 cm) and distant from (> 5 cm) from the primary tumor. They found that in the colon, lymph node location is more important than lymph node number because metastatic lymph nodes were present mostly in the peritumoral area. This suggested that lymph nodes should be initially recovered from the pericolic fat close to the tumor. If there are less than 4 tumor-positive lymph nodes and less than 12 tumor-negative lymph nodes examined in total, only then additional lymph nodes should be retrieved from the distal fraction for potential upstaging. In the rectum, systematic sampling of close and distant lymph nodes seemed mandatory because in some cases, metastases were detected only in distant lymph nodes, particularly in patients who had undergone neoadjuvant radiotherapy.

Additionally, interim results from Bilchik *et al.*⁴⁷ assessing sentinel lymph nodes for the presence of occult tumor cells, suggests the presence of micrometastases in sentinel lymph nodes to increase the risk for disease recurrence.

Importantly, quantitative restriction of lymph node sampling and sentinel lymph node mapping in CRC does not substitute a complete oncologic resection and thus far all lymph nodes will continued to be examined with conventional hematoxylin and eosin staining.

In conclusion, there is need for better prognostication in stage II CRC patients since disease recurrence will occur in up to 30% of these patients and the administration of adjuvant chemotherapy to all node-negative CRC patients is controversial. Our study results show that immunohistochemistry combined with two level analyses of lymph nodes is helpful in detecting macrometastases and micrometastases which showed prognostic relevance. Isolated tumor cells were of no prognostic importance.

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CHAPTER SIX

THE FEASIBILITY AND RELIABILITY OF SENTINEL NODE MAPPING IN COLORECTAL CANCER

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ABSTRACT

Background: Sentinel node mapping (SNM) has been introduced in colorectal cancer (CRC) to improve staging by facilitating occult tumor cell (OTC) assessment in lymph nodes that are most likely to be tumor-positive. In this paper, studies on the feasibility and reliability of SNM in CRC are reviewed.

Methods: A literature search was conducted in the National Library of Medicine by using the keywords colonic, rectal, colorectal, neoplasm, adenocarcinoma, cancer and sentinel. Additional articles were identified by cross-referencing from papers retrieved in the initial search.

Results: There is a large variation in identification rates and false-negative rates mainly due to the learning curve effect, differences in SNM technique and tumor T stage.

Conclusions: We conclude that SNM in CRC is technically feasible. Standardization of SNM procedures is mandatory to resolve the debate on the reliability of sentinel lymph node status for predicting the tumor status of all lymph nodes. Only then can adjuvant treatment of patients upstaged by OTC detection in sentinel nodes be justified.

INTRODUCTION

Lymph node metastases are one of the most important predictors of survival in patients with resectable colorectal cancer (CRC). In most countries, patients with curatively resected CRC without nodal tumor involvement do not receive adjuvant systemic therapy, since meaningful survival benefits have not yet been proven.^{1,2} However, up to 30% of these patients will develop locoregional recurrence or distant metastases.³ A recent study shows that stage II CRC patients who receive 5-fluorouracil have a significantly lower risk of disease recurrence.⁴ Nevertheless, adjuvant systemic treatment of all node-negative CRC patients would lead to overtreatment and unnecessary complications due to adverse effects of adjuvant systemic therapy. Providing only the high-risk patients with adjuvant therapy prevents this and leads to a better quality of life and an optimized cure rate for these patients. The high-risk node-negative CRC patients may be identified through the detection of occult tumor cells (OTC) in lymph nodes. OTC comprise of micrometastases and isolated tumor cells that are usually not detected with conventional hematoxylin and eosin (HE) staining. Micrometastases are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and isolated tumor cells either as single tumor cells or as clusters of tumor cells of 0.2 mm or less.⁵⁻⁷ Focused examination methods such as serial sectioning, step sectioning, immunohistochemistry, polymerase chain reaction and reverse transcriptase

polymerase chain reaction increase the likelihood of finding OTC. Examining all regional lymph nodes with these methods would be preferred, but is expensive and time consuming and therefore not feasible in daily practice.

Sentinel node mapping (SNM), also known as lymphatic mapping, offers a potential solution. During an SNM procedure sentinel nodes (SNs), i.e. lymph nodes onto which the tumor drains directly, are retrieved.⁸ These SNs have the highest chance of harboring tumor cells.⁹⁻¹² When SNs are free from tumor, nonsentinel nodes (NSNs) are also expected to be tumor-negative. By examination of SNs only, which reflect the status of the whole regional lymph node basin, OTC detection becomes attainable as the number of lymph nodes to be examined is greatly reduced.

The SNM concept originated in 1923 when Braithwaite^{13;14} evaluated the flow of lymphatic vessels near the ileocaecal valve using indigo carmine. He used the terms "lymphatic mapping" and "glands sentinel". The term "sentinel node" was initially used in 1960 by Gould *et al.*¹⁵ in their study to detect lymphatic metastases in parotid carcinoma. In 1977, Cabanas *et al.*¹⁶ performed lymphangiograms in penile carcinoma to search for a specific lymph centre where the lymphatic vessels drained to. They referred to this specific lymph centre as the "sentinel lymph node". The SNM procedure was further developed in melanoma by Morton *et al.*⁸ and in breast cancer by Guiliiano *et al.*¹⁷ The aim of SNM in melanoma and breast cancer is to limit postoperative morbidity by preventing an unnecessary lymphadenectomy. A lymphadenectomy will be performed when the usually perioperatively examined SNs harbor tumor cells. In CRC, however, lymph nodes near the tumor are routinely resected en bloc with the primary tumor. Lymphadenectomy in CRC is a standardized procedure, generally without complications or comorbidity and a one-stage surgical procedure is preferred in abdominal surgery. Thus, the main advantage of SNM in CRC, is OTC assessment in a limited number of lymph nodes. A second important advantage of in vivo SNM in CRC is the detection of aberrant lymphatic drainage patterns that may occur in up to 14% of the patients, leading to an adjustment of the initial resection plan.¹⁸

There are a growing number of publications on the role of SNM in CRC. This review assesses the feasibility and reliability of SNM in CRC. Differences in the SNM techniques used are emphasized.

METHODS

A literature search was conducted with PubMed software in the National Library of Medicine, containing articles from 1953 until 2004. The following keywords were used in appropriate combinations: colonic or rectal or colorectal neoplasm, adenocarcinoma or cancer and sentinel. Papers with anal cancer in the title were excluded and the language was restricted to English. All hits from this PubMed search were individually checked, and included only if they addressed the subject of this review. Additional articles were identified by cross-referencing from papers retrieved in the initial search.

Among the SNM studies considered in this review, several variables were used to indicate the feasibility and reliability of this procedure. The identification or success rate was calculated as the number of SNM procedures where at least one blue node was found, divided by the total number of SNM procedures. True positives (TP) were defined as cases where the SNs were positive and the NSNs were positive or negative. True negatives (TN) were defined as cases where both the SNs and NSNs were negative. False-negative cases (FN) were defined as cases where the SNs were negative whereas the NSNs were positive. The FN rate was calculated using the formula $FN/(FN+TP)$. Sensitivity was defined as the complement of the false-negative rate $[TP/(TP+FN)]$. Negative predictive value was calculated using the formula $TN/(TN+FN)$. The upstaging percentage represented the cases where lymph nodes, tumor-negative by conventional HE staining, became tumor-positive when additional examination methods were used. The upstaging percentage was calculated by dividing the number of HE-negative cases found to be tumor-positive with additional examination techniques by the total number of HE-negative cases investigated with additional examination techniques.

Sentinel node mapping studies in colorectal cancer

In vivo versus ex vivo sentinel node mapping

In contrast to SNM in breast cancer and melanoma, the regional lymphatic vessels and lymph nodes in CRC are routinely resected along with the primary tumor. Hence, ex vivo injection of a tracer in CRC is possible. During an in vivo SNM procedure there is still pressure to push the tracer into lymphatic vessels. When performing an ex vivo SNM procedure, the injected site should be gently massaged for approximately 2 to 5 minutes to push the tracer into lymphatic vessels.¹⁹ A disadvantage of the ex vivo procedure is the inability to detect aberrant lymphatic drainage patterns. However, during an ex vivo SNM the patient is not at risk of allergic reactions or falsely low pulse oximeter readings due to injection of isosulphan blue or patent blue dye. Furthermore, ex vivo SNM has the advantage of precise injection of dye into the submucosa. In some studies the tracer was injected in four quadrants around the tumor.¹⁹ However, this method seems to be associated with accidental exclusion of draining lymphatic vessels and the tracer should therefore be injected circumferentially around the tumor.²⁰

Tracers used for sentinel node mapping

There are several types of tracers²¹ that can be used when performing an SNM procedure; these include blue dye, radioactive or fluorescent tracer. Blue dyes that are being used are lymphazurin isosulphan blue, patent blue, methylene blue or Evans blue.²² Other dyes that can be used are indigo carmine²² and indocyanine green.²³ Among radioactive tracers²⁴ there are technetium labelled-sulphur colloid, antimony, tin colloid, human serum albumin and technetium-labelled phytate, each with different particle sizes.^{25;26} In the United States, lymphazurin isosulphan blue and technetium sulphur colloid are mostly used.²⁷

Patent blue²⁸, technetium sulphur colloid²⁹ and technetium human serum albumin³⁰ were used in Europe. The Japanese surgeons prefer the radioactive tracer technetium tin colloid for SNM in CRC.³¹

The size of blue dye particles is smaller than the radioactive colloid particles. Therefore, dyes show a shorter transit time through the lymphatic vessels with an increasing risk of staining of the second tier lymph nodes. However, blue dye is inexpensive and relatively easy to use. Furthermore, no protection measures have to be taken and the infiltrated lymphatic vessels and lymph nodes are easily visualized without additional equipment. Unfortunately, isosulphan blue and patent blue dye have been associated with allergic reactions in 1% to 1.6% of patients.³²⁻³⁸ These allergic reactions varied from urticaria and hypotension to life-threatening anaphylactic shock. Both dyes are also known to lead to falsely low pulse oximeter readings during the course of surgery.^{32;39-42} Thus far, methylene blue and fluorescent tracer have not been used in CRC. The former, however, was associated with skin lesions at the injection site in breast cancer patients when using combined deep parenchymal and intradermal injections.⁴³

In 19^{10;12;28;44-59} of the 25 studies published on SNM in CRC (*Table 1*), blue dye was used as a tracer, usually injected in vivo. Four studies reported SNM using both blue dye and radioactive tracer^{27;30;60;61} and in two studies only radioactive tracer was used.^{29;31} The volume of blue dye used ranged from 0.5 to 2 ml except in three studies^{46;55;61} where up to 5 ml blue dye was used. The moment of injection was usually in vivo and the site of injection was mostly subserosally and sometimes submucosally during an endoscopy or ex vivo procedure. In all but one study⁵³ using blue dye, the identification time between injection of the tracer and labelling of SNs ranged from 1 to 60 minutes. In the study by Gandy *et al.*⁵³, however, the resected specimens were fixed in formalin for 48 hours followed by the search for blue nodes. After identification, blue nodes were processed separately because blue dye is removed during routine histological processing. The studies using radioactive tracers injected preoperatively or perioperatively to detect SNs showed an identification time ranging from 26 minutes to 20 hours. Different types of radioactive tracer were used in these studies. The first use of a radioactive tracer in SNM in CRC was reported in 2000 by Kitagawa *et al.*⁶² When using a radioactive tracer additional equipment such as a gamma probe is required to detect the radioactivity level of the lymph nodes. No reports linking radioactive tracers to allergic reaction or interference with patient monitoring have been published. Merrie *et al.*⁶⁰ used patent blue dye combined with the radioactive tracer technetium colloidal antimony sulphide in 21 SNM procedures. Eighty-one percent of the hot nodes were identified by blue dye and 51% of the blue nodes were identified by the radioactive tracer. The lower number of blue nodes that were also radioactive was explained by the rapid passage of blue dye through the lymph nodes to the following nodes. For the hot nodes that did not show blue dye it was proposed that the blue dye had passed through the node without leaving a stain, or that these nodes were more immunologically active, resulting in greater uptake of the radioactive tracer.

The average number of blue nodes was 6.2 and the average number of hot nodes was 3.9.

Saha *et al.*²⁷ showed that a combination of isosulphan blue dye and the radioactive tracer technetium sulphur colloid (TSC) may improve the identification and accuracy rate of SNM in CRC. The SNM with blue dye was successful in 100% of 57 patients *versus* 89% when using the radioactive tracer. Blue dye detected 152 SNs, the radioactive tracer detected 100 SNs, and both modalities detected 96. Of the SNs detected by blue dye only, 10.7% had nodal metastases, whereas 19.8% of SNs detected by both modalities had nodal metastases ($P = 0.028$). This suggests that using both type of tracers leads to a higher accuracy rate of the SNM. However, Patten *et al.*⁶¹, who used the same combination of isosulphan blue dye and TSC, showed no increase in sensitivity of detection of tumor-positive SNs compared to the use of blue dye alone. Additionally, Saha *et al.*²⁷ reported a lower average number of SNs (2.7 vs 3.5 SNs) and a lower FN rate (16% vs 50%) compared to Patten *et al.*⁶¹ These studies show discrepancies in the additional value of a radioactive tracer.

As yet there is no consensus on the best tracer or combination of tracers to be used.

Identification rates

The identification rate of the 25 studies ranged from 58% to 100% with an average rate of 89% (*Table 1*). Factors that might have contributed to the failed SNM procedures were incomplete circumferential injection around the tumor²⁸ or insufficient volumes of tracer for large tumors⁵⁵ and inclusion of advanced tumors.²⁹ Also a learning curve has been confirmed by studies reporting an identification rate of almost 100% if the first SNM procedures were not included in the analysis.^{10;52} In conclusion, SNM in CRC is technically feasible.

False-negative rates

The FN rate ranged from 0% to 63% with an average of 33% (*Table 1*). Use of both blue dye and radioactive tracer led to an average FN rate of 28% with a range of 0 to 50%. The two studies using only radioactive tracer showed FN rates of 18% and 56%. High FN rates might be explained by extensive nodal replacement and large tumors. These factors can cause occluded lymphatic vessels leading to lymph drainage through an alternative route.^{28;61} Another contributing factor to FN cases might be a long identification time between the injection of the tracer and the identification of SNs. On the other hand, one would expect that limitation of the time between injection of the tracer and resection of the bowel specimen should suffice because of pressure loss preventing the dye from travelling further. However, blue dye travels fast and might have already left the real SNs by the time that the specimen has been resected and the pathologist looks for blue nodes. This phenomenon might also explain the wide range of detected SNs in some studies (*Table 1*).

Table 1. Overview of sentinel node mapping studies in colorectal cancer

References	Year	No of patients	SNM procedure	Identification ^a rate (%)	False-negative rate ^a (%)	Sensitivity ^a (%)	Negative predictive value ^a (%)	No of LNs ^b (range)	No of SNs ^b (range)	No of HE-negative patients	Upstaging percentage ^c (%)
Joosten ²⁸	1999	50	in vivo	70	60	40	56	14 ^d (2-37)	3 ^d (1-16)	15	13
Cserni ¹⁴	1999	25	in vivo	96	38	62	69	15.5 (2-34)	4 (0-12)	11	-
Waters ^{e,45}	2000	22	in vivo	91	0	100	100	11.6	1	15	7
Feig ⁴⁶	2001	48	in vivo	98	63	38	76	13 (4-46)	2.6 (0-7)	31	13
Saha ¹⁰	2001	203	in vivo	98	15	85	95	-	(1-4)	144	19
Merrie ^{f,60}	2001	25 (26 tumors)	in vivo	88	45	55	71	17 ^d (4-52)	3 ^d (0-8)	-	-
Esser ⁴⁷	2001	31	in vivo	58	33	67	94	15	1.7 (1-5)	15	-
Broderick-Villag ^{c,48}	2002	50 (51 tumors)	in vivo or ex vivo	92	50	50	73	7.8 (1-17)	1.5 (1-5)	27	-
Evangelista ⁴⁹	2002	11	in vivo	91	50	50	89	-	(1-3)	8	1
Nastro ³⁰	2002	8	in vivo	75	0	100	100	-	-	4	50
Fitzgerald ⁵⁰	2002	26	ex vivo	88	40	60	90	14.8	2.5	18	11
Bendavid ⁵¹	2002	20	in vivo	90	13	88	91	-	3.9 (0-5)	10	50
Paramo ⁵²	2002	55	in vivo	82	7	93	97	12	1.9 (1-4)	30	20
Kitagawa ³¹	2002	56	in vivo	91	18	82	88	23.9	3.5 (0-8)	29	-
Gandy ⁵³	2002	19	in vivo	63	17	83	86	17 ^d (7-27)	7.1	6	-
Roseano ⁵⁴	2003	23	in vivo or ex vivo	100	50	50	90	14.6 (7-35)	2.5 (1-8)	19	0
Vieh ⁵⁵	2003	31	in vivo	87	50	50	71	21 ^d (5-40)	2 ^d (1-8)	15	0
Saha ^{27,72}	2004	57	in vivo	100	16	84	93	12.4	2.7 (1-4)	38	-
Demirbas ⁵⁶	2004	41	ex vivo	90	0	100	100	8.5	3 (1-10)	17	12
Bertoglio ⁵⁷	2004	26	in vivo	92	22	78	88	12.6 (6-18)	2.9 (1-3)	15	-
Bembeneke ^{4,29}	2004	48	in vivo	96	56	44	77	18 ^d (9-69)	4 ^d	-	-
Patten ⁶¹	2004	57	in vivo	98	50	50	76	17.9	3.5 (0-11)	34	15
Braat ⁵⁸	2004	35	in vivo or ex vivo	94	20	80	92	9 (1-23)	1.7 (1-4)	25	8
Wong ^{9,12}	2004	124	ex vivo	97	47	53	74	30 (5-111)	3.8 (1-21)	66	27
Bertagnoli ⁵⁹	2004	72	in vivo	92	58	42	75	17.3	2.1	42	0

SNM, sentinel node mapping; LNs, lymph nodes; SNs, sentinel nodes; HE, hematoxylin eosin; -, not done or unknown; ^a FN rate, sensitivity and negative predictive value are calculated considering HE staining results unless mentioned otherwise; ^b Values are mean unless otherwise indicated; ^c The upstaging percentage was calculated by dividing the number of HE-negative cases found tumor-positive with additional examination techniques by the total number of HE-negative cases investigated with additional examination techniques; ^d median; ^e FN rate, sensitivity and negative predictive value are calculated considering HE staining and upstaging results; ^f FN rate, sensitivity and negative predictive values are from the article; ^g When considering the results obtained from the IHC analysis of the lymph nodes from the HE node-negative patients, the false-negative rate was 28%, the sensitivity was 72% and the negative predictive value was 91%.

Ten of the 25 studies reported a FN rate of 20% or less (*Table 1*). Four of these 10 studies were the only studies that considered the first four blue nodes, usually marked *in vivo*, to be the real SNs.^{10;27;52;58} Bendavid *et al.*⁵¹ considered the four to five blue nodes closest to the tumor as SNs. Waters *et al.*⁴⁵ and Demirbas *et al.*⁵⁶ reported average numbers of SNs of 1 and 3, respectively. The study by Kitagawa *et al.*³¹ showed a range of SNs from 0 to 8. Gandy *et al.*⁵³ considered all blue nodes as SNs and reported an average number of 7.1 SNs. The majority of the 15 SNM studies reporting a FN rate higher than 20%, considered all blue or radioactive nodes as SNs leading to wider ranges of SNs. Up to 21 SNs were found as shown in *Table 1*.¹² To consider the first blue nodes as the real SNs has limitations. A subject for debate is the fact that blue nodes are usually found by inspection.^{52;58} However, it is assumed that SNs are often locoregional nodes, which are usually located near the serosa within the mesocolic fatty tissue. This suggests that with the aforementioned way of selecting blue nodes, there is a chance of missing real SNs, and selecting second tier lymph nodes. Therefore, it might be expected that immediate examination of the entire mesocolon by inspection, palpation and incisions and considering the first to fourth blue nodes closest to the tumor, might be more reliable.²⁰

Studies in CRC show higher FN rates than studies in melanoma and breast cancer, when considering HE staining results. Three SNM studies in melanoma show FN rates of 0%, 5% and 8%.^{8;63-65} All 20 SNM studies in breast cancer recently reviewed by Kelly *et al.*⁶⁶ showed an average FN rate of 8% (range 0% to 19%). The difference in FN rate between SNM in CRC and SNM in melanoma or breast cancer might be due to the larger tumors found in the colon and the rectum and also to the dissimilarity in lymphatic drainage pattern. Furthermore, in melanoma and breast cancer, enlarged suspected lymph nodes that have not taken up dye are easier to find by inspection and palpation of the lymph nodes.

In summary, we conclude that there is a large variation in FN rates usually ascribed to the differences in SNM technique and tumor T stage. Most studies show that SNM in CRC is usually unreliable in patients with lymph nodes containing macrometastases detected with HE staining. All studies regarding the first four blue nodes as the real SNs showed low FN rates.

Sentinel node mapping in rectal cancer

In vivo SNM in rectal cancer with blue dye is technically difficult because of the anatomic location of the rectum deep within the narrow pelvis. A clear view of the tumor site is therefore not always feasible. Additionally manual injection of the tracer into the perimuscular fatty tissue is troublesome and real-time observation of the fast transit of blue dye into SNs is almost impossible. However, submucosal injection of blue dye during an *ex vivo* SNM procedure followed by immediate incisions in the perimuscular fatty tissue to find the blue nodes is possible.¹² Another way is lymphoscintigraphy by preoperative or perioperative submucosal injection of radioactive tracer through endoscopy.^{29;31}

When using a radioactive tracer, there is no need for real-time observation due to the longer transit time and the detection of hot nodes through a probe.

Kitagawa *et al.*³¹ included patients with sigmoid colon or rectal cancer and Bembenek *et al.*²⁹ included only rectal cancer patients. Both used a radioactive tracer that was injected submucosally during preoperative endoscopy. The identification rates of these studies were similar, but the FN rates differed. The first study reported an identification rate of 91% and a FN rate of 18% and the latter reported an identification rate of 96% and a FN rate of 56%. Ninety percent of the patients of the study by Bembenek *et al.*²⁹ had locally advanced cancer (endosonographically uT3/4) of whom 86% had undergone neoadjuvant radiochemotherapy. Kitagawa *et al.*³¹ included clinically localized sigmoid colon and rectal cancer patients. Fifty-two percent of these patients were diagnosed with T1 or T2 tumor stage. All four FN cases in this study were seen in patients with T3 tumors. The difference in patient tumor stage may explain the difference in FN rates between these two studies.

Lymphoscintigraphy might be more applicable in cases of rectal cancer because of the ability to detect aberrant lymph drainage patterns to extramesorectal lymph nodes, which might be considered an indication for lateral lymphadenectomy. Some researchers claim that lateral lymphadenectomy is unnecessary because even if there are radioactive extramesorectal lymph nodes, these are usually tumor-free.⁶⁷ Nevertheless, Cutini *et al.*⁶⁸ reported that one of two patients in their study with radioactive extramesorectal lymph nodes had metastases in these nodes. Kitagawa *et al.*³¹ showed direct lymph drainage to lateral lymph nodes in 10% of their rectal cancer patients. These numbers confirm the clinical importance of lymphoscintigraphy in rectal cancer patients.

Sentinel node mapping during a laparoscopic procedure versus open surgery

Without elaborating on SNM during laparoscopic colon resection (LCR), we would like to remark briefly that there is evidence that SNM during LCR is technically feasible and yields similar identification and accuracy rates as SNM during open colon resection.^{69;70} SNM during a LCR can be useful for patients with small early-stage tumors.

Occult tumor cell detection in sentinel nodes

It should be kept in mind that the SNM procedure in CRC is an aid to OTC detection in lymph nodes. Therefore, it is necessary to study the reliability of SNM mapping in HE node-negative patients. Smith *et al.*⁷¹ reported a power calculation determining the number of HE node-negative patients required to prove that SNM upstages patients by selective identification of nodes rather than by chance. When assuming that the proportion of HE node-negative patients that are truly node-positive is 30%, a total of at least 34 HE node-negative patients would be required in order to upstage all 30% of these patients.

If the percentage of patients that were truly node-positive is 20% of all HE node-negative patients, then at least 130 HE node-negative patients would need to be mapped in order to show statistical significance of SNM in upstaging of patients. *Table 1* shows four studies with 34 or more HE node-negative patients. SNs in three of these studies were differently examined than the NSNs.^{10;27} Only the study by Wong *et al.*¹² investigated SNs and NSNs in an identical fashion. They found a FN rate of 47% when considering the lymph node metastases found in HE stained sections. However, the FN rate was 28% when the SNs and NSNs from the HE node-negative patients were examined with step sectioning and immunohistochemistry. Additionally, Wong *et al.*¹² reported an overall presence of tumor cells in 4.7% of SNs and in 0.3% of NSNs ($P < 0.001$) confirming the SN concept. By examining SNs and NSNs from 34 CRC patients with immunohistochemistry, Turner *et al.*⁹ showed that SNs are significantly more likely to contain tumor cells than NSNs ($P < 0.001$; odds ratio, 9.64; 95% confidence interval, 4.94 – 19.60). However, they did not examine the SNs and NSNs identically. The SNs were examined on four levels and the NSNs were examined on three levels and even though the SNs of all the patients were HE negative, six patients actually had HE positive NSNs. In some studies FN rates were calculated considering the results obtained with HE staining of all lymph nodes and focused examination of only SNs.⁵¹ FN rates decreased when results from upstaging techniques of SNs were included in the calculation. This might relate to the fact that in most FN cases the real SNs could not take up the tracer because of occluded lymphatic vessels due to metastases. The likelihood of detecting OTC in false-negative SNs from such a specimen is increased because tumor cells had already started to metastasize to these nodes too.

We should bear in mind that the intended benefit of SNM in CRC is mainly to facilitate in OTC assessment. This suggests that the study of Wong *et al.*¹², reporting that the SN concept is applicable to HE node-negative CRC, needs confirmation.

CONCLUSIONS

SNM in CRC is technically feasible. However, there is a large variation in identification rates and false-negative rates usually ascribed to the learning curve effect, differences in SNM technique and tumor T stage. Most studies show that SNM in CRC is usually unreliable in patients with lymph nodes containing macrometastases detected with HE staining. All studies regarding the first four blue nodes as the real SNs showed low false-negative rates. We should bear in mind that SNM in CRC is mainly intended to facilitate OTC assessment, suggesting that the few studies reporting that the SN concept is applicable to HE node-negative CRC need confirmation. A uniform protocol regarding the technical aspects of SNM is mandatory. Therefore, in the current situation, OTC assessment combined with SNM has to be considered merely an experimental diagnostic strategy. The formulation of transatlantic working parties may aid in tackling this problem by

focusing on standardization of techniques and subsequent adherence to surgical and pathological protocols. Only then might high-risk HE node-negative patients be identified reliably with OTC detection in SNs, and subsequently be provided with adjuvant therapy.

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CHAPTER SEVEN

DISSEMINATED TUMOR CELLS IN BONE MARROW OF PATIENTS WITH COLORECTAL LIVER METASTASES PREDICT SURVIVAL

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ABSTRACT

Background: A number of patients with colorectal liver metastases who undergo locoregional treatment, show progressive disease after surgery. These patients might benefit from additional systemic chemotherapy. The aim of this study was to identify these high-risk patients by bone marrow analysis for the presence of disseminated tumor cells.

Patients and methods: Bone marrow was aspirated prior to surgery from 81 colorectal cancer patients scheduled for liver tumor resection or isolated liver perfusion and 59 control patients who were operated on for benign diseases. Samples were analyzed with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for the expression of cytokeratin 20 (CK20) and with immunocytochemistry (CK-ICC) combined with automated microscopy by use of anti-cytokeratin antibodies A45-B/B3.

Results: Bone marrow samples in 7 of 43 control patients and 26 of 69 colorectal liver metastases patients tested positive with the CK20 RT-PCR (Chi-square test, 16% vs 38%, $P = 0.016$). The CK-ICC test was positive in 6 of 59 control patients and in 15 of 69 colorectal liver metastases patients (Chi-square test, 10% vs 22%, $P = 0.078$). In a multivariate Cox regression analysis a positive CK20 RT-PCR test (hazard ratio 2.5, 95 per cent confidence interval 1.2 to 5.2, $P = 0.014$) and a serum carcinoembryonic antigen level higher than 200 micrograms per liter (hazard ratio 2.4, 95 per cent confidence interval 1.0 to 5.9, $P = 0.045$) were independent predictors for a reduced disease-related survival. No correlations between survival and a positive CK-ICC test were found.

Conclusions: Disseminated tumor cells in bone marrow of patients with colorectal liver metastases detected by CK20 RT-PCR is associated with a worse clinical outcome. However, also numerous control patients had positive bone marrow both by RT-PCR and immunocytochemistry. Therefore research on more specific antibodies or molecular markers is required. Detection of disseminated tumor cells in bone marrow by CK20 RT-PCR may aid in improving individual patient's treatment.

INTRODUCTION

Locoregional treatment of colorectal cancer metastases confined to the liver contributes to improved survival and can even lead to cure. Surgical resection is as yet the only effective curative treatment.^{1;2} Patients with unresectable liver metastases can be treated with isolated hepatic perfusion.³ The reported 3-year survival of patients treated with surgical liver resection ranges from 57% to 73%^{4;5} and patients treated with isolated hepatic perfusion show a 3-year survival of 37%.⁶

A high percentage of patients who have undergone locoregional treatment for colorectal liver metastases show progressive disease, i.e. progression within the liver and/or extrahepatic relapse, within a relatively short period of time after locoregional treatment. These high-risk patients might benefit from additional systemic treatment.⁷ Although colorectal cancer usually does not metastasize to the bone, disseminated tumor cells found in bone marrow could predict disease outcome since their presence might represent the aggressive nature of the tumor.^{8;9}

The aim of this study was to assess whether the presence of disseminated tumor cells in bone marrow from patients who undergo locoregional treatment of colorectal liver metastases is associated with a worse outcome of disease. The presence of disseminated tumor cells in bone marrow was analyzed using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for cytokeratin 20 (CK20) and immunocytochemistry (CK-ICC) combined with automated microscopy by using antibodies A45-B/B3 which recognize cytokeratins 8, 18 and 19.

PATIENTS AND METHODS

Patients

Between October 2001 and May 2005, a total of 81 consecutive patients with colorectal liver metastases scheduled for resection or isolated hepatic perfusion at the Department of Surgery at the Leiden University Medical Center were included. All patients had previously undergone a resection of their primary colorectal tumor and underwent preoperative chest and abdominal computed tomography (CT) to confirm metastatic disease confined to the liver. CT was performed using a helical scanner (SR7000 or AVE; Philips Medical System, Best, The Netherlands). Non-enhanced and contrast-enhanced (150 ml non-ionic agent, 350 mg iodine per ml, using power injection at 3 ml/s via the antecubital vein, fixed delay 60 s) images of the liver were obtained. After scanning the liver, the remainder of the abdomen was scanned. 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;6;10} During follow-up, CT-scans were made 4, 8 and 12 months after surgery and then after every 12 months until 3 years after surgery except for patients who underwent an isolated hepatic perfusion. These patients were permanently followed by CT-scans. Patients who did not undergo any intervention and patients who showed disease progression and could not be surgically treated, were referred to a medical oncologist for further treatment. Bone marrow samples from 59 control patients who were operated on for benign diseases such as inflammatory bowel diseases ($n = 31$), diverticulosis/-itis ($n = 8$), cholelithiasis or cholecystitis ($n = 13$), hernia inguinalis or cicatricialis ($n = 6$) or endometriosis externa ($n = 1$) were assessed. Approval from the Ethical Committee for this study was granted and informed written consent was obtained from all patients. No complications were seen after bone marrow aspiration.

Bone marrow aspiration

Twenty to 30 ml of bone marrow was aspirated from the anterior iliac crest prior to surgery under general anesthesia. Prior to 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 using ficoll gradient centrifugation and aliquoted to isolate RNA for the RT-PCR analysis or to make cytospin-slides to stain with ICC.

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from mononuclear cells by Trizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Random primed cDNA was synthesized from 1 µg of total RNA using the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche, Indianapolis, USA). 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 (Applied Biosystems, Foster City, California, USA). The forward sequence of the CK20 primer was 5'-gagctgagacgcacctccc-3' and the reverse sequence was 5'-gggctccagagagctcaac-3' leading to a product with an amplicon length of 132 base pairs. The sequence of the probe was 5'-TET-accaaggcccgttacagcagccagt-TAMRA-3'. The low-copy housekeeping gene porphobilinogen deaminase was used as an internal control. The forward primer sequence for porphobilinogen deaminase was 5'-ggcaatgcggctgcaa-3' and the reverse primer sequence was 5'-gggtaccacgcgaatcac-3' leading to a product with an amplicon length of 63 base pairs. The probe sequence for porphobilinogen deaminase was 5'-TET-ctcatctttgggctgtttcttcgcc-TAMRA-3'. For each patient two RNA samples resulting in cDNA samples were processed. Five microliters of cDNA (equivalent to 0.05 µg total RNA) were used per amplification. For all PCRs the following PCR conditions were used: after activation of the polymerase (10 min. at 95°C) 55 cycles of 15 sec at 95°C, 30 sec at 65°C and data collection at an additional 30 sec at 65°C were performed. Probe concentrations were 120 nM for porphobilinogen deaminase and 160 nM for all other markers, reporter dye is TET and quencher TAMRA for all probes. Per reaction 300nM of each primer was used. Magnesium concentrations were 4 mM for porphobilinogen deaminase and CK20. Quantitative PCR was performed on an ABI PRISM® 7700 Sequence Detector System (Applied Biosystems) using the qPCR™ Core Kit (Eurogentec, Seraing, Belgium) and analyzed using SDS v. 1.7 software package (Applied Biosystems).

The sensitivity of these tests was determined by spiking tumor cells from the colon cancer cell line CCL-218 in bone marrow from a patient undergoing surgery for a benign disease. Serial five-fold dilutions were made with a total number of 2×10^6 cells per sample. Each sample was amplified four times to determine the reproducibility of the detection. CK20 RT-PCR resulted in positive results for bone marrow samples with one CCL-218 cell.

Cytospin-slides and immunocytochemistry

Mononuclear cells were washed twice with phosphate buffered saline (PBS), and diluted in PBS to a concentration of 0.5×10^6 cells per ml (containers from Greiner Bio-One). Four ml of this suspension were evenly spread onto Histobond® adhesion micro slides (Marienfeld, Lauda-Königshofen, Germany) through cytocentrifugation in centrifugation buckets (home made by the Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands). Centrifugation was at 190g for 10 minutes in a swing-out rotor, with a controlled start and brake (Hettich, Tuttlingen, Germany) resulting in 2 million cells per glass slide. Slides were dried overnight at 37°C and stored at -70°C. The slides were stained with primary antibodies A45-B/B3 (diluted 1:100), directed against cytokeratins 8, 18 and 19 (Micromet AG, Munich, Germany) or with isotype control antibodies directed against an irrelevant antigen, MOPC21, as a negative control staining (diluted 1:200) (BD Pharmingen, Erembodegem, Belgium). A detailed protocol in a comparative immunostaining study of bone marrow specimens has been published before by Pantel *et al.*¹¹ This staining resulted in a red precipitate in the cytoplasm of cytokeratin-positive cells. The slides were counterstained with hematoxylin (Mayer's Hemalum; Merck, Darmstadt, Germany) to visualize nuclear morphology. The detection limit of CK-ICC was previously found to be up to one tumor cell among 1.87×10^6 hematopoietic cells.¹²

Automated microscopy

The stained slides were analyzed using the ARIOL SL-50 automated microscope® (Applied Imaging Corporation, San Jose, CA). One slide stained for cytokeratin and one negative control (MOPC21) slide were analyzed per patient. The features of this system have been previously published.¹³

Data definition

A PCR sample was 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. In case of immunocytochemistry combined with automated microscopy, cytokeratin-positive cells were confirmed by a pathologist (AMvL) 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 Tumor Cell Detection.^{14;15} 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.

Statistical analysis

Statistical analysis was carried out using SPSS software, version 12.0.1 (SPSS Inc, Chicago, Illinois, USA). Numerical data are presented as mean $\pm$ standard deviation. Endpoint was disease-related survival. Correlation between clinicopathologic features and test results was assessed by a Chi-square test. Disease-related survival was considered from the day of surgery to the day of death due to disease or censored at most recent follow-up visit. Patients who did not undergo locoregional treatment, who showed extrahepatic 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. Cox regression analysis was used to calculate univariate and multivariate hazard ratio's (HR) with corresponding 95 per cent confidence intervals (CI) and p-values (P). Differences in disease-related survival between the groups were presented as mean $\pm$ standard error and were compared by the logrank test. A p-value of less than 0.05 was considered statistically significant. Factors with a (overall) p-value less than 0.10 in the univariate analysis were entered into the multivariate logistic or Cox regression model.

RESULTS

Patient and treatment characteristics

Patient characteristics and the type of surgical intervention are shown in *Table 1*. Twenty-two of 81 patients did not undergo the planned locoregional treatment because of the presence of extrahepatic disease ($n = 14$) or insufficient vital liver tissue ($n = 5$). Resection could not be performed in three patients because of the high number of metastases ($n = 2$) or the location of the metastasis to the portal vein ($n = 1$). In one patient who had undergone complete resection of two liver metastases, a resected suspicious lesion on the diaphragm was a metastasis. Four patients had undergone resection of lung metastases and 30 patients had received systemic chemotherapy for liver disease before locoregional treatment. The time interval between the end of the chemotherapy and surgery ranged from 3 weeks to 13 months with a median of 10 weeks. Patients were successfully followed until October 2008. Sixty-five (80%) of 81 patients died during follow-up. The median follow-up from the date of diagnosis of the primary tumor of patients alive ($n = 16$) was 5.4 years with a range of 3.6 to 10.7 years. From the date of liver surgery of patients alive ($n = 16$), the median follow-up was 4.2 years with a range of 3.1 to 6.9 years.

Results of quantitative RT-PCR and CK-ICC (immunocytochemistry combined with automated microscopy)

Due to a sometimes low number of harvested mononuclear cells, bone marrow of not all 59 control patients and 81 colorectal liver metastases patients could be analyzed with both RT-PCR and CK-ICC. Bone marrow samples of 43 control patients were analyzed with RT-PCR and bone marrow samples of all 59 control

patients were analyzed with immunocytochemistry combined with automated microscopy. Samples of 81 colorectal liver metastases patients were analyzed with RT-PCR ($n = 69$) and or immunocytochemistry combined with automated microscopy ($n = 69$).

Bone marrow samples in 7 of 43 control patients and 26 of 69 colorectal liver metastases patients tested positive with the CK20 RT-PCR (Chi-square test, 16% vs 38%, $P = 0.016$). CK20 expression was seen in 5 (25%) of 20 patients who underwent resection, 15 (56%) of 27 patients who underwent an isolated hepatic perfusion and 6 (27%) of 22 patients who underwent no intervention.

By using CK-ICC, bone marrow samples tested positive in 6 of 59 control patients and in 15 of 69 colorectal liver metastases patients (Chi-square test, 10% vs 22%, $P = 0.078$). Bone marrow from control patients was found positive due to the presence of tumor cells ($n = 1$), candidate tumor cells ($n = 1$) or apoptotic cells ($n = 4$). For patients with liver metastases, bone marrow analysis resulted in the presence of tumor cells ($n = 4$), candidate tumor cells ($n = 7$) or apoptotic cells ($n = 7$) (*Figure 1*). A positive CK-ICC test was seen in 5 (23%) of 22 patients who underwent a resection, 3 (10%) of 31 patients who underwent an isolated hepatic perfusion and 7 out of 16 (44%) patients who underwent no intervention. Bone marrow of 43 control patients and of 57 colorectal liver metastases patients

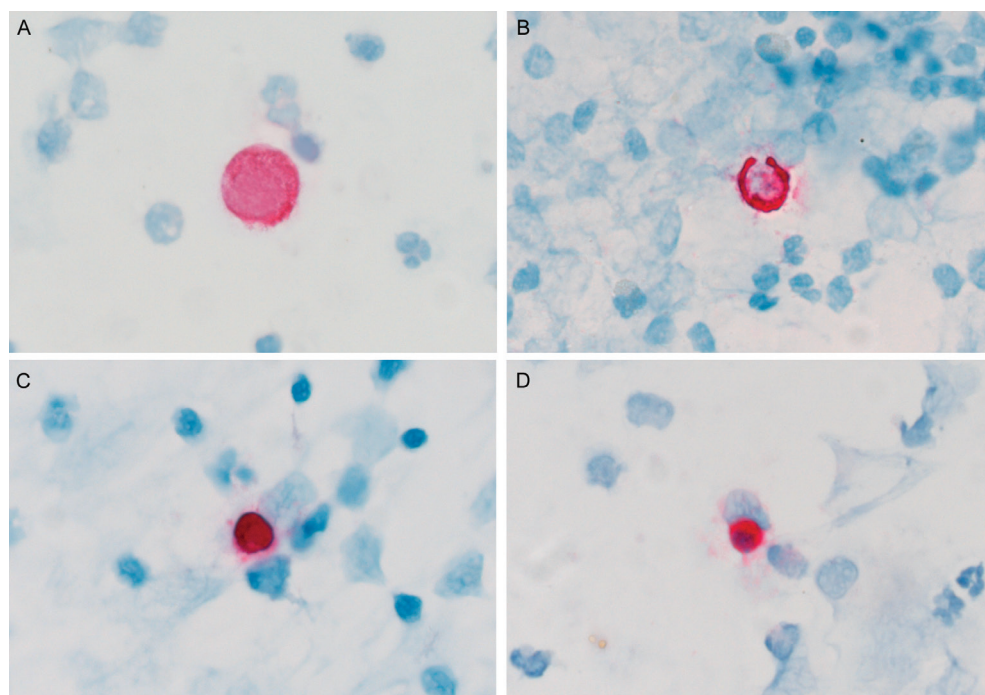


Figure 1. Cytokeratin-positive cells in preoperatively aspirated bone marrow, considered as (A) a tumor cell (B) a candidate tumor cell (C) an apoptotic cell and (D) a hematopoietic cell according to the guidelines of the European ISHAGE Working Group for Standardization of Tumor Cell Detection.¹⁴

Table 1. Patient characteristics (n=81)

Characteristics	n	%
Sex		
Male	57	70
Female	24	30
Mean age (yrs) ^a	58.6 ± 8.9	
TNM stage of primary tumor		
1	4	5
2	9	11
3	21	26
4	47	58
Nodal status of primary tumor		
Negative	27	33
Positive	54	67
Time span between PT and LM (months)		
< 12	58	72
≥ 12	23	28
Preoperative lung metastases ^b		
No	77	95
Yes	4	5
Preoperative systemic chemotherapy		
No	51	63
Yes	30	37
Serum CEA level ^c		
≤ 200 µg/l	64	79
> 200 µg/l	16	20
not assessed	1	1
Number of liver metastases ^c		
< 4	22	27
≥ 4	59	73
Maximal diameter of liver metastases (cm) ^c		
≤ 5	59	73
> 5	22	27
Perioperative extrahepatic metastases		
No	66	81
Yes	15	19
Surgical intervention		
Resection	26	32
Isolated liver perfusion	33	41
No intervention	22	27

PT, primary tumor; LM, liver metastases; CEA, carcinoembryonic antigen; ^a Presented as mean ± standard deviation; ^b Lung metastases had been resected before locoregional treatment of liver metastases; ^c Serum CEA level was dichotomized according to Fong *et al.*⁴ and the number of metastases and maximal diameter were dichotomized according to Nordlinger *et al.*¹⁰

were analyzed by both CK-ICC and CK20 RT-PCR. The data obtained from patients and controls that were analyzed using both tests, showed no significant correlation between the CK-ICC test and the CK20 RT-PCR test (Chi-square test, $P = 0.948$ and $P = 0.811$, respectively), which may relate to the difference in techniques and markers used.

Correlation with clinicopathological parameters

With CK20 RT-PCR significantly more patients diagnosed with four or more liver metastases had bone marrow samples tested positive as compared to patients with less than four liver metastases (Chi-square test, 23 of 50 vs 3 of 19, $P = 0.021$). With CK-ICC significantly more patients with extrahepatic metastases at time of surgery had a positive bone marrow as compared to patients without extrahepatic metastases (Chi-square test, 6 of 12 vs 9 of 57, $P = 0.009$). No significant difference in extrahepatic metastases at the time of surgery was seen between CK20 RT-PCR positive and negative patients (Chi-square test, 4 of 26 vs 11 of 43, $P = 0.320$).

No significant difference in the CK-ICC test (Chi-square test, $P = 0.067$) or CK20 RT-PCR (Chi-square test, $P = 0.683$) was seen between the patient group with preoperative chemotherapy and the patient group without preoperative chemotherapy.

Survival analysis

We analyzed whether a positive CK-ICC or CK20 RT-PCR test could predict disease-related survival. Twenty-two patients who did not undergo the planned locoregional treatment and four treated patients who died because of none disease-related causes were excluded for analysis leading to a total of 55 patients.

An univariate analysis for disease-related survival showed a serum carcinoembryonic antigen (CEA) level higher than 200 micrograms per liter, four or more liver metastases, isolated liver perfusion and a positive CK20 RT-PCR test to be prognostic factors (*Table 2*). These variables were entered in the multivariate analysis. A positive CK20 RT-PCR test (HR 2.5, 95% CI 1.2 to 5.2, $P = 0.014$) and a serum CEA level higher than 200 micrograms per liter (HR 2.4, 95% CI 1.0 to 5.9, $P = 0.045$) were demonstrated to be independent predictors for reduced disease-related survival in a multivariate analysis. From the diagnosis of the primary tumor, a median disease-related survival of 4.6 years (range 1.5 to 10.7 years) was seen for patients with a negative CK20 RT-PCR test ($n = 25$) compared to 3.5 years (range 1.0 to 6.2 years) for patients with a positive CK20 RT-PCR test ($n = 18$) (Logrank test, $P = 0.010$). A serum CEA level of 200 micrograms or less per liter ($n = 45$) led to a median disease-related survival of 4.2 years (range 1.1 to 10.7 years) compared to 2.1 years (range 1.0 to 4.6 years) if serum CEA higher than 200 micrograms ($n = 10$) (Logrank test, $P = 0.001$).

Table 2. Univariate analysis for disease-related survival (n=55)^a

Characteristics	Disease-related survival		
	HR	95% CI	P
TNM stage of primary tumor			.680 ^b
1	1		
2	0.7	(0.2-3.0)	.653
3	0.6	(0.2-2.3)	.487
4	0.5	(0.2-1.7)	.277
Nodal status of primary tumor			
Positive vs negative	1.4	(0.7-2.8)	.303
Time span between PT and LM (months)			
< 12 vs ≥ 12	1.3	(0.7-2.7)	.429
Preoperative systemic chemotherapy			
No vs yes	0.7	(0.4-1.3)	.214
Serum CEA level ^c			
> 200 µg/l vs ≤ 200 µg/l	3.2	(1.5-6.6)	.002
No of liver metastases ^c			
≥ 4 vs < 4	2.1	(1.0-4.4)	.038
Maximal diameter of liver metastases (cm) ^c			
> 5 vs ≤ 5	1.2	(0.6-2.3)	.583
Surgical intervention			
Resection	1		
Isolated liver perfusion	2.1	(1.1-4.0)	.025
CK20 RT-PCR test in bone marrow (n=43)			
Positive vs negative	2.6	(1.3-5.5)	.009
CK-ICC test in bone marrow (n=49)			
Positive vs negative	0.9	(0.3-2.3)	.800

PT, primary tumor; LM, liver metastases; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; HR, hazard ratio; CI, confidence interval; The second category is used as the reference category; ^a Patients who did not undergo locoregional treatment (n=22) or patients with extrahepatic disease at the time of surgery (n=15) or patients who died due to none disease-related causes (n=6) were excluded from this analysis; ^b Overall P value; ^c Serum CEA level was dichotomized according to Fong *et al.*⁴ and the number of metastases and maximal diameter were dichotomized according to Nordlinger *et al.*¹⁰

DISCUSSION

This study shows that a positive CK20 RT-PCR test in bone marrow and a serum CEA level higher than 200 micrograms per liter were independent predictors of reduced disease-related survival. We found similar results as published by Koch *et al.*¹⁶ who examined bone marrow samples from 25 patients with colorectal liver metastases who underwent surgical resection. They found a positive CK20 RT-PCR test to be an independent prognostic factor for recurrence-free survival. Vlems *et al.*¹⁷ analyzed bone marrow from 22 patients undergoing hepatic resection for colorectal liver metastases. They showed that patients without treatment with chemotherapy before liver resection did not have CK20 RT-PCR positive bone marrow samples.

In contrast, in our study no difference in RT-PCR test results was observed between patients with and without preoperative chemotherapy. This finding may be explained by the use of quantitative RT-PCR in our study compared to conventional RT-PCR in their study. Another explanation might be the time interval between the end of the chemotherapy and surgery. In our study, a median of 3 months was found compared to one month in the study by Vlems *et al.*¹⁷ The additional value of our study compared to the above-mentioned studies lies in the sensitive detection techniques we used i.e. quantitative RT-PCR and CK-ICC and in the number of patients studied. Although, our patient study group did not consist of patients treated with resection only but also with isolated hepatic perfusion, we found similar results in the patient group who underwent resection of colorectal liver metastases.

Previous studies have developed a prognostic clinical MSKCC score (Memorial Sloan-Kettering Cancer Center) to assist in colorectal cancer patient selection for liver tumor resection.^{4,10,16} This clinical score consists of the following clinicopathological parameters predicting a worse clinical outcome: a positive nodal status of the primary tumor, less than 12 months between the primary tumor and the liver metastases, 4 or more liver metastases, metastases diameter more than 5 cm and serum CEA level higher than 200 micrograms per liter. When our results are confirmed in a larger patient set, the analysis of bone marrow samples could be added to this clinical score. Moreover, in cases in which the preoperative evaluation concluded that resection was not feasible and isolated hepatic perfusion was the only option, results from bone marrow analysis might be useful in making treatment decisions. Since curation or significant improvement of survival by isolated hepatic perfusion has yet to be unequivocally proven one might consider a strategy without surgical locoregional treatment in those cases that have a high risk for a reduced survival or to combine it upfront or after surgery with systemic treatment. Recently was reported that perioperative additional treatment with oxaliplatin, leucovorin and 5-fluorouracil improved progression free survival over surgery alone in patients whose metastases were actually resected.¹⁸

No relation between a positive CK-ICC test and disease-related survival was seen in our study whereas a positive CK-ICC test did correlate with the presence of extrahepatic disease at the time of surgery. Also, no correlation was seen between the CK-ICC test and the CK20 RT-PCR test. Several facts must be taking into consideration in trying to explain the difference between CK-ICC and RT-PCR test results. First, different markers were used for the tests. Primers specific for CK20 for RT-PCR and antibodies recognizing CK8, 18 and 19 for CK-ICC were used. Second, RNA which more likely represents active production of cytokeratin might be produced by colon-cancer-initiating cells whereas CK-ICC may detect differentiated tumor cells with already intracellular produced cytokeratin.¹⁹ Recently, it was shown that undifferentiated colon-cancer-initiating cells which did not show CK20 protein expression were responsible for the tumorigenic potential

whereas differentiated colon cancer cells immunohistochemically stained with anti-CK20 antibodies had lost their ability to form tumors in immunocompromised mice.²⁰ For breast cancer, tumor cells detected with the CK-ICC test are found to be of major prognostic significance.²¹ The bone marrow of these cancer patients not just reflects the spread of metastases but is also a 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.²² Nonetheless, we found the CK20 RT-PCR test in bone marrow of patients with colorectal liver metastases to be of prognostic value.

Curiously, bone marrow from a number of control patients also tested positive with both the CK-ICC and the CK20 RT-PCR tests. These control patients did not have a malignant disease. Other groups report false positive percentages as high as 22%-61%.¹⁵ Contamination or cross-reactivity of the monoclonal antibody with related epitopes present on hematopoietic cells or circulating normal epithelial cells can be responsible. For immunocytochemistry the morphological examination of immunostained cells is therefore essential and for this reason standardized by the International Society for Cellular Therapy Committee (ISHAGE).^{14;15} Several studies have shown that careful morphological examination is essential to identify the prognostic relevance of cytokeratin positive cells.^{21;23} In our study, we saw a correlation between a positive CK-ICC test and the presence of extrahepatic disease at the time of surgery but no prognostic significance was found of a CK-ICC test in the patient group who underwent locoregional treatment for their colorectal liver metastases.

In conclusion, the presence of disseminated tumor cells in bone marrow detected with CK20 RT-PCR, is associated with a worse clinical outcome. Disseminated tumor cells in bone marrow detected with the CK-ICC test failed to show prognostic significance. CK20 RT-PCR may therefore be a more valuable diagnostic tool than the CK-ICC test in patients with colorectal liver metastases. The fact, however, that numerous control patients had positive bone marrow both by RT-PCR and CK-ICC stresses the need for research on more specific molecular markers or antibodies. The analysis of preoperatively aspirated bone marrow from patients with colorectal liver metastases for the presence of disseminated tumor cells may have additional value to the current clinicopathological characteristics in identifying high-risk patients who may benefit from additional systemic treatment.

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CHAPTER EIGHT

SIALYL LEWIS X EXPRESSION AND LYMPHATIC MICROVESSEL DENSITY IN PRIMARY TUMORS OF NODE-NEGATIVE COLORECTAL CANCER PATIENTS PREDICT DISEASE RECURRENCE

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ABSTRACT

Background: Up to 30% of curatively resected colorectal cancer patients with tumor-negative lymph nodes, show disease recurrence. We assessed whether these high-risk patients can be identified by examining primary tumors for the following blood and lymphatic vasculature markers: A) sialyl Lewis X (sLeX), vascular endothelial growth factor (VEGF)-C and VEGF-D expression; B) blood and lymphatic microvessel density (BMVD/LMVD); and C) the presence of blood and lymphatic vessel invasion.

Patients and methods: Thirty-six cases (disease recurrence within 5 years) and 72 controls (no disease recurrence for at least 5 years) were selected in a case-control design. Tumor sections were stained by antibodies CSLEX1 (sLeX), anti-VEGF-C, anti-VEGF-D, anti-CD31 (BMVD) or D2-40 (LMVD) to determine the parameters as mentioned above.

Results: A multivariate analysis showed sLeX expression and high LMVD (odds ratio 5.1, 95% confidence interval 1.3-20.0 and odds ratio 3.1, 95% confidence interval 1.0-10.0, respectively) to be independent factors predicting disease recurrence. Expression of sLeX correlated with liver metastases ($P = 0.015$). A high LMVD was related to regional intra-abdominal or intrapelvic metastases in lymph nodes and distant metastases other than in the liver and lungs such as peritoneum, bones, brain and adrenal glands ($P = 0.004$). A high BMVD in the invasive front correlated with lung metastases ($P = 0.018$).

Conclusions: We show that high-risk node-negative colorectal cancer patients can be identified by primary tumor assessment for sLeX expression and LMVD. Our results are consistent with the notion that both lymphatic and hematogenous metastasis play a role in colorectal cancer.

INTRODUCTION

In most countries, patients with curatively resected colorectal cancer (CRC) without nodal tumor involvement do not receive adjuvant systemic therapy since meaningful survival benefits have not been proven yet.^{1;2} However, 10 to 30 percent of these patients show disease recurrence.^{3;4} Although, a small survival benefit has been reported in stage II CRC patients who received 5-fluorouracil⁵, adjuvant systemic treatment of all node-negative CRC patients will lead to overtreatment and unnecessary complications due to adverse effects of the treatment. Providing only high-risk patients with adjuvant systemic therapy prevents unnecessary treatment and leads to an optimized cure rate. Lymph node-negative CRC patients at risk for disease recurrence may be identified by tumor features known to determine the process of invasion and metastasis which was the focus of this study.

Invasion and metastasis can be facilitated by proteins which stimulate tumor cell attachment to host cellular or extracellular matrix determinants, proteolysis of extracellular matrix host barriers such as the basement membrane, tumor cell migration and formation of distant metastases.⁶ Tumor cells are expected to express similar molecules as leucocytes to interact with blood and lymphatic microvessel endothelium. Normally, the blood group-related antigen sialyl Lewis X (sLeX) is located on the membrane of neutrophils and binds to the endothelial leukocyte adhesion molecule-1 also known as E-selectin which leads to neutrophil extravasation and migration into tissue.⁷ In cancer, the interaction of sLeX on tumor cells and E-selectin was shown to mediate adhesion of tumor cells to endothelial cells.⁸ Previous publications have reported high sLeX expression on tumor cells to be a prognostic factor in colorectal cancer.^{9;10}

The vascular endothelial growth factor (VEGF) family has been reported to be involved in the formation of blood and lymphatic microvessels¹¹ through which tumor cells disseminate. The VEGF family includes five ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor and three receptors: VEGFR-1, VEGFR-2, and VEGFR-3.¹² Studies have shown that VEGF-C and VEGF-D, which are ligands for VEGFR-3, can induce tumor lymphangiogenesis and lymphatic metastasis.^{13;14}

Also, the number and tumor invasion of blood and lymphatic microvessels have been shown to play a role in the process of metastasis.¹⁵⁻¹⁷

In this study, we examined whether disease recurrence in lymph node-negative CRC patients could be predicted by assessing their primary tumors for: A) the expression of angiogenic and lymphangiogenic factors, sLeX, VEGF-C, and VEGF-D; B) blood and lymphatic microvessel density; and C) the presence of blood and lymphatic vessel invasion.

PATIENTS AND METHODS

Patients

Between January 1981 and December 2001, 1044 patients underwent surgery for a primary CRC at the Leiden University Medical Center. For the present study, a selection was made from the total group of patients with tumor-negative lymph nodes (N0) and without metastases (M0) at the time of surgery (n = 506). Patients who were operated on their first CRC in another hospital, or who were diagnosed with another invasive malignancy before or within five years after the date of diagnosis of the primary colorectal carcinoma, and patients who developed a local recurrence were excluded for the present study. Cases (n = 36) were defined as patients who suffered from regional or distant recurrent disease at least three months after but within five years after the date of diagnosis of primary CRC. Cases developed liver metastases (n = 23), and/or lung metastases (n = 8), and/or regional metastases which were considered intra-abdominal or intrapelvic metastases in lymph nodes or in connective tissue (n = 7), and/or peritoneal metastases (n = 5), and/or other distant metastases (n = 8). The latter consists of brain, bone or adrenal metastases.

Controls were patients who did not develop locoregional or distant disease within five years after diagnosis of primary CRC. For each case two controls were matched for TNM stage, date of incidence and date of birth, leading to a total number of 72 controls. Of patients with rectal carcinoma, two had received preoperative and three had received postoperative radiotherapy. None of the patients had received adjuvant chemotherapy.

A tumor was considered mucinous when more than 50% of its volume consisted of mucinous component. Tumor growth pattern at the invasive margin was classified as either expanding also known as pushing or infiltrative based on the predominant morphology as defined by Jass *et al.*¹⁸ Clinicopathologic characteristics of patients are shown in *Table 1*.

Immunohistochemistry

Four micron sections were cut from formalin-fixed paraffin-embedded tumors, transferred to aminopropylethoxysilane (APES) coated slides, and dried overnight at 37°C. One section of each tumor was stained by hematoxylin and eosin (HE). Five sections of each tumor were respectively stained with CSLEX1 also known as CD15s (Becton Dickinson Pharmingen, San Jose, California, 1:150), a monoclonal antibody that binds to sLeX, polyclonal anti-VEGF-C (Zymed, San Francisco,

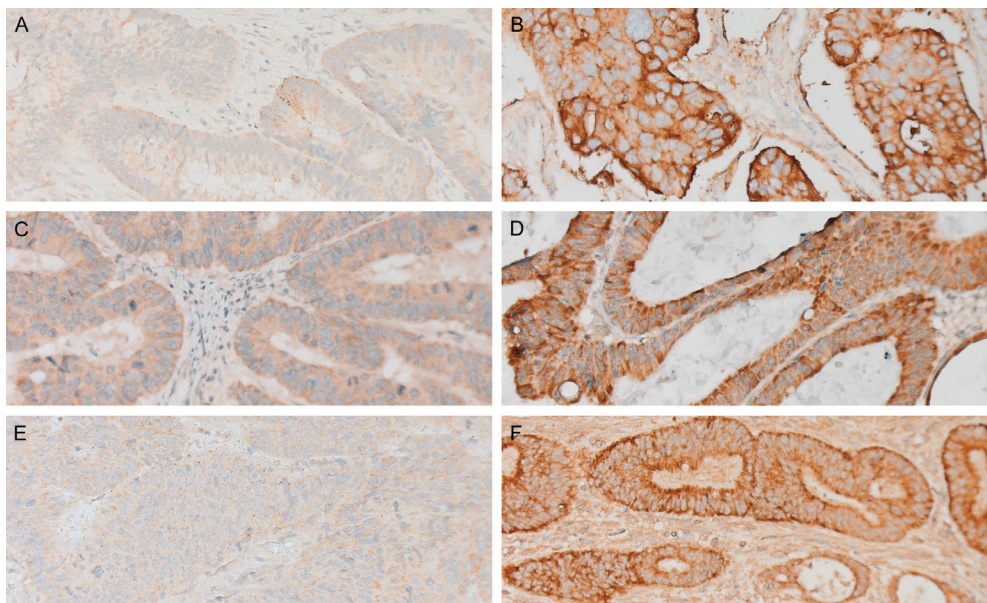


Figure 1. CSLEX1, VEGF-C and VEGF-D expression in colorectal tumors. A tumor negatively stained (A) and positively stained (B) for CSLEX1; this tumor showed both membrane and cytoplasmic brown staining. A tumor with negative (C) and positive cytoplasmic brown staining (D) for VEGF-C; similar faint cytoplasmic brown staining as shown in negatively stained tumors for VEGF-C was seen in phosphate buffered saline controls for the VEGF-C staining; this background staining could be clearly differentiated from positive cytoplasmic brown staining for VEGF-C. A tumor with negative (E) and positive cytoplasmic brown staining (F) for VEGF-D; a brown stromal background staining was seen.

California, 1:100), monoclonal anti-VEGF-D (Research and Development Systems, Minneapolis, USA, 1:400), monoclonal antibody anti-CD31 (Dako, Glostrup, Denmark, 1:300), or monoclonal antibody D2-40 (Signet Laboratories, Dedham, MA, USA, 1:100). All primary antibodies were diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumine (BSA). As negative control the sections were incubated with only PBS/1% BSA. For the immunohistochemical staining procedure, the sections were deparaffinized in xylene. Endogenous peroxidase was blocked in methanol containing 0.3% hydrogen peroxide at room temperature for 20 minutes. Subsequently the sections were rehydrated. After washing in PBS, antigen retrieval treatment for the antibodies CSLEX1, anti-VEGF-C and anti-VEGF-D was done by boiling the sections in 1 mM EDTA (ethylene diamine tetra acetic acid, pH 8.0) solution during 10 minutes. Antigen retrieval for anti-CD31 and D2-40 was done by boiling the sections in a 0.01 M sodium citrate solution (pH 6.0) during 10 minutes. After applying the primary antibodies, the sections were incubated overnight at room temperature. Detection of the antigen was achieved by incubating the sections for 30 minutes with the biotinylated rabbit-anti-mouse conjugate (Dako, Glostrup, Denmark, 1:200) or with biotinylated swine-anti-

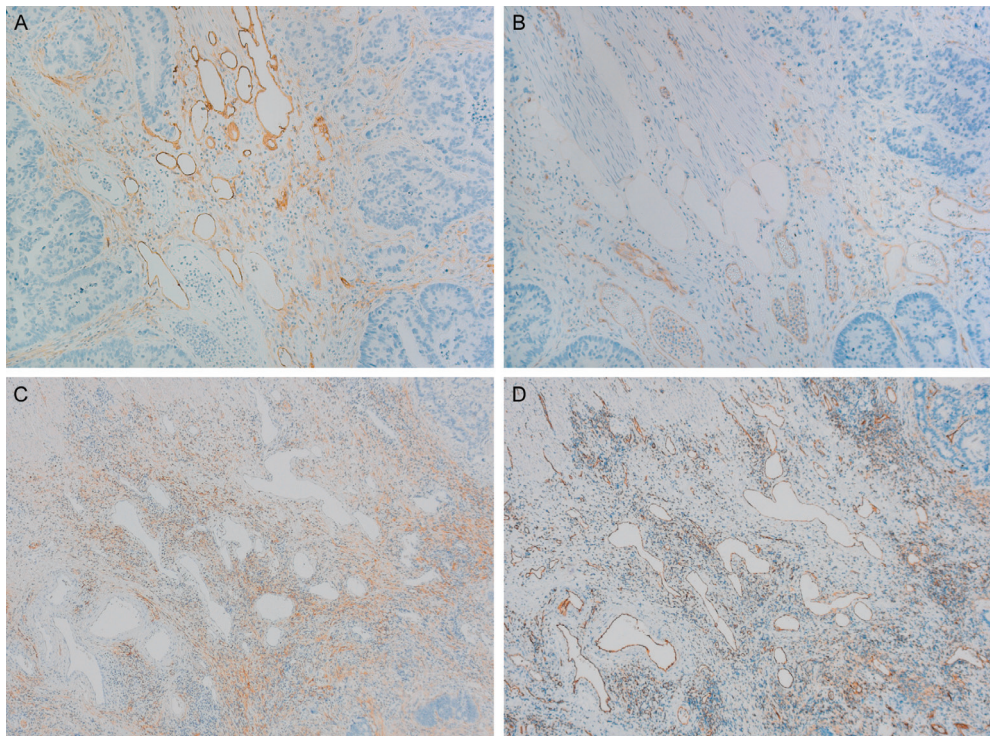


Figure 2. Visualization of lymphatic and blood vessels in colorectal tumors. Lymphatic vessels were stained brown when using immunohistochemistry by the antibodies D2-40 (A). These lymphatic vessels showed no staining by immunohistochemistry when using antibodies CD31 (B). Blood vessels were not recognized by D2-40 (C) but were brown stained by CD31 (D).

rabbit conjugate (Dako, Glostrup, Denmark, 1:400) for VEGF-C and incubation for 30 minutes with a streptavidin-biotin-peroxidase conjugate (DAKO, Glostrup, Denmark, 1:100). For the staining with anti-CD31 and D2-40, detection of the antigen was achieved with Envision (Dako, Glostrup, Denmark). The sections were developed in 3,3-di-amino-benzidine tetrahydrochloride (DAB) substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide leading to brown-staining of the antigen. Counterstaining was done with Mayer's Hematoxylin. For microscopic analysis the sections were dehydrated and mounted in pertex. Tumor cells positive for sLeX, VEGF-C or VEGF-D and vessels positive for CD31 or D2-40 stained brown.

Quantification

Slides were evaluated by using a standard light microscope. At least two investigators (Doekhie, Morreau, Speetjens or Dekker) independently examined each staining and discordant cases were re-evaluated to reach consensus. Tissue sections which were too much damaged by the staining procedure were excluded from further analysis, leading to a variable number of examined slides (*Table 2 and 3*).

Sialyl Lewis X staining with CSLEX1 showed immunoreactivity of apical cell membranes, cytoplasm of tumor cells and secretory material in luminal spaces as reported previously.⁹ For the sLeX expression the percentage of cytoplasmic stained tumor cells and apical membrane stained tumor cells were separately estimated at 100x magnification. VEGF-C and VEGF-D expression showed cytoplasmic localization. In these stainings, the percentage of cytoplasmic stained tumor cells was estimated, also at 100x magnification.

Microvessel density was assessed in sections stained by anti-CD31 and D2-40 antibodies, as described previously.¹⁵ Three fields with the highest vascular density (hot spots) were identified both within intratumoral stroma and the invasive front if present, using 50x and 100x magnification. Subsequently, the number of vessels was counted using 200x magnification after applying a 10x10 grid on the hot spots corresponding to an area of 0.25 mm². All vessels hitting two of the sides of the grid were included in the count, whereas vessels hitting the other two sides of the grid were excluded from the count, using the principles of Gundersen *et al.*¹⁹ Microvessels in the submucosa served as internal controls in assessing the quality of staining for CD31 and D2-40. Any immunoreactive endothelial cell or endothelial cell cluster that was clearly separated from the adjacent microvessels was considered as a single countable microvessel. The occasionally found immunoreactive lymphocytes, macrophages, and plasma cells were excluded on the basis of the staining pattern and cell morphology. Regions of necrosis were excluded from analysis.

Blood and lymphatic vessel invasion was assessed in HE stained sections and in sections stained with antibodies directed against CD31 or D2-40.

In HE stained sections, the presence of tumor cells in luminal spaces lined by endothelial cells in peritumoral stroma was scored as blood or lymphatic vessel invasion. On immunohistochemically stained sections, the presence of tumor cells inside a CD31 or D2-40 stained vessel was considered as blood or lymphatic vessel invasion.

Statistical analysis

Statistical analysis was carried out using SPSS software, version 12.0.1 (SPSS Inc, Chicago, IL). For both blood and lymphatic vessels, statistical analysis was performed using the maximum number of counted vessels in the hot spots as was also done in a recent study in which the same antibodies were used.²⁰ Numerical data are presented as mean $\pm$ standard deviation or as median and range in case of skewness. The clinicopathologic features of cases and controls were compared either by a Chi-square test, Student T-test or Mann-Whitney test. Univariate and multivariate odds ratio's (OR), 95% confidence intervals (95% CI) and p-values (P) were calculated by applying logistic regression analysis with patient's clinical outcome as dependent variable. Parameters with a (overall) p-value less than 0.10 in the univariate analysis were entered into the multivariate logistic regression model.

RESULTS

Standard patient and histopathological characteristics

No significant difference in sex, T stage, tumor size, tumor location, tumor differentiation, mucinous tumors, number of harvested lymph nodes and serum CEA level was seen between the case and control group. We did see that patients from the case group were significantly more often identified with tumors showing an infiltrative growth pattern in comparison to patients from the control group (Chi-square test, 41% vs 21%, $P = 0.031$). Patient characteristics are shown in *Table 1* and univariate analysis of clinicopathological characteristics is shown in *Table 4*.

Expression of sialyl Lewis X, VEGF-C and VEGF-D

Membrane and cytoplasmic staining for CSLEX1 were scored separately. Membrane and cytoplasmic staining respectively was seen in 91 and 74 of 99 examined CSLEX1 stained sections. Significantly more cases than controls showed cytoplasmic staining for CSLEX1 (Chi-square test, 91% vs 67%, $P = 0.012$) (*Table 2*). No difference in membrane staining was seen between the case and control group.

More cases than controls were identified with cytoplasmic positively stained tumor cells for VEGF-C (Chi-square test, 44% vs 27%, $P = 0.093$) and VEGF-D (Fisher's exact test, 21% vs 6%, $P = 0.039$) (*Table 2*). Representative immunohistochemical stainings for CSLEX1, VEGF-C and VEGF-D are shown in *Figure 1*. Cytoplasmic background staining was seen in negative controles for the VEGF-C staining.

Tumors negative for VEGF-C showed similar background staining which could be clearly differentiated from positive staining for VEGF-C (*Figure 1C, D*). The univariate analysis of these angiogenesis and lymphangiogenesis-related tumor cell markers is shown in *Table 2*.

Blood and lymphatic microvessel density

We choose to count blood and lymphatic microvessels in three hot spots (i.e. fields with the highest vascular density) located in intratumoral stroma and three hot spots at the invasive front. Blood microvessels stained by anti-CD31 antibodies were seen in tumor stroma or the invasive front in all of 96 analyzed primary tumors (100%) and lymphatic microvessels stained by D2-40 antibodies were seen in 78 of 96 (81%) analyzed primary tumors. As previously shown^{21;22}, we found that anti-CD31 antibodies did not stain D2-40 positive lymphatic vessels and vice versa (*Figure 2*). We counted more blood microvessels (39 ± 12 ; median 37; range 12 to 80) than lymphatic microvessels (14 ± 9 ; median 15; range

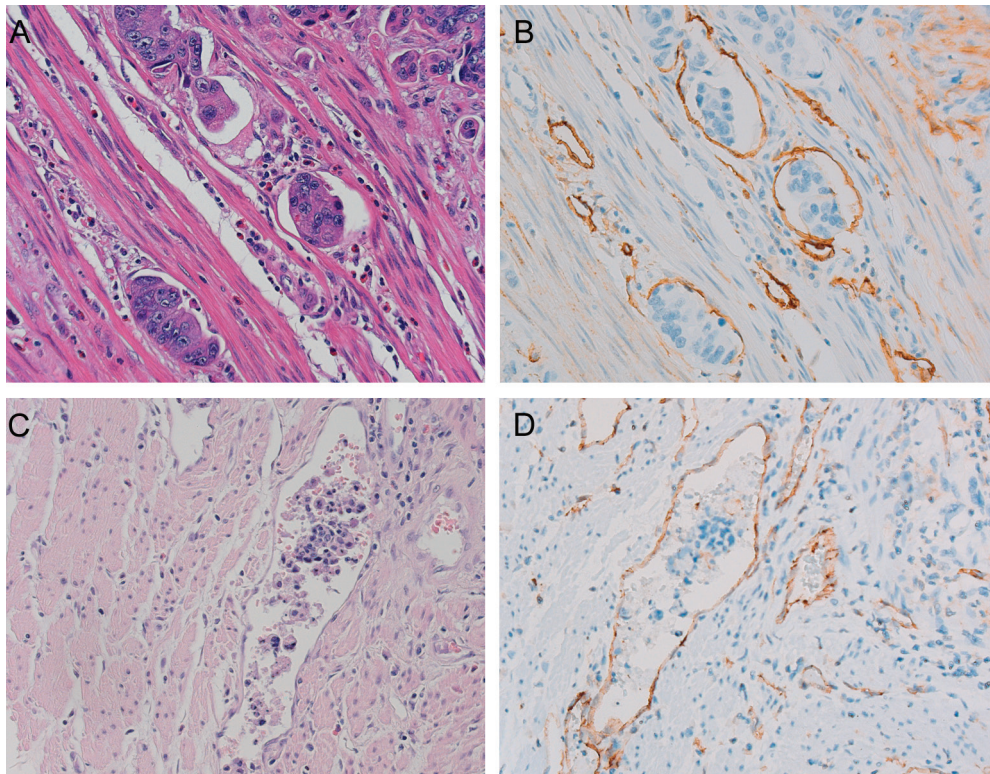


Figure 3. Tumor cell invasion in lymphatic and blood vessels in colorectal tumors. Lymphatic vessel invasion seen on a hematoxylin and eosin stained section (**A**) and immunohistochemically stained section by the antibodies D2-40 (**B**); lymphatic vessels were brown stained by D2-40. Blood vessel invasion seen on a hematoxylin and eosin stained section (**C**) and immunohistochemically stained section by the antibodies CD31 (**D**); blood vessels were brown stained by CD31.

Table 1. Patient and primary tumor characteristics

Characteristics	All patients (n=108)		Cases (n=36)		Controls (n=72)		P ^a
	n	%	n	%	n	%	
Sex							
Female	53	49	16	44	37	51	.496
Male	55	51	20	56	35	49	
Age (years) ^b	67 ± 12		67 ± 12		67 ± 12		.911 ^c
TNM stage ^d							
I	6	6	2	6	4	6	1.000 ^e
II	102	94	34	94	68	94	
T stage ^f							
T2	6	6	2	6	4	6	.126
T3	88	82	26	72	62	86	
T4	14	13	8	22	6	8	
Tumor size (cm) (n=104) ^{b, g}	5.1 ± 2.0		4.9 ± 1.9 (n=34)		5.2 ± 2.1 (=70)		.539 ^c
Tumor location							
Colon (coecum – sigmoid)	89	82	30	83	59	82	.858
Rectum (rectosigmoid – rectum)	19	18	6	17	13	18	
Differentiation							
Good	27	25	9	25	18	25	.912
Moderate	68	63	22	61	46	64	
Poor	13	12	5	14	8	11	
Mucinous							
No	98	91	32	89	66	92	.728 ^e
Yes	10	9	4	11	6	8	
Growth pattern (n=97) ^h							
Expanding or pushing	70	72	20	60	50	79	.031
Infiltrating	27	28	14	41	13	21	
Number of lymph nodes ⁱ	6 (1-26)		6 (1-18)		6 (1-26)		.096 ^j
Preoperative serum CEA level (n=43) ^k							
< 6 µg/l	30	70	5	56	25	74	.417 ^e
≥ 6 µg/l	13	30	4	44	9	26	

CEA, carcinoembryonic antigen; ^a Chi-square test of cases vs controls, unless mentioned otherwise;^b Presented as mean ± standard deviation; ^c Student T-test; ^d According to the 6th edition of the TNM classification³⁹; ^e Fisher's exact test; ^f If T2 and T3 stage were combined, the P value was 0.043; this comparison was therefore used in the logistic regression; ^g Tumor size could not be found in pathology reports from four patients; ^h Patients were excluded from analysis if no invasive front was found in primary tumor sections; ⁱ Presented as median and range between brackets; ^j Mann-Whitney test;^k Serum CEA had been determined in only 43 of 108 patients as it was not a standard procedure.

Table 2. Sialyl Lewis X and vascular endothelial growth factor C and D cytoplasmic staining in primary colorectal tumors (n=99)^a

	All patients		Cases		Controls		Univariate analysis			
	n	%	n	%	n	%	P ^b	OR	95% CI	P
CSLEX1										
Negative	25	25	3	9	22	33	.012	1		
Positive	74	75	29	91	45	67		4.7	(1.3-17.2)	.019
VEGF-C										
Negative	67	68	18	56	49	73	.093	1		
Positive	32	32	14	44	18	27		2.1	(0.9-5.1)	.096
VEGF-D										
Negative	88	89	26	79	62	94	.039 ^c	1		
Positive	11	11	7	21	4	6		4.2	(1.1-15.5)	.033

VEGF, vascular endothelial growth factor; OR, odds ratio; CI, confidence interval; ^a Primary tumors from three controls could not be retrieved from the archive and tissue sections which were destroyed after staining were excluded from analysis leading to a lower number of examined slides; ^b Chi-square test of cases vs controls, unless mentioned otherwise; ^c Fisher's exact test.

0 to 42). Patients with more than 46 blood microvessels in the invasive front (high BMVD) were more often seen in the case than in the control group (Fisher's exact test, 25% vs 10%, $P = 0.077$), but it did not reach significance (*Table 3*). Significantly more patients with more than 20 lymphatic microvessels in tumor stroma or invasive front (high LMVD) were seen in the case group than in the control group (Chi-square test, 33% vs 14%, $P = 0.027$) (*Table 3*).

Concluding, overall more blood microvessels than lymphatic microvessels were observed. In the case group, a higher number of blood and lymphatic microvessels was seen compared to the control group.

Blood and lymphatic vessel invasion

Blood and lymphatic vessel invasion detected on HE stained sections will be referred to as morphological vaso-invasion. In 49 of 108 (45%) primary tumors, morphological vaso-invasion was found.

Lymphatic vessel invasion was seen in 8 of 96 (8%) D2-40 stained tumor sections (*Figure 3A, B*) and blood vessel invasion was only seen in one of 96 (1%) sections stained with antibodies directed against CD31 (*Figure 3C, D*). Seven of the 8 patients with lymphatic vessel invasion detected on the D2-40 stained sections had been recognized on the HE stained sections and the one tumor with blood vessel invasion was also seen on the HE stained section. These results show that morphological vaso-invasion scored on HE stained sections overestimated the actual blood and lymphatic vessel invasion as seen after immunohistochemical staining.

Table 3. Microvessel density and microvessel tumor invasion in primary colorectal tumors^a

	All patients		Cases		Controls		Univariate analysis			
	n	%	n	%	n	%	P ^b	OR	95% CI	P
BMVD invasive front (n=90)^{c, d}										
Low	76	84	24	75	52	90	.077 ^e	1		
High	14	16	8	25	6	10		2.9	(0.9-9.3)	.074
LMVD tumor stroma or invasive front (n=91)^{c, f}										
Low	72	79	22	67	50	86	.027	1		
High	19	21	11	33	8	14		3.1	(1.1-8.8)	.032
Blood vessel invasion (n=96)										
Absent	95	99	32	97	63	100	.344 ^e	NA		
Present	1	1	1	3	0	0				
Lymphatic vessel invasion (n=96)										
Absent	88	92	28	85	60	95	.119 ^e	1		
Present	8	8	5	15	3	5		3.6	(0.8-16)	.096

BMVD, blood microvessel density; LMVD, lymphatic microvessel density; OR, odds ratio; CI, confidence interval; NA, not assessable; ^a Primary tumors from three controls could not be retrieved from the archive and tissue sections which were destroyed after staining were excluded from analysis leading to a variable number of examined slides; blood microvessels were stained with anti-CD31 antibodies and lymphatic vessels were stained with D2-40 antibodies; ^b Chi-square test of cases vs controls, unless mentioned otherwise; ^c Maximum number of counted vessels from hot spots; invasive front was not present on all sections; ^d High: greater than 46; ^e Fisher's exact test; ^f High: greater than 20.

Relation between studied variables

We assessed mutual correlation between the different markers that were investigated. We found that 10 of 11 tumors showing positive staining for VEGF-D were also positive for VEGF-C (Chi-square test, $P < 0.001$).

Significantly more patients with positively stained tumor cells for VEGF-C showed a high LMVD compared to patients negative for VEGF-C (Chi-square test, 11 of 31 vs 8 of 59, $P = 0.015$) indicating an association between VEGF-C expression and lymphangiogenesis. Sialyl Lewis X expression seemed to be related to an infiltrating type of tumor growth as significantly more of these tumors showed cytoplasmic positive staining with CSLEX1 (Chi-square test, 24 of 27 vs 46 of 68, $P = 0.034$) compared to tumors with an expanding growth pattern. Also significantly more infiltrating tumors showed morphological vaso-invasion compared to expanding tumors (Chi-square test, 19 of 27 vs 27 of 70, $P = 0.005$). This correlation suggests a role of infiltrating tumor growth in the overestimation of blood and lymphatic vessel invasion in the morphological vaso-invasion score.

Table 4. Univariate analysis of clinicopathological characteristics

Characteristics	Univariate analysis		
	OR	95% CI	P
Sex			
Female	1		
Male	1.3	(0.6-3.0)	.497
Age (years)	1.0	(0.97-1.03)	.910
T stage			
T2 and T3	1		
T4	3.1	(1.0-9.9)	.050
Tumor size (cm) (n=104)	0.9	(0.8-1.2)	.536
Tumor location			
Colon (coecum – sigmoid)	1		
Rectum (rectosigmoid – rectum)	0.9	(0.3-2.6)	.858
Differentiation			.913 ^a
Good	1		
Moderate	1.0	(0.4-2.5)	.927
Poor	1.3	(0.3-4.9)	.750
Number of lymph nodes	0.9	(0.9-1.0)	.132
Mucinous			
No	1		
Yes	1.4	(0.4-5.2)	.640
Growth pattern			
Expanding or pushing	1		
Infiltrating	2.7	(1.1-6.7)	.034
Preoperative serum CEA level (n=43)			
< 6 µg/l	1		
≥ 6 µg/l	2.2	(0.5-10.2)	.303

OR, odds ratio; CI, confidence interval; CEA, carcinoembryonic antigen; ^a Overall P value.

Logistic regression analysis

All variables with a P value lower than 0.10 in the univariate analysis (*Tables 2, 3 and 4*) were entered in a forward step multivariate analysis. T stage, tumor growth pattern, CSLEX1-, VEGF-C- or VEGF-D-expression, high BMVD, high LMVD and lymphatic vessel invasion were entered in the multivariate analysis. The independent factors predicting disease recurrence that remained were sLeX tumor expression by CSLEX1 and a high LMVD (OR 5.1, 95% CI 1.3-20.0 and OR 3.1, 95% CI 1.0-10.0, respectively).

Correlation with site of metastases

The site of the metastases was registered in our hospital, which enabled us to assess its correlation with the different variables tested. Primary tumors with CSLEX1 expression significantly more often led to disease recurrence in the liver compared to primary tumors negative for CSLEX1 (Chi-square test, 20 of 74 vs one of 25, $P = 0.015$). Liver metastases were also significantly more often seen in the patients with infiltrative tumors than in patients with expanding tumors (Chi-square test, 11 of 27 vs 11 of 70, $P = 0.008$).

Patients with a high BMVD in the invasive front developed lung metastases significantly more often than patients with a low BMVD in the invasive front (Fisher's exact test, 4 of 14 vs 4 of 76, $P = 0.018$).

The chance to develop regional, peritoneal or metastases to the brain, bones or adrenal glands was significantly higher in patients with high LMVD compared to patients with low LMVD (Fisher's exact test, 8 of 19 vs 8 of 72, $P = 0.004$). This chance also significantly increased with the T stage (Fisher's exact, T4 stage: 6 of 14 vs T2 or T3 stage: 12 of 94, $P = 0.012$) and decreased with the number of harvested lymph nodes (Fisher's exact test, 0 of 22 in patients with 12 or more examined lymph nodes vs 18 of 86 in patients with less than 12 lymph nodes, $P = 0.021$). Moreover, no correlation was seen between a high LMVD, T4 stage or 12 or more harvested lymph nodes. When entering these three variables in a multivariate analysis, a high LMVD remained as an independent predictor for regional, peritoneal or metastases to distant sites other than liver and lungs (OR 7.3, 95% CI 2.0-27.4, $P = 0.003$).

DISCUSSION

In this study, we show sLeX expression and a high LMVD of the primary tumor to be independent risk factors for disease recurrence in curatively resected CRC patients with tumor-negative lymph nodes. Our results confirm data from Nakagoe *et al.*⁹ showing that lymph node-negative CRC patients with sLeX expression detected with CSLEX1 have a worse prognosis. Moreover, we showed a significant correlation between sLeX expression and liver metastases as previously reported by others.^{23;24} We saw a correlation between sLeX expression and infiltrative tumor growth pattern and showed the latter also to correlate with disease recurrence.¹⁸ The reproducibility of tumor growth pattern assessment has been shown to be problematic²⁵ which suggests growth pattern to be an unreliable prognostic marker in contrast with sLeX immunohistochemical detection. Our results suggest that sLeX expression plays a role in infiltrative tumor growth and in facilitating the hematogenous spread of tumor cells through blood microvessels via the portal vein to the liver.

The lymphatic system has also been believed to be one of the most important pathways for tumor cell dissemination as it is expected that tumor cells can enter lymphatic microvessels easier than blood microvessels because the former show a discontinuous or completely absent basement membrane and

are devoid of pericytes.²⁶ Years of research have resulted in several lymphatic endothelial cell specific markers.²⁶ In this study, we used D2-40 which was reported to be more sensitive in detecting lymphatic endothelium than Prox1, LYVE-1 and podoplanin.²⁷ We found a high LMVD detected with D2-40 to be an independent risk factor for disease recurrence. Similar findings were seen by Matsumoto *et al.*²⁸ who used the anti-podoplanin antibody to detect lymphatic microvessels in primary tumors of 106 stage I to IV CRC patients. They showed a high LMVD and lymphatic vessel invasion to correlate with a poor outcome but only the former remained as an independent predictor in the multivariate analysis. Saad *et al.*²⁰ examined BMVD and LMVD in 90 stage I to IV CRC patients by using anti-CD31 and D2-40 antibodies, respectively. They observed a significant correlation between LMVD and liver metastases, but they did not analyze other types of distant metastases. In our study, a high LMVD was found not to correlate with liver metastases or lung metastases but with regional intra-abdominal or intrapelvic metastases in lymph nodes and other distant metastases such as peritoneum, bones, brain and adrenal glands. We suggest that a high LMVD leads to tumor cell dissemination through lymphatic microvessels into intra-abdominal or pelvic lymph nodes. The lymphatic system finally returns lymph to the systemic blood circulation via the thoracic duct leading to metastases in the bones, brain and other distant sites.

Additionally, a high BMVD correlated with disease recurrence restricted to the lungs. This may explain the variability in published studies regarding the prognostic relevance of BMVD as it probably depends on the number of patients in those particular studies who suffered from disease recurrence in the lungs. Accordingly, a recent meta-analysis showed a high BMVD, detected by using antibodies directed against CD31 or CD34, to significantly predict poor clinical outcome.²⁹

We assessed tumor invasion in blood and lymphatic vessels on HE stained sections and referred to this as morphological vaso-invasion. By comparing morphological vaso-invasion and blood and lymphatic vessel invasion scored on immunohistochemically stained sections, we found the former to be overestimated as reported previously by others.³⁰ Analysis suggested that scoring of morphological vaso-invasion does not distinguish between blood or lymphatic vessel invasion and an infiltrative tumor growth pattern. Previous studies have shown that artifactual tissue retraction around tumor islands complicate and overestimate true lymphovascular invasion.^{16;17} Immunohistochemical staining of blood and lymphatic vessels with specific antibodies resolves this problem and enables objective estimation of tumor invasion in vessels.

To our knowledge, we are the first to identify a multiple set of tumor markers each correlating with a different preferential site of metastasis in CRC. Our results support two types of mechanisms involved in metastasis. First, there is the mechanical way of tumor cell dissemination through blood and lymphatic microvessels, resulting in tumor cell arrest in the narrow capillary network in different organs including lymph nodes. There they may proliferate and develop clinically evident metastases.

Hematogenous and lymphatic metastasis is expected to increase with the number of blood and lymphatic microvessels. This may explain the correlation between a high BMVD and lung metastases and the correlation between a high LMVD and regional intra-abdominal or intrapelvic metastases in lymph nodes with eventually distant metastases.

In addition to this mechanical entrapment of tumor cells, also biological processes determine outgrowth of metastases as explained by the “seed and soil” theory.^{31;32} This theory suggests that a subpopulation of tumor cells with metastatic potential, recently identified as colon-cancer-initiating cells³³, disseminate through the whole body. These cells proliferate and differentiate to form clinically evident metastases at preferential sites depending on local molecular interaction among which availability of local growth factors. In our study, the correlation between sLeX expression and liver metastases may be explained by this pathway. The interaction of the antigen sLeX on tumor cells and E-selectin on endothelial cells was shown to mediate adhesion of tumor cells to endothelial cells⁸ possibly facilitating tumor cell invasion in blood microvessels, extravasation and migration into tissue. Additionally, colorectal tumor cells showing sLeX expression might prefer the liver to grow out to form clinically evident metastases due to interaction with local E-selectin.³⁴

It is important to realize that this study regards a small patient group but even in these small patient numbers we showed significant findings consistent with current thoughts in terms of hematogenous and lymphatic metastasis. Furthermore, only 20% of our patient group had undergone an adequate lymph node harvest of at least 12 lymph nodes as recommended by the American Joint Committee on Cancer Staging³⁵, which is a limitation of our study. Nevertheless, similar numbers were reported by Johnson *et al.*³⁶ and Baxter *et al.*³⁷ who showed adequate lymph node evaluation in 22% of 569 CRC specimens respectively 37% of 116995 CRC patients. They reported the effect of different pathology assistants, older age and rectal cancer to affect the number of lymph nodes retrieved.^{37;38}

In summary, our study shows that high-risk lymph node-negative CRC patients can be identified by assessing the primary tumor for sLeX expression with CSLEX1 and LMVD. Our results have to be validated in prospective studies which should also evaluate whether these high-risk CRC patients may benefit from adjuvant systemic therapy. Moreover, our results are consistent with the notion that both a lymphatic and hematogenous route plays a role in CRC.

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CHAPTER NINE

SUMMARY AND GENERAL DISCUSSION

SUMMARY

Cancer is a leading cause of death worldwide, accounting for 7.9 million cases in 2007 which comprises around 13% of all deaths.¹ The number of cancer-related deaths is expected to increase with an estimated 9 million in 2015. Overall cancer mortality is mainly caused by solid tumors arising from lung, stomach, liver, colorectum and breast tissue. In this thesis, we will focus on gastric and colorectal cancer with the emphasis on the latter.

Gastric cancer is the second most common cancer worldwide with a varying incidence, being higher in Japan and some parts of South America (> 40 per 100,000) and lower in Western Europe and the United States (< 15 per 100,000).² Environmental exposure rather than genetic factors play a role in the predisposition to gastric cancer.³

Colorectal cancer (CRC) is the third most prevalent cancer worldwide with an estimation of 1.2 million new cases and 630,000 deaths in 2007.⁴ The incidence of CRC is higher in developed countries, making it the second most common cancer in Europe.⁵ The risk for developing CRC increases with age as a result of accumulation of (epi)genetic mutations, with more than 90% of new cases being diagnosed in patients older than 50 years. Most cases of CRC occur in sporadic forms of which about 20% have some component of familial risk.⁶ Approximately 5 to 10% are hereditary.

Surgery is the first choice of treatment in gastric cancer and CRC. After surgery, patients receive adjuvant systemic treatment depending on disease stage. Disease stage is based on the Tumor-Node-Metastasis (TNM) classification system which is one of the most well established prognostic factors in solid tumors.^{7;8} After the introduction in 1987, the TNM system has been revised every few years to allow the incorporation of new evidence. Unfortunately, this staging system does not provide prediction of prognosis for the individual patient leading to both over- and undertreatment.

This thesis assesses whether gastric cancer and CRC patients with minimal metastatic disease at the time of surgery who are at risk for disease recurrence, can be identified by detection of occult tumor cells (OTC) in lymph nodes or bone marrow, or by analyzing the primary tumor for angiogenic and lymphangiogenic markers.

Chapter 1 presents a general introduction and describes the background and outline of this thesis. In short, the TNM staging classification represents the routes of tumor spread namely local invasion (T), lymphatic spread (N) and hematogenous dissemination (M). With the current TNM classification, mostly macroscopical tumor spread is determined by using conventional hematoxylin and eosin stainings and radiological techniques. Therefore, numerous patients who are at risk for disease recurrence after supposedly curative resection are not recognized. By exploring the presence of minimal disease in lymph nodes or

bone marrow or by applying biological markers in the primary tumor related to tumor cell spread, a more individualized portrait of patient's prognosis might be achieved. The first adjustments have been made in the last revision of this staging system since micrometastases (MM; > 0.2 mm and ≤ 2 mm) and isolated tumor cells (ITC; ≤ 0.2 mm) in lymph nodes were incorporated.

In **chapter 2** we investigate whether the presence of OTC in originally considered tumor-negative lymph nodes from curatively resected gastric cancer patients can predict disease recurrence. Also, the value of automated microscopy is evaluated. Forty cases (disease recurrence) and 41 controls (no disease recurrence for at least 5 years) were selected from the previously published multicenter Dutch D1-D2 Gastric Cancer trial.⁹ One tissue section was immunohistochemically stained by anticytokeratin antibodies CAM5.2 and examined by conventional and automated microscopy. There was no significant difference in the presence of OTC, MM or ITC between the case and control groups ($P = 0.658$; $P = 0.691$; $P = 0.887$, respectively). However, significantly more cases than controls presented with 20% or more OTC-positive lymph nodes (Chi-square test, 9 (24%) vs one (3%), $P = 0.015$). A multivariate logistic regression analysis showed that examination of less than five lymph nodes (OR 13.8, 95% CI 1.6-120.6, $P = 0.018$) was the only significant independent risk factor for disease recurrence, especially for locoregional disease recurrence (OR 20.4, 95% CI 2.2-190.8, $P = 0.008$). A similar analysis for distant disease recurrence showed a percentage of 20% or more OTC-positive lymph nodes to be the only significant independent risk factor (OR 15.6, 95% CI 1.6-151.4, $P = 0.018$). The sensitivity of immunohistochemistry evaluated by microscopy to identify cases with 20% or more OTC-positive lymph nodes increased from 8% for conventional microscopy to 22% for automated microscopy (McNemar's test, $P = 0.063$). Our results suggest that locoregional disease recurrence might be the result of lymph nodes that were not removed. In addition, when 20% or more of the lymph nodes are OTC-positive, tumor cells may already have travelled to other sites in the body, eventually leading to distant metastases.

Additionally, this chapter presents an overview of previously published immunohistochemistry studies regarding the clinical relevance of OTC in lymph nodes of patients with gastric cancer. There was an extensive variation in the antibodies that were used and in the reported upstaging percentages. Ten of 16 studies reported a negative impact of the presence of OTC in lymph nodes on the clinical outcome.¹⁰⁻¹² Similar to our study, Siewert *et al.*¹³ also reported clinical significance of the percentage of OTC-positive lymph nodes.

Chapter 3 presents an overview of studies regarding the detection methods and clinical relevance of OTC in lymph nodes in CRC.

Three out of four studies using polymerase chain reaction (PCR) for OTC detection,

showed its prognostic relevance. Certain DNA regions that might have contained tumor-specific mutations in the genes p53 or K-ras were amplified by the PCR assay in these studies. Disadvantages such as a non-consistent occurrence of p53 and K-ras mutations in CRC and the large number of codons or even exons in which mutations can be detected, will prevent PCR from becoming a ubiquitously utilized application for OTC detection.

All six studies using reverse-transcriptase polymerase chain reaction (RT-PCR) to detect OTC in lymph nodes showed a negative impact of OTC on patient's prognosis.¹⁴⁻¹⁹ However, two realtime RT-PCR studies failed to show any significance of OTC in lymph nodes.^{20;21} We concluded that OTC in lymph nodes detected with RT-PCR show prognostic value provided that fresh or frozen lymph nodes, the markers CEA, CK20 and GCC and the optimal number of PCR cycles are used.

Only 9 out of 28 immunohistochemistry studies showed a significantly worse clinical outcome in patients with lymph nodes containing OTC. Particular studies using anticytokeratin antibodies CAM5.2, reported OTC-positive lymph nodes as clinically relevant.

We found upstaging percentages through OTC assessment and the prognostic relevance of OTC in lymph nodes to vary among studies, particularly in the immunohistochemistry studies, which was related to differences in techniques used to detect OTC.

In **chapter 4**, we assess the value of multiple sectioning of lymph nodes combined with immunohistochemical staining in detecting prognostic relevant OTC in CRC patients. The slides were examined by automated microscopy. Although OTC in lymph nodes detected by RT-PCR, more often are of clinical value, the hospital infrastructure for this technique is more demanding due to the need for fresh frozen tissue. Additionally, morphologic assessment of tissue samples analyzed, is warranted. This study was performed on histologically tumor-negative paraffin-embedded lymph nodes previously studied by Liefers *et al.*¹⁴ with RT-PCR for CEA for the detection of OTC. They found a five-year survival rate of 50% for the OTC-positive group and 91% for the negative group ($P = 0.02$).

Lymph node tissue blocks from 20 patients were sectioned (five consecutive sections of 10 levels at 200 μm interval or until no material was left) and immunohistochemically stained using antibodies against cytokeratin, AE1/AE3. The stained slides were analyzed using rapid high capacity flatbed scanning. OTC were detected in all of six (100%) patients with disease recurrence, compared to eight of 14 (57%) patients who did not develop a recurrence (Chi-square test, $P = 0.055$). Five of six (83%) patients who developed disease recurrence were OTC-positive detected by CEA RT-PCR and three of 14 (21%) who showed no recurrence were OTC-positive.

These findings showed that multilevel sectioning combined with immunohistochemical staining followed by automated microscopy had a higher sensitivity

in predicting disease recurrence than RT-PCR (100% vs 83%) but was lower in specificity (43% vs 79%). In this study, no distinction was made between MM and ITC but groups of cells (≥ 2) were separately noted and these were seen in all six patients with disease recurrence and in five of 14 (36%) patients without disease recurrence. Additionally, all patients with disease recurrence were identified after analysis of four levels at 200 μm distance.

Chapter 5 addresses whether detailed examination of lymph nodes by multilevel sectioning and immunohistochemical staining can improve prognostication. Thirty-six cases (disease recurrence within 5 years) and 72 controls (no disease recurrence for at least 5 years) were selected. Tissue sections from paraffin-embedded lymph nodes from four levels at 200 μm interval were immunohistochemically stained with anticytokeratin antibodies, AE1/AE3. These slides were analyzed using conventional and automated microscopy for the presence of tumor cells.

Altogether, the case group showed more MM ($n = 3$) than the control group ($n = 1$). There was no difference in the presence of ITC between the case and control group. Analysis of a second level had led to the additional detection of one case with MM ($n = 1$), one case with macrometastasis ($n = 1$) and two cases and seven controls with ITC ($n = 9$). Examining more than two levels only resulted in detection of additional ITC. All MM and macrometastasis were detected by conventional analysis and automated microscopy only led to detection of more ITC. In a multivariate logistic regression analysis, the presence of macrometastases or MM (OR 34.5, 95% CI 2.7-440, $P = 0.006$), T4 stage (OR 2.9, 95% CI 0.4-23.5, $P = 0.040$) and number of lymph nodes (OR 0.9, 95% CI 0.8-1.0, $P = 0.025$) were independent predictors for disease recurrence. Messerini *et al.*²² and Bilchik *et al.*²³ who also distinguished between MM and ITC reported similar detection rates for MM (9.9% and 3%, respectively). Additionally, the first study also showed prognostic relevance of MM and none for ITC.

Detailed examination of lymph nodes in CRC by multilevel sectioning combined with immunohistochemical staining or RT-PCR in search for ITC is expensive and laborious given the enormous number of lymph nodes that can be harvested. Identification of sentinel lymph nodes (SNs) and selective detailed analysis of only these nodes, is suggested as a possible approach to circumvent this extra workload. Sentinel nodes are the first lymph nodes to drain the primary tumor and therefore have the highest chance of harboring tumor cells (*see introduction, Figure 1*). In **chapter 6**, twenty-five studies on the feasibility and reliability of sentinel node mapping (SNM) in CRC are reviewed with the emphasis on differences in the SNM techniques used. There was a large variation in identification rates and false-negative (FN) rates, ranging from 58% to 100% (average 89%) and 0 to 60% (average 33%), respectively. Factors that might have contributed to failed SNM procedures were incomplete circumferential injection around the tumor,

insufficient volumes of tracer for large tumors, inclusion of advanced tumors and rectal tumors. A major component reported was the learning curve effect, leading to a much higher identification rate of almost 100% in some studies if results from the early phase of the learning curve were not included in the analysis. High FN rates may be explained by extensive nodal replacement and large tumors, which can occlude lymphatic vessels leading to lymph drainage through an alternative route or by a long identification time between injection of the tracer and identification of the SNs, as blue dye travels fast and might have already reached a second echelon lymph nodes. Also, a low FN rate of 20% or less was mostly seen in studies where the number of SNs was limited to the first four blue nodes or the four or five blue nodes closest to the tumor whereas studies reporting a FN rate higher than 20% had a higher number of SNs. The majority of the latter studies considered all blue nodes or radioactive nodes as SNs leading to a wider range of SN. In melanoma and breast cancer lower FN rates are seen. The difference might be in larger tumors found in the colon and rectum and also in the dissimilarity in lymphatic drainage pattern.

Limitations in rectal cancer are the close vicinity of the primary tumor and the pararectal lymph nodes resulting in overlapping radioactivity. Moreover, incising mesorectal tissue during the operation is a contraindication for oncologic adequate removal of rectal cancer. Neoadjuvant radiotherapy and advanced tumors can also hamper the identification of SNs. These factors make SNM in rectal cancer more difficult and of low clinical value.

Two colon cancer studies showed that SNs are significantly more likely to contain tumor cells than nonsentinel nodes^{24;25} whereas conflicting results were seen in a third study published in 2006 in which no difference between SNs and nonsentinel nodes in regard to the frequency of MM or ITC involvement was seen.²⁶ One SNM study in colon cancer reported a higher rate of disease recurrence in patients with SNs containing macrometastases or MM detected by hematoxylin and eosin staining or RT-PCR.²⁷ Nevertheless, the reliability of SNM in colon cancer has not been clarified yet.

In CRC, disseminated tumor cells (DTC) are found in bone marrow although usually no clinically evident metastases develop in bone.^{28;29} We suspect that the presence of DTC in bone marrow might represent the aggressive nature of the tumor and could therefore be used to predict disease recurrence. This is evaluated in **chapter 7** by assessing whether the presence of DTC in bone marrow from 81 patients with colorectal liver metastases who were scheduled for surgical resection or isolated hepatic perfusion, is associated with a worse outcome of disease. The presence of DTC in bone marrow was analyzed by using quantitative RT-PCR for the expression of cytokeratin 20 (CK20) and or immunocytochemistry (CK-ICC) combined with automated microscopy by use of A45-B/B3, anti-cytokeratin antibodies. Bone marrow samples in 26 of 69 (38%) patients tested positive with the CK20 RT-PCR and samples in 15 of 69 (22%) patients tested positive with the CK-ICC test.

In a multivariate Cox regression analysis a positive CK20 RT-PCR test (HR 2.5, 95% CI 1.2 - 5.2, $P = 0.014$) and a serum CEA level greater than 200 micrograms per liter (HR 2.4, 95% CI 1.0 - 5.9, $P = 0.045$) were independent predictors for a reduced disease-related survival. A positive CK-ICC test did not relate to a worse survival but correlated with the presence of extrahepatic disease at the time of surgery (Chi-square test, $P = 0.009$). No correlation was seen between the CK20 RT-PCR test and the CK-ICC test. Factors that might explain this are the difference in markers used for these tests or RNA detected by RT-PCR may more likely represent active production of cytokeratin whereas CK-ICC may detect dormant or apoptotic cells.³⁰

We found similar results as published by Koch *et al.*³¹ who examined bone marrow samples from 25 patients with colorectal liver metastases who underwent surgical resection. They found a positive CK20 RT-PCR test to be an independent prognostic factor for recurrence-free survival. Previously, a prognostic clinical MSKCC score (Memorial Sloan-Kettering Cancer Center) was proposed by Nordlinger *et al.*³² and Fong *et al.*³³ to select patients who will have optimal chances for cure after hepatic resection. This clinical score consists of the following clinical and pathological risk factors: a positive nodal status of the primary tumor, less than 12 months between the primary tumor and the liver metastases, four or more liver metastases, a maximum diameter of the metastases of more than 5 cm and serum CEA level higher than 200 micrograms per liter. Given our results and others, the CK20 RT-PCR might be an interesting variable to be added to the MSKCC score.

In general, the spread of tumor cells occurs through local invasion and or hematogenous routes and or lymphatic routes. To reach lymph nodes or other tissue compartments in the body, the tumor cells have to overcome many boundaries at the site of the primary tumor. This occurs with the aid of various proteins. In **chapter 8** the markers related to tumor cell spread through blood and lymphatic vessels are studied for their prognostic significance. Specifically, we examine whether disease recurrence in lymph node-negative CRC patients can be predicted by assessing their primary tumors for the expression of the angiogenic and lymphangiogenic factors sialyl Lewis X (sLeX), vascular endothelial growth factor (VEGF)-C, and VEGF-D, and for blood and lymphatic microvessel density (BMVD and LMVD), and for the presence of blood and lymphatic vessel invasion. This is performed in a case-control design studying the same patient group as in **chapter 5**. Thirty-six cases (disease recurrence within 5 years) and 72 controls (no disease recurrence for at least 5 years) were selected. Tumor sections were stained by antibodies CSLEX1 also known as CD15s (sLeX), anti-VEGF-C, anti-VEGF-D, anti-CD31 (BMVD) or D2-40 (LMVD) to determine the parameters as mentioned above.

A multivariate analysis showed sLeX expression and high LMVD (OR 5.1, 95% CI 1.3-20.0 and OR 3.1, 95% CI 1.0-10.0, respectively) to be independent factors predicting disease recurrence. Expression of sLeX correlated with liver metastases ($P = 0.015$).

A high LMVD was related to regional intra-abdominal or intrapelvic metastases in lymph nodes and distant metastases other than in the liver and lungs such as peritoneum, bones, brain and adrenal glands ($P = 0.004$). A high BMVD in the invasive front correlated with lung metastases ($P = 0.018$).

When comparing the results in **chapter 5 and 8**, the following is noticed. There is a significant correlation between the presence of macrometastases or MM and lymphatic vessel invasion (Fisher's exact test, $P = 0.001$ and $P = 0.014$, respectively) but none with a high number of LMVD (Fisher's exact test, $P = 0.170$) (data not published). Also, lymph node metastases are related to distant metastases (Fisher's exact test, $P = 0.001$).

This study is one of the first studies that combine a multiple set of tumor markers each correlating with a hematogenous or lymphatic tumor spread as previously described by Pantel and Brakenhoff (*see introduction, Figure 3*).³⁴ Additionally, these results imply two types of mechanisms involved in metastasis (passive and active intravasation) as previously described by the group of Jain (*see introduction, Figure 2*).³⁵ First, there is the mechanical way of tumor cell dissemination through blood and lymphatic microvessels, resulting in tumor cell arrest in the narrow capillary network in different organs including lymph nodes. There they might proliferate and develop clinically evident metastases. This may explain the correlation between a high BMVD and lung metastases and the correlation between a high LMVD and regional intra-abdominal or intrapelvic metastases in lymph nodes with eventually distant metastases.

Moreover, also biological processes determine outgrowth of metastases as reflected in the "seed and soil" theory.^{36;37} This theory suggests that a subpopulation of tumor cells with metastatic potential, recently identified as colon-cancer-initiating cells³⁰, disseminate through the whole body. These cells proliferate and differentiate to form clinically evident metastases at preferential sites depending on local molecular interaction among which availability of local growth factors. In our study, the interaction of sLeX on tumor cells and E-selectin on endothelial cells might have facilitated tumor cell invasion in blood microvessels, extravasation and migration into distant tissue. As blood microvessels drain to the portal vein, the tumor cells arrive in the liver where their sLeX expression might prefer the liver to grow out to form clinically evident metastases due to interaction with local E-selectin.³⁸

GENERAL DISCUSSION

Chapters 2 to 5 assess the detection of OTC in lymph nodes and its clinical relevance. OTC in lymph nodes from gastric cancer patients (**chapter 2**) are of clinical value if they are present in at least 20% of the lymph nodes whereas in CRC (**chapter 5**), MM and not ITC are found to be of clinical relevance. The difference between these studies in OTC relevance might be explained by the higher number of harvested lymph nodes in gastric cancer compared to CRC (median 15 with a range of one to 92 vs median 6 with a range of one to 26).

Besides being a staging tool, lymphadenectomy has also been reported to have therapeutic value.³⁹⁻⁴¹ In CRC and bladder cancer, improved survival was shown in both node-negative and node-positive patients when an increased number of lymph nodes was examined.^{39;40} The results of our study in gastric cancer patients (**chapter 2**) support the therapeutic value of a higher number of dissected lymph nodes as a significantly higher number of harvested lymph nodes was seen in less than 20% OTC-positive group (n = 27) compared to the greater or equal than 20% OTC-positive group (n = 10) (Mann-Whitney test, median 18 (range 6 to 92) vs median 8.5 (range one to 35); P = 0.021). This again corresponds to the survival benefit of patients with N2 disease⁴² who underwent a D2 dissection as previously reported.⁴³

We do not expect the number of levels examined to be a factor for the difference between chapter 2 and 5 as the percentage of OTC-positive lymph nodes after analysis of the first level in CRC also did not differ between cases and controls. Furthermore, the anticytokeratin antibodies CAM5.2 and AE1/AE3 had been compared and no difference in staining pattern was observed (data not published).

In **chapter 4**, we do not differentiate between ITC and MM. More patients are seen with OTC detected by multilevel sectioning and immunohistochemical staining in patients who have developed disease recurrence than in patients without recurrence, but it does not reach significance (P = 0.055). When disregarding single tumor cells as relevant, the sensitivity of the test remains 100% and the specificity rises from 43% to 64% in predicting disease recurrence. Nevertheless, the specificity of the RT-PCR test is still higher (79%). This may relate to the fact that RT-PCR detects RNA which represents active cells whereas immunohistochemistry detects proteins which might also be in apoptotic tumor cells.

An additional finding in the study presented in **chapter 5** are four macrometastases in the first sectioning level which had not been recognized as such on the conventional hematoxylin and eosin stained sections. They were reported as vascular invasion or tumor deposits without lymphoid tissue. These patients had been considered as node-negative and did not receive adjuvant chemotherapy. The combination of serial sectioning and immunohistochemical brown-staining of tumor cells might lead to a more clear differentiation from surrounding lymphoid tissue. A recent review by Nagtegaal *et al.*⁴⁴ shows that various studies have tried to determine the importance of tumor deposits based on contour, size and origin but all fail to provide an evidence base to substantiate its use in the TNM system. Detailed analysis of lymph nodes may aid in examining tumor deposits in perirectal and pericolic fat.

To further elucidate the role of MM and ITC in lymph nodes, it would be advisable for further studies to follow the guidelines recently provided by Turner *et al.*⁴⁵ These guidelines regard criteria for clusters of tumor cells, measurements of OTC and a more extensive definition of ITC and MM.

In their study, the variability of pathologists in their distinction between MM and ITC decreased after examining the set of clearly defined histologic criteria. Agreement improved from 76% to 97% leading to a reproducible nodal classification.

Given our results and others, MM are predictors of disease recurrence in CRC and therefore correctly used in the current TNM staging classification. There is no clarity on ITC as yet. Stroma analysis, staining for apoptosis, laser capture followed by single cell analysis⁴⁶ or other techniques might clarify their relevance. Also, automated microscopy might be helpful in detection of OTC.

Both in **chapter 2 and 5**, the number of examined lymph nodes are of clinical relevance. In **chapter 5** that regards archival material and includes rectal cancer patients, only 20% of the patients had undergone the recommended examination of at least 12 lymph nodes by the American Joint Committee on Cancer (AJCC).⁸ Similar findings are seen in other studies investigating large archival material.^{47;48} Higher numbers of lymph nodes have been found in prospective studies investigating OTC detection²³ implying a more dedicated search for lymph nodes. Fat clearance techniques increases the number of harvested lymph nodes, especially small lymph nodes less than 5 mm.^{49;50} However, these methods are time consuming, expensive and impractical as they involve noxious volatile agents. Recently, the use of modified Davidson's fixative instead of conventional formalin fixation, showed an increase in lymph node yield, especially of smaller lymph nodes.⁵¹ An alternative might be injection of blue dye which also leads to detection of small lymph nodes.²³ Nevertheless, at best 50% of patients in the United States undergo adequate lymph node evaluation for colon cancer.^{47;52} Risk factors reported for < 12 lymph nodes examined are male sex, older age, earlier T stage tumor, low-volume hospitals and preoperative (chemo)radiotherapy.^{47;52;53} Studies have suggested a wide range for adequate nodal evaluation but guidelines from the National Comprehensive Cancer Network, the College of American Pathologists, the National Cancer Institute and the AJCC all recommend the examination of 12 or more lymph nodes because this is a feasible and effective threshold.^{8;54-56} To improve the number of harvested lymph nodes, guidelines from the Santa Monica Conference in 2007, are recommended. If less than 12 lymph nodes are found on initial examination, the pathologist should reexamine the specimen, possibly including microscopic examination of extramural soft tissue. A harvest of less than 12 lymph nodes, should be documented in the pathology report including the type and extent of reexamination undertaken. Also, the surgeon bears responsibility for submitting both sufficient tissue for examination and a meticulous description of the surgical resection. As Jass *et al.*⁵⁷ claim "there is no unacceptably low number of lymph nodes for an individual dissection, but the mean number of lymph nodes in a series of colon cancer dissections should approximately be between 12 and 15. In the case of rectal cancer with the increasing use of neoadjuvant radiotherapy, the mean number may be lower than 12 despite intensive search.

Summarizing, the number of examined lymph nodes is a prognostic factor proven in numerous studies. Accordingly, it might also be a factor to include in a revised TNM system to aid in the decision-making regarding adjuvant systemic treatment.

CLINICAL PERSPECTIVES

Although surgical resection is the cornerstone in the treatment of both gastric cancer and CRC, surgery alone is not able to cure patients with minimal metastatic disease. These patients at risk for disease recurrence might benefit from additional systemic treatment and therefore their identification is a major issue. Detection and treatment of minimal metastatic disease is of increasing importance given the expected rise in the incidence of early-stage gastric and colorectal cancer patients through the use of gastroscopy, fecal occult blood tests and colonoscopy. Additionally, curative surgical resection of isolated colorectal liver or lung metastases is more often becoming an option for treatment. Also in these patients, determination of minimal metastatic disease may direct further treatment. This thesis shows prognostic significance of occult tumor cells in lymph nodes or bone marrow and of primary tumor markers related to lymphatic and hematogenous spreading in patients who had undergone a supposedly curative resection, and, therefore, may indicate the presence of minimal metastatic disease.

For years, chemotherapy failed to show survival benefit in gastric cancer.⁵⁸ In 2001, the Intergroup 0116 trial showed adjuvant chemoradiotherapy (5-fluorouracil plus leucovorin and radiation) to improve overall survival in gastric cancer.⁵⁹ Also, a Japanese trial confirmed an increase in overall survival after adjuvant chemotherapy with an oral fluoropyridimine S-1.⁶⁰ The MAGIC trial (Medical Research Council Adjuvant Gastric Infusional Chemotherapy) showed perioperative chemotherapy (pre- and postoperative epirubicin, cisplatin and 5-fluorouracil) to result in improved progression-free and overall survival.⁶¹ Therefore, the gastric cancer patient group with minimal metastatic disease at risk for disease recurrence might optimally benefit from adjuvant chemotherapy. Currently, patient's prognosis after neo-adjuvant chemotherapy and curative surgery followed by postoperative chemoradiotherapy or chemotherapy is investigated in the CRITICS (ChemoRadiotherapy after Induction chemoTherapy In Cancer of the Stomach) study which is being performed in the Netherlands.

Until recently, adjuvant systemic therapy in colon cancer had consisted of 5-fluorouracil and leucovorin.⁶² Now, results with capecitabine, an oral fluoropyrimidine, suggests that 5-fluorouracil can be replaced by capecitabine.⁶³⁻⁶⁶ Also, the MOSAIC (Multi-center International Study of Oxaliplatin/5-fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer) trial showed that addition of oxaliplatin improved disease-free survival of patients with stage III disease.⁶⁷ The value of adjuvant chemotherapy for patients with stage II disease remains controversial because evidence is inconsistent that adjuvant 5-fluorouracil-based chemotherapy is associated with a better overall survival

compared to surgery alone.⁶⁸⁻⁷⁰ However, subgroups of patients with stage II CRC at risk for disease recurrence including T4 stage, perforation, complete obstruction⁷¹ and given the results of this thesis, the presence of minimal metastatic disease, might benefit from adjuvant chemotherapy to a similar extent as shown for stage III CRC patients. Nevertheless, future studies have to prove this.

Results of other studies investigating the survival benefit in gastric cancer and CRC patients treated with biological agents attacking several pathways such as bevacizumab and cetuximab have to be awaited.⁷²⁻⁷⁴

Concluding, considerable numbers of patients who underwent curative surgical resection of their gastric or colorectal tumor are at risk for disease recurrence. In this thesis, we have shown that patients treated for gastric or colorectal cancer who are at risk for disease recurrence can be identified through occult tumor cell detection in lymph nodes, disseminated tumor cell detection in bone marrow and analysis of angiogenic and lymphangiogenic features of the primary tumor. These findings can be implemented in new strategies for identification of high risk patients, leading to individualized therapy preventing over- and undertreatment.

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CHAPTER TEN

NEDERLANDSE SAMENVATTING

SAMENVATTING

Wereldwijd overleden in 2007 7,9 miljoen mensen aan kanker, ongeveer 13 procent van het totaal aantal overledenen. De verwachting is dat dit aantal zal stijgen naar 9 miljoen in 2015.¹ Maagkanker en dikkedarmkanker zijn twee veelvoorkomende vormen van kanker met een hoge mortaliteit. Deze ziekten staan centraal in dit proefschrift.

De incidentie van maagkanker varieert van hoog in Japan en bepaalde delen van Zuid-Amerika (> 40 per 100.000) tot laag in West-Europa en de Verenigde Staten (< 15 per 100.000).² Omgevingsfactoren spelen bij het krijgen van maagkanker een grotere rol dan genetische factoren.³

In 2007 werden naar schatting ongeveer 1,2 miljoen nieuwe patiënten met dikkedarmkanker gediagnosticeerd.⁴ Deze vorm van kanker komt vaker voor in ontwikkelde landen. De kans op het ontwikkelen van dikkedarmkanker stijgt met de leeftijd als gevolg van een cumulatie van (epi)genetische mutaties. Meer dan 90 procent van de nieuwe gevallen werd gediagnosticeerd bij patiënten ouder dan 50 jaar. De meeste gevallen van dikkedarmkanker komen voor als sporadische vorm waarvan ongeveer 20 procent een familiaircomponent (twee of meerdere eerste- of tweedegraads familieleden met dikkedarmkanker) heeft.⁵ Daarnaast is ongeveer 5 tot 10 procent autosomaal dominant erfelijk.

Chirurgie staat bij de behandeling van maagkanker en dikkedarmkanker op de eerste plaats, aangezien dit de meeste kans op genezing biedt. Patiënten worden, afhankelijk van het ziektestadium waarin zij verkeren, aanvullend behandeld met chemotherapie. Het ziektestadium wordt bepaald met behulp van het classificatiesysteem voor tumor-regionale lymfekliermetastasen-afstandsmetastasen (TNM) (zie *inleiding, tabel 2 en 3*). Dit is de best beschreven prognostische factor bij solide tumoren.^{6;7} Het TNM-classificatiesysteem is sinds de introductie in 1987 meerdere malen aangepast naar gelang actuele resultaten. Desondanks kan dit stadiëringssysteem de prognose van de individuele patiënt nog niet voorspellen, met als gevolg over- en onderbehandeling.

In dit proefschrift wordt onderzocht of het mogelijk is patiënten met maagkanker of dikkedarmkanker met minimale gemetastaseerde ziekte ten tijde van de operatie die risico lopen de ziekte terug te krijgen, te identificeren door detectie van occulte tumorcellen (OTC) in lymfeklieren of beenmerg of door analyse van angiogenese en lymfangiogenese eigenschappen van de primaire tumor.

Hoofdstuk 1 behandelt de achtergrond en inhoud van dit proefschrift. De TNM-classificatie representeert de onderling gerelateerde routes van tumorverspreiding, namelijk lokale invasie (T), verspreiding via lymfevaten (N) en metastasering op afstand via bloedvaten (M). Met de huidige TNM-classificatie wordt met name macroscopische tumorverspreiding gedetecteerd door conventionele hematoxyline-eosinekleuringen en radiologische technieken. Vandaar dat de patiënten die risico lopen de ziekte terug te krijgen, na zogenoemde curatieve resectie niet worden herkend. Door te zoeken naar minimale ziekte in lymfeklieren of beenmerg of door

biologische markers in de primaire tumor gerelateerd aan tumormetastasering te onderzoeken, kan een preciezer beeld verkregen worden van de prognose van de individuele patiënt. De eerste stappen hierin zijn al ondernomen in de laatste revisie van het TNM-classificatiesysteem, waarbij minimale ziekten zoals micrometastasen (MM; $> 0,2$ mm en ≤ 2 mm) en geïsoleerde tumorcellen (ITC; $\leq 0,2$ mm) in lymfeklieren zijn toegevoegd aan macrometastasen (> 2 mm) die met conventionele hematoxyline-eosinekleuringen meestal wel worden gevonden.

In **hoofdstuk 2** is onderzocht of de aanwezigheid van OTC in oorspronkelijk als tumornegatief bestempelde lymfeklieren van curatief geopereerde maagkankerpatiënten de terugkeer van de ziekte kan voorspellen. Daarnaast is de aanvullende waarde van geautomatiseerde microscopie onderzocht. Veertig patiënten die de ziekte terugkregen (cases) en 41 patiënten die minimaal vijf jaar ziektevrij bleven (controlegroep), werden geselecteerd uit de eerder gepubliceerde multicenter Nederlandse D1-D2 maagkankertrial.⁸ Eén weefselcoupe werd immunohistochemisch gekleurd door anticytokeratine antilichamen CAM5.2 en daarna bekeken door conventionele en geautomatiseerde microscopie.

Uit het onderzoek bleek geen significant verschil in de aanwezigheid van OTC, MM of ITC tussen de cases en de controlegroep ($P = 0,658$ respectievelijk $P = 0,691$ respectievelijk $P = 0,887$). In significant meer cases zat echter 20 procent of meer OTC-positieve lymfeklieren (Chi-squaretest, negen (24 procent) *versus* één (3 procent), $P = 0,015$). Uit een multivariate regressieanalyse blijkt dat het onderzoeken van minder dan vijf lymfeklieren (OR 13,8; 95% CI 1,6 - 120,6; $P = 0,018$) de enige significante onafhankelijke risicofactor is voor het ontwikkelen van een recidief, met name voor een locoregionaal recidief (OR 20,4; 95% CI 2,2 - 190,8; $P = 0,008$). Een soortgelijke analyse voor afstandsmetastasen toont een percentage van 20 procent of meer OTC-positieve lymfeklieren als enige significante risicofactor (OR 15,6; 95% CI 1,6 - 151,4; $P = 0,018$). De sensitiviteit van immunohistochemie gevolgd door microscopie ter identificatie van cases met 20 procent of meer OTC-positieve lymfeklieren nam toe van 8 procent bij conventionele microscopie naar 22 procent bij geautomatiseerde microscopie (McNemar's test, $P = 0,063$).

Deze resultaten suggereren dat locoregionale recidieven het gevolg zijn van tumorpositieve lymfeklieren die niet zijn verwijderd. Als 20 procent of meer van de lymfeklieren OTC-positief zijn, hebben de tumorcellen zich al verspreid naar andere delen van het lichaam, wat uiteindelijk resulteert in afstandsmetastasen. Dit hoofdstuk geeft ook een overzicht van eerder gepubliceerde soortgelijke studies bij patiënten met maagkanker. Er is een uitgebreide variatie in antilichamen en in de gerapporteerde percentages van OTC-positieve lymfeklieren. Tien van zestien studies rapporteren een slechtere prognose bij aanwezigheid van OTC in lymfeklieren.⁹⁻¹¹ Siewert *et al.*¹² vermeldt ook klinische relevantie van het percentage OTC-positieve lymfeklieren, wat in overeenstemming is met deze studie.

Hoofdstuk 3 geeft een overzicht van studies waarin detectiemethoden en klinische relevantie van OTC in lymfeklieren bij patiënten met dikkedarmkanker worden onderzocht. Drie van de vier studies waarbij polymerase chain reaction (PCR) wordt gebruikt voor detectie van OTC, laten de prognostische relevantie hiervan zien. Bepaalde DNA-gebieden die tumorspecifieke mutaties in de genen p53 en K-ras kunnen bevatten, werden geamplificeerd door de PCR-test in deze studies. Nadelen zoals het niet consistent voorkomen van p53- en K-ras-mutaties bij dikkedarmkanker en het enorme aantal codons of zelfs exonen waarin mutaties kunnen voorkomen, zijn de reden dat PCR geen alom gebruikte methode zal worden voor detectie van OTC.

Alle zes studies die reverse-transcriptase polymerase chain reaction (RT-PCR) toepasten om OTC in lymfeklieren te detecteren, vermelden een slechtere prognose bij OTC-detectie.¹³⁻¹⁸ Wel hebben twee veel gevoeliger real-time RT-PCR-studies geen klinisch belang aangetoond van OTC in lymfeklieren.^{19;20} Er kan geconcludeerd worden dat OTC in lymfeklieren gedetecteerd met RT-PCR van prognostische waarde zijn, als gebruikgemaakt wordt van verse of ingevroren lymfeklieren, de markers CEA, CK20 en GCC en een optimaal aantal PCR-cycli.

Slechts negen van 28 immunohistochemiestudies rapporteren een significant slechtere prognose bij patiënten met OTC-positieve lymfeklieren. Met name studies die gebruikmaakten van anticytokeratine antilichamen CAM5.2, vermelden dat OTC-positieve lymfeklieren klinisch relevant zijn. Er is geconcludeerd dat percentages OTC-positieve lymfeklieren en de prognostische relevantie van OTC varieerden tussen de studies. Dit wordt met name gezien in de immunohistochemie studies, wat gerelateerd kan worden aan verschillen in detectietechnieken.

In **hoofdstuk 4** is de waarde geëvalueerd van immunohistochemische kleuringen van multipale lymfekliercoupes voor OTC-detectie bij patiënten met dikkedarmkanker. De glaasjes werden gescreend met geautomatiseerde microscopie. Hoewel OTC in lymfeklieren gedetecteerd door RT-PCR vaker van prognostisch belang zijn, is de infrastructuur in de ziekenhuizen voor deze techniek niet geschikt, doordat verse of ingevroren lymfeklieren nodig zijn voor een optimaal resultaat. Verder is tot nog toe morfologisch onderzoek van lymfeklieren obligaat. Deze studie is verricht op histologisch als tumornegatief bestempelde in paraffine ingebedde lymfeklieren, die Liefers *et al.*¹³ al eerder hebben onderzocht op de aanwezigheid van OTC met RT-PCR, gebruikmakend van de marker CEA. Zij vonden een vijfjaarsoverleving van 50 procent voor de OTC-positieve groep en 91 procent voor de negatieve groep ($P = 0,02$).

Coupes van paraffineblokken van twintig patiënten werden gesneden (vijf opeenvolgende coupes van tien niveaus op een afstand van tweehonderd micrometer of tot het weefsel opgesneden was) en immunohistochemisch gekleurd met anticytokeratine antilichamen AE1/AE3. De gekleurde glaasjes werden met de flatbedscanner gescreend. OTC werden gevonden in alle zes (100

procent) patiënten die een recidief hadden ontwikkeld, vergeleken met acht van veertien (57 procent) patiënten die ziektevrij waren gebleven (Chi-squaretest, $P = 0,055$). Volgens de OTC-detectie met CEA RT-PCR waren vijf van zes (83 procent) patiënten die een recidief hadden ontwikkeld, OTC-positief en drie van veertien (21 procent) die ziektevrij waren gebleven, testen OTC-positief.

Deze resultaten tonen aan dat het onderzoeken van meerdere coupes van lymfeklieren op verschillende niveaus, gecombineerd met immunohistochemische kleuringen en gevolgd door geautomatiseerde microscopie, een hogere sensitiviteit in het voorspellen van recidieven heeft dan RT-PCR (100 *versus* 83 procent). Tegelijkertijd geeft het wel een lagere specificiteit (43 *versus* 79 procent).

In deze studie is er niet gedifferentieerd tussen MM en ITC, maar er zijn wel groepjes met tumorcellen (≥ 2) separaat gescoord en gezien in alle zes patiënten met een recidief en in vijf van veertien (36 procent) patiënten die ziektevrij bleven. Bijkomend moet vermeld worden dat alle patiënten met een recidief werden geïdentificeerd na analyse van vier lymfeklierniveaus op een afstand van tweehonderd micrometer.

In **hoofdstuk 5** staat de vraag centraal of met gedetailleerd onderzoek van lymfeklieren door immunohistochemische kleuringen van coupes van meerdere niveaus de prognose van patiënten met dikkedarmkanker beter kan worden voorspeld. Hiervoor werden 36 cases (terugkeer van ziekte binnen vijf jaar) en 72 controles (ten minste vijf jaar geen terugkeer van ziekte) geselecteerd. Er zijn paraffinecoupes van lymfeklieren gesneden op vier niveaus met een afstand van tweehonderd micrometer en immunohistochemisch gekleurd met anticytokeratine antilichamen AE1/AE3. Deze glaasjes werden geanalyseerd met conventionele en geautomatiseerde microscopie.

In totaal heeft de groep met cases meer MM ($n = 3$) dan de controlegroep ($n = 1$). Er is geen verschil in aanwezigheid van ITC tussen de case en de controlegroep. Analyse van een tweede lymfeklierniveau heeft geleid tot de additionele detectie van één case met MM ($n = 1$), één case met macrometastase ($n = 1$) en twee cases en zeven controles met ITC ($n = 9$). Het onderzoeken van meer dan twee niveaus heeft alleen geresulteerd in detectie van additionele ITC. Alle MM en macrometastasen zijn gevonden door conventionele microscopie. Geautomatiseerde microscopie heeft alleen geleid tot de detectie van meer ITC. In een multivariate regressieanalyse zijn de aanwezigheid van macrometastasen of MM (OR 34,5; 95% CI 2,7 – 440; $P = 0,006$), T4 stadium (OR 2,9; 95% CI 0,4 – 23,5; $P = 0,040$) en minder onderzochte lymfeklieren (OR 0,9; 95% CI 0,8 – 1,0; $P = 0,025$) onafhankelijke voorspellers voor terugkeer van ziekte. Messerini *et al.*²¹ en Bilchik *et al.*²², die ook onderscheid maakten tussen MM en ITC, rapporteren soortgelijke lage detectiepercentages voor MM (9,9 respectievelijk 3 procent). Ook liet de eerste studie prognostische relevantie zien van MM en niet voor ITC. In de studie van Bilchik *et al.*²² is deze analyse niet gedaan.

Gedetailleerd onderzoek van lymfeklieren bij patiënten met dikkedarmkanker met immunohistochemische kleuringen van coupes van meerdere niveaus of met RT-PCR op zoek naar OTC is duur en arbeidsintensief, rekening houdend met het grote aantal lymfeklieren dat kan worden geïsoleerd. Identificatie van schildwachtklieren en selectieve analyse van alleen deze lymfeklieren zijn voorgesteld als een mogelijke wijze om deze werklust te vermijden. Schildwachtklieren (SWK's) zijn de eerste lymfeklieren waar de primaire tumor naar draineert, daarom hebben zij de meeste kans op het bevatten van tumorcellen (zie *inleiding*, *figuur 1*).

In **hoofdstuk 6** wordt een overzicht gegeven van 25 studies die de haalbaarheid en de betrouwbaarheid van de SWK-procedure bij dikkedarmkanker hebben beschreven. Er bleek een grote variatie in identificatiepercentages^a en fout-negatieve(FN-)percentages^b, variërend van 58 tot 100 procent (gemiddeld 89 procent) respectievelijk 0 tot 60 procent (gemiddeld 33 procent). Factoren die hebben kunnen bijdragen tot mislukte SWK-procedures waren: incomplete injectie rondom de tumor, insufficiënte hoeveelheden tracer^c voor grote tumoren, includeren van vergevorderde tumoren en rectale tumoren. Een belangrijke factor was het leercurve-effect, dat leidde tot een hoger identificatiepercentage van bijna honderd procent in sommige studies als de resultaten van de eerdere fase in de leercurveperiode niet werden geïnccludeerd in de analyse.

Hoge FN-percentages kunnen worden verklaard door uitgebreide tumoringroei in lymfeklieren en grote tumoren. Daardoor kunnen lymfevaten worden afgesloten, leidend tot lymfedrainage via een andere route. Ook een lange identificatietijd tussen injectie van de tracer en identificatie van de SWK's kan een rol spelen. Blauwe kleurstof verspreidt zich namelijk snel en kan daardoor al een tweede echelon lymfeklieren hebben bereikt. Bovendien werd een laag FN-percentage van 20 procent of minder meestal gezien in studies waar het aantal SWK's beperkt bleef tot de eerste vier blauwe lymfeklieren of de vier of vijf blauwe klieren die het dichtst bij de tumor waren. Terwijl studies met een hoger FN-percentage (> 20 procent) een hoger aantal schildwachtklieren hadden gerapporteerd. Het overgrote deel van de laatstgenoemde studies had alle blauwe of radioactieve lymfeklieren beschouwd als SWK's leidend tot een brede variatie in aantal SWK's. Bij melanomen en borstkanker worden lagere FN-percentages gezien. Het verschil zou kunnen liggen in de grotere tumoren bij dikkedarmkanker en ook in het verschil in het lymfedrainagepatroon.

Beperkingen bij endeldarmkanker zijn onder andere de korte afstand tussen

^a Identificatiepercentage is het aantal SWK-procedures waarmee ten minste één SWK (radioactief of blauw) is gevonden, gedeeld door het totaal aantal SWK-procedures.

^b Fout-negatiepercentage is het aantal gevallen waarbij de SWK's tumornegatief zijn terwijl de niet-SWK's tumorpositief zijn, gedeeld door het aantal gevallen met tumorpositieve lymfeklieren.

^c Tracer kan blauwe kleurstof zijn of radioactief materiaal dat rondom de tumor wordt ingespoten, waarna het naar de SWK's wordt getransporteerd via de lymfebanen.

primaire tumor en lymfeklieren resulterend in overlappende radioactiviteit van de tracer. Daarnaast mag het weefsel rondom de endeldarm tijdens de operatie niet worden ingesneden in verband met het adequaat verwijderen van endeldarmkanker. Bestraling voor de operatie en vergevorderde tumoren kunnen ook de identificatie van SWK's verstoren. Deze factoren maken de SWK-procedure in endeldarmkanker ingewikkeld en klinisch van minder belang.

Twee studies bij patiënten met dikkedarmkanker hebben aangetoond dat SWK's significant vaker tumorcellen bevatten dan niet-SWK's.^{23;24} Een derde studie laat tegenstrijdige resultaten zien, waarbij er geen verschil in de aanwezigheid van MM of ITC werd gezien tussen SWK's en niet-SWK's.²⁵ Eén SWK-studie bij patiënten met dikkedarmkanker meldde een hoger percentage van terugkeer van de ziekte bij patiënten met SWK's die macrometastasen of MM bevatten.²⁶ Toch is de betrouwbaarheid van de SWK-procedure bij dikkedarmkanker nog niet eenduidig.

Hoewel er bij patiënten met dikkedarmkanker meestal geen botmetastasen optreden, worden er wel tumorcellen in het beenmerg gevonden.^{27;28} Mogelijk geven deze tumorcellen in het beenmerg de agressieve aard van de tumor weer en kan detectie van deze tumorcellen in beenmerg gebruikt worden om terugkeer van ziekte te voorspellen. In **hoofdstuk 7** werd dit onderzocht door na te gaan of de aanwezigheid van tumorcellen in het beenmerg van 81 patiënten met levermetastasen, afkomstig uit de dikke darm, die gepland stonden voor resectie of leverperfusie, een slechte prognose kon voorspellen. De aanwezigheid van tumorcellen in beenmerg is onderzocht met kwantitatieve RT-PCR, waarbij gebruik werd gemaakt van cytokeratine 20 (CK20), en of immunocytochemie (CK-ICC) gecombineerd met geautomatiseerde microscopie. Hierbij werden A45-B/B3 anticytokeratine antilichamen gebruikt. 26 beenmergmonsters van 69 (38 procent) patiënten zijn positief met de CK20-RT-PCR-test en monsters van 15 van 69 (22 procent) patiënten testen positief met de CK-ICC-test. Uit een multivariate Cox-regressieanalyse blijkt dat een positieve CK20-RT-PCR-test (HR 2,5; 95% CI 1,2 - 5,2; P = 0,014) en een serum-CEA-niveau hoger dan tweehonderd microgram per liter (HR 2,4; 95% CI 1,0 - 5,9; P = 0,045) onafhankelijke voorspellers zijn voor een kortere ziektegerelateerde overleving. Een positieve CK-ICC-test correleert niet met een slechtere overleving, maar wel met de aanwezigheid van extrahepatische ziekte tijdens de operatie (Chi-squaretest, P = 0,009). Er is geen correlatie tussen de CK20-RT-PCR-test en de CK-ICC-test. Factoren die dit kunnen verklaren, zijn het verschil in gebruikte markers voor deze testen of de mogelijkheid dat de RT-PCR actief delende cellen detecteert, terwijl CK-ICC cellen in rust of in afbraak detecteert.²⁹

Deze resultaten corresponderen met die van Koch *et al.*³⁰ Zij onderzochten beenmerg van 25 patiënten met levermetastasen afkomstig uit de dikke darm, die chirurgische resectie ondergingen. In deze studie was een positieve CK20-RT-PCR ook een onafhankelijke prognostische factor voor recidiefvrije overleving.

Eerder is een prognostische klinische MSKCC-score (Memorial Sloan-Kettering Cancer Center) samengesteld door Nordlinger *et al.*³¹ en Fong *et al.*³² om patiënten te selecteren die optimaal baat hebben bij een leverresectie. Deze klinische score bestaat uit de volgende klinische en pathologische eigenschappen: een positieve lymfeklierstatus van de primaire tumor, minder dan twaalf maanden tussen de primaire tumor en het ontstaan van levermetastasen, vier of meer levermetastasen, maximale diameter van metastasen groter dan vijf centimeter en een serum-CEA-niveau groter dan tweehonderd microgram per liter. Elke eigenschap is één punt en hoe hoger het aantal punten hoe slechter de prognose voor de patiënten. Gezien deze resultaten en die van anderen zou de CK20-RT-PCR een aanvullende variabele kunnen zijn voor de MSKCC-score.

Tumorcellen verspreiden zich in het algemeen door locale invasie en/of hematogene en/of lymfogene routes. Om lymfeklieren of andere weefselcompartimenten in het lichaam te bereiken, moeten de tumorcellen verscheidene obstakels rondom de primaire tumor overwinnen. Dit gebeurt met behulp van verschillende eiwitten. In **hoofdstuk 8** is de prognostische relevantie onderzocht van markers gerelateerd aan tumorverspreiding via bloed- en lymfevaten. Er is met name bekeken of ziekterugkeer bij dikkedarmkankerpatiënten met tumornegatieve lymfeklieren voorspeld kon worden door de primaire tumor te onderzoeken op expressie van angiogenese en lymfangiogenese factoren sialyl-Lewis-X (sLeX), vasculaire endotheliale groeifactor (VEGF)-C en VEGF-D, en op bloedvat- en lymfevatdichtheid (BVD en LVD), en op de aanwezigheid van tumorcelinvasie van bloed- en lymfevaten. Dit is onderzocht met een case-controlestudieopzet, waarbij dezelfde patiëntengroep als in **hoofdstuk 5** werd bestudeerd. 36 cases (ziekteterugkeer binnen vijf jaar) en 72 controles (ten minste vijf jaar ziektevrij) werden geselecteerd. Coupes van de tumor werden gekleurd met de antilichamen CSLEX1 oftewel CD15s (sLeX), anti-VEGF-C, anti-VEGF-D, anti-CD31 (BVD) of D2-40 (LVD) om de parameters te bepalen zoals hierboven vermeld.

Een multivariate analyse toont aan dat sLeX-expressie en een hoog LVD (OR 5,1; 95% CI 1,3 - 20 respectievelijk OR 3,1; 95% CI 1 - 10) onafhankelijke voorspellers zijn voor terugkeer van ziekte. Expressie van sLeX correleert met levermetastasen ($P = 0,015$). Een hoog LVD is gerelateerd aan regionale intra-abdominale of intrapelviene lymfekliermetastasen en afstandsmetastasen op andere locaties dan de lever en longen zoals peritoneum, skelet, hersenen en bijnieren ($P = 0,004$). Een hoog BVD in het invasieve front van de tumor correleert met longmetastasen ($P = 0,018$).

Bij vergelijking van de resultaten in **hoofdstuk 5 en 8** valt op dat er een significante correlatie is tussen de aanwezigheid van macrometastasen of MM en lymfevatinvasie door tumorcellen (Fisher's exacttest, $P = 0,001$ respectievelijk $P = 0,014$), maar geen correlatie met een hoge LVD (data niet gepubliceerd). Daarnaast zijn de lymfekliermetastasen gerelateerd aan afstandsmetastasen (Fisher's exacttest, $P = 0,001$).

Deze studie is een van de eerste waarbij meerdere tumormarkers worden bestudeerd die elk correleren met hematogene of lymfogene tumoruitzaaiing zoals eerder beschreven door Pantel en Brakenhoff (*zie inleiding, figuur 3*).³³ Bovendien suggereren de resultaten dat er twee mechanismen betrokken zijn bij invasie (passieve en actieve intravasatie), die uiteindelijk resulteren in afstandsmetastasen, zoals eerder beschreven door de groep van Jain (*zie inleiding, figuur 2*).³⁴

Deze resultaten zijn als volgt te verklaren. Ten eerste is er de mechanische wijze van tumorcelverspreiding door bloed- en lymfevaten, waarbij de tumorcellen vastlopen in het smalle capillaire netwerk in verschillende organen inclusief die van de lymfeklieren. Hier kunnen de tumorcellen prolifereren en klinisch evidente metastasen vormen. Dit kan de correlatie tussen een hoge BVD en longmetastasen verklaren én de correlatie tussen een hoog LVD en regionale intra-abdominale of intrapelviene lymfekliermetastasen met uiteindelijk afstandsmetastasen.

Ten tweede is er ook een biologisch proces dat de invadering en metastasering bepaalt en ten grondslag ligt aan de 'seed-en-soil' theorie.^{35;36} Deze theorie suggereert dat een subpopulatie van tumorcellen met het vermogen tot metastasering, recent geïdentificeerd als 'colon-cancer-initiating' cellen²⁹, zich door het hele lichaam verspreidt. Deze cellen prolifereren en differentiëren om klinisch evidente metastasen te vormen op voorkeursplaatsen afhankelijk van de locale interactie met beschikbare groeifactoren. In deze studie heeft de interactie tussen sLeX op de tumorcellen en E-selectine op de endotheelcellen faciliterend gewerkt op de invasie van de tumorcellen in de bloedvaten, extravasatie en migratie in de lever met lokaal uitgroei door interactie met E-selectine ter plekke.³⁷

CONCLUSIE

Chirurgie is feitelijk de eerste keuze van behandeling bij maagkanker en dikkedarmkanker. Afhankelijk van het TNM-stadium worden patiënten aanvullend behandeld met chemotherapie. Een beduidend aantal patiënten dat curatief geopereerd is aan maagkanker of dikkedarmkanker en volgens het TNM-stadiëringssysteem geen aanvullende therapie behoeft, krijgt echter de ziekte terug. De resultaten uit dit proefschrift tonen aan dat deze hoogrisicopatiënten kunnen worden geïdentificeerd door OTC-detectie in lymfeklieren en beenmerg en door analyse van de bloed- en lymfateviëenschappen van de primaire tumor. Deze methoden om hoogrisicopatiënten te identificeren kunnen bijdragen aan een meer persoonsgebonden behandeling, waardoor over- en onderbehandeling van individuele patiënten met maagkanker en dikkedarmkanker kunnen worden voorkomen.

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LIST OF ABBREVIATIONS

APC	adenomatous polyposis coli
BMVD	blood microvessel density
BRCA	breast cancer gene
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CI	confidence interval
CIN	chromosomal instability
DCC	deleted in colon cancer
CK	cytokeratin
CK-ICC	cytokeratin-immunocytochemistry
CK20	cytokeratin 20
CRC	colorectal cancer
CT	computed tomography
CTC	circulating tumor cells
DCC	deleted in colorectal cancer
DNA	desoxy-ribonucleic acid
DTC	disseminated tumor cells
EGFR	epidermal growth factor receptor
5-FU	5-Fluorouracil
FAP	familial adenomatous polyposis
FN rate	false-negative rate
GCC	guanylyl cyclase C
HE	hematoxylin and eosin
HR	hazard ratio
ICC	immunocytochemistry
IHC	immunohistochemistry
ITC	isolated tumor cells
LMVD	lymphatic microvessel density
MCC	missing in colon cancer
MM	micrometastasis
MSI	microsatellite instability
OR	odds ratio
OTC	occult tumor cells
PBGD	porphobilinogen deaminase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
SD	standard deviation
sLeX	sialyl Lewis X
SN	sentinel node
SNM	sentinel node mapping
SPSS	statistical package for social sciences
TNM	tumor node metastasis
VEGF	vascular endothelial growth factor

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CURRICULUM VITAE

Fania Doekhie is geboren op 12 december 1973 in Nickerie, Suriname. In juli 1991 behaalde zij haar vwo-diploma aan de VHK (vwo/havo/kweekschool) scholengemeenschap in Nickerie, met de hoogste landelijke score.

In september 1991 is Fania begonnen aan de studie Medische Biologie aan de Vrije Universiteit (VU) van Amsterdam. Haar eerste stage was op de afdeling Gastro-enterologie van het VU Medisch Centrum, onder supervisie van prof. dr. A.S. Peña. Vervolgens heeft zij onder begeleiding van dr. T.D. de Gruijl stage gelopen op de afdeling Pathologie van het VU Medisch Centrum. In 1996 heeft zij haar doctoraal diploma behaald na een extra derde stage in San Francisco op de afdeling Gastro-enterologie in het Veteran Affairs Medical Center. Ook kreeg zij in dat jaar de Unileverprijs uitgereikt voor geleverde prestaties tijdens haar studie. Daarnaast is Fania in 1996 begonnen aan de studie Geneeskunde, opnieuw aan de VU. Tijdens die studie heeft ze in 1996 en in 1998 enkele maanden in deeltijd gewerkt als onderzoeker op respectievelijk de afdeling Celbiologie en de afdeling Gastro-intestinale Immunogenetica van het VU Medisch Centrum. In oktober 2000 behaalde zij haar artsexamen, waarna zij in januari 2001 is begonnen met het promotieonderzoek dat is beschreven in dit proefschrift. Dit onderzoek vond plaats op de afdeling Heelkunde van het Leids Universitair Medisch Centrum onder leiding van prof. dr. R.A.E.M. Tollenaar, prof. dr. C.J.H. van de Velde, prof. dr. J.H.F. Falkenburg en dr. P.J.K. Kuppen.

Van mei 2005 tot juni 2008 heeft Fania gewerkt als arts-assistent, eerst op de afdeling Heelkunde in het Universitair Medisch Centrum Nijmegen en vervolgens bij het Rijnstate Ziekenhuis in Arnhem. Hierna heeft zij gewerkt bij het Julius Centrum, een divisie van het Universitair Medisch Centrum Utrecht, in het kader van het CAPITA-onderzoek: een vaccinatiestudie bij senioren. Sinds november 2008 werkt Fania op de spoedeisende hulp in het Flevoziekenhuis in Almere. Na haar promotie zal zij zich bij haar echtgenoot in Suriname voegen en aldaar haar werkzaamheden voortzetten.

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