Lymph node staging in colon cancer

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Not to be absolutely certain is one of the essential things in rationality

Bertrand Russell

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Chapter 1

Introduction, aim and outline of the thesis

Introduction

Adequate surgical resection including en-bloc removal of the involved colon segment and associated mesenteric lymph nodes as well as accurate pathological examination of resected lymph nodes are prerequisites for accurate tumor staging in colon cancer. Staging of patients based on the pathological tumor, node, metastasis (pTNM) classification system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), is important for both selection of patients for adjuvant treatment and prediction of long-term survival.[1] The single most important determinant of prognosis in patients with localized colon cancer is the presence of nodal metastases at the time of surgical treatment. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but only 45-50 % for those with node positive tumors (stage III).[2] In patients with stage III colon cancer adjuvant chemotherapy improves survival considerably.[3-5] In addition, a recent meta-analysis showed that there might be a benefit of adjuvant treatment in high-risk stage II colon cancer patients.[6] Therefore, it is highly important to accurately reflect the status of the regional lymph nodes.

The fact that about 20% of the patients without lymph node metastases develop recurrent disease after apparently curative surgery, leads to the question if there might have been understaging at the time of the primary operation.[7] It is possible that in this group of patients small lymph node metastases have been missed. This may be due to an inadequate surgical lymphadenectomy or inadequate pathological examination.[8] According to international guidelines meticulous pathological examination of at least 12 lymph nodes is warranted for adequate staging of patients with colon carcinoma.[1] However, several studies showed that the minimal number of lymph nodes necessary for correct staging varied considerably from 6 to 18 to as many as possible in the study of Goldstein et al.[8-12]

In depth pathological examination of lymph nodes by immunohistochemical staining for cytokeratin or reverse transcriptase-polymerase chain reaction (RT-PCR) may reveal micrometastases that could have been missed by routine haematoxylin & eosin (H&E) examination. There have been conflicting results on the impact of micrometastases and/or tumor DNA in mesenteric lymph nodes on survival.[13,14] [15,16] [17-20] Several authors have reported a decreased survival rate when micrometastases are detected in colon carcinoma.[13,14] [15,16] The possible benefit of adjuvant therapy in this group of patients is therefore not clear yet.

Ultrastaging techniques are time consuming, labour intensive and costly. For optimal staging, in depth examination of only the sentinel lymph node (SLN) could be helpful. The technique of the sentinel node biopsy was first described and performed by Cabanas (1977) in penile carcinoma.[21] Morton et al. and Giuliano et al. introduced the sentinel node biopsy for staging patients in general practice in melanoma and breast cancer.[22,23] In colon cancer, the SLN's are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumor.[24] They are the most likely to harbor metastatic disease when present, enabling focused examination with multilevel microsectioning of the SLN's to provide a more efficient and cost-effective detection of micrometastases. In addition, patterns of aberrant lymphatic drainage can be visualized with sentinel lymph node mapping, which may lead to a more extended resection.[25]

Aim and outline of this thesis

Main goal of this thesis is to investigate the current problems with lymph node staging in colon cancer and to describe possible improvements in lymph node sampling in order to make a better selection of patients eligible for adjuvant treatment.

Chapter 2 starts with a population based study in which the impact of the number of examined lymph nodes in colon cancer on survival is studied. In addition, the tumor and patient factors important for the number of harvested lymph nodes were examined.

In **Chapter 3**, the effect of lymph node fixation with modified Davidson's fixative (mDF) on the number of examined nodes and lymph node status is described.

Chapter 4, 5 and 6 describe the sentinel node procedure in colon cancer. Chapter 4 presents a pilot study on the feasibility of the procedure for patients with localized colon cancer. In chapter 5, we studied the accuracy of the SLN procedure in a multi-centre setting with a special focus on nodal upstaging and aberrant lymphatic drainage. Chapter 6 deals with validation of the procedure, tested with RT-PCR examination of all tumour negative lymph nodes. The main goal of this part of the study is to validate a method in which it would be sufficient to examine only the SLN's with ultrastaging methods in stead of all H&E negative lymph nodes.

In **Chapter 7**, a review is presented in which an overview of the history of adjuvant chemotherapy in colon cancer is given with a special attention to the effects of chemotherapy in high risk stage II patients.

The discussion of the aforementioned studies as well as future perspectives are presented in **Chapter 8** , while **Chapter 9** contains a Dutch summary.

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Chapter 2

Impact of the number of histologically examined lymph nodes on prognosis in colon cancer: a population-based study in the Netherlands.

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Abstract

Purpose: To study the impact on survival of the reported number of lymph nodes at pathological examination of colon specimens.

Methods: This is a retrospective review on the data of 2,281 patients with localized colon cancer. The effect of tumor characteristics and surgical and pathological factors on the number of lymph nodes and examined lymph node numbers on nodal status and survival were analyzed.

Results: The number of examined nodes increased with T-stage, left sided tumors and mucinous morphology, but decreased with age. The proportion of node-positive patients (N_+) increased with a larger number of nodes. A high number of examined nodes and high T-stage affected nodal status. The 5-year overall survival was 51.3% for N_+ versus 68.2% for node-negative (N_0) patients. N_0 patients had a significantly higher 5-year crude and relative survival when more lymph nodes were examined. This was not found for the N_+ group and for all patients combined.

Conclusions: T-stage, localization and patient age were predictive for the number of nodes examined. A higher number of examined nodes was associated with an increase in node-positivity. The survival benefit can be explained by stage migration. Eventually this may lead to an overall survival benefit, as more patients are classified as node positive, and therefore will receive adjuvant therapy.

Introduction

Colorectal carcinoma (CRC) is the most common gastro-intestinal malignancy and the second leading cause of cancer related deaths in the world. Each year, worldwide, nearly one million cases are newly diagnosed and 500.000 patients die of this disease.[1] Adequate surgical lymphadenectomy and pathological evaluation of resected lymph nodes are prerequisites for accurate tumor staging. The primary treatment for colon cancer is a radical surgical resection including en-bloc removal of the involved colon segment and associated mesenteric lymph nodes. Staging of patients based on the pathological tumor, node, metastasis (pTNM) classification system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), is important for selecting patients for adjuvant treatment and for prediction of long-term survival.[2] The single most important determinant of prognosis in patients with localized colon cancer is the presence of nodal metastases at the time of surgical treatment. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but only 45-50 % for those with node positive tumors (stage III).[3] In patients with stage III colon cancer adjuvant chemotherapy improves survival considerably.[4-6] In addition, a recent meta-analysis showed that there might be a benefit of adjuvant treatment in high-risk stage II colon cancer patients.[7] Therefore, it is highly important to accurately reflect the status of the regional lymph nodes. The number of removed nodes in a surgical specimen may depend on the extent and diligence in identifying nodes at the pathological examination.[8-17] In this population based survey of nodal staging in colon cancer we emphasize the influence of the number of histologically examined lymph nodes on nodal stage, and it's impact on survival.

Patients and Methods

Patients

All patients were treated for colon cancer in the Northern Netherlands between January 1998 and December 2002. Exclusion criteria were exploratory surgery only and an incomplete pathological report not mentioning the number of examined lymph nodes. Since the number of nodes is influenced by pre-operative radiotherapy which is routinely used for rectal cancer in The Netherlands, patients with rectal cancer, defined as a tumor situated within 15 cm distance from the anus, were also excluded.[18] Furthermore, patients with distant metastatic (M1) disease, patients with in-situ carcinomas and patients treated with a polypectomy were excluded as the surgical and pathological approach for these patients may have differed from standard recommendations. Also excluded were patients who underwent a (sub)total colectomy, patients with non-adenocarcinomas as well as

patients with a previous diagnosis of invasive cancer, other than non-melanoma skin cancer.

Data collection by the cancer registry

The data was retrospectively collected. Patients were selected from the regional cancer registry of the Comprehensive Cancer Centre North-Netherlands. This registry covers the Northern part of the Netherlands, a main rural area with a population of about 2.1 million, served by 16 community hospitals, one university medical centre and seven pathology laboratories. PALGA, the nationwide Dutch network and registry of histo- and cytopathology, regularly submits reports of newly diagnosed malignancies to the registry. The national hospital discharge databank, which receives discharge diagnoses of admitted patients from all Dutch hospitals, completes case ascertainment. After notification, trained registry personnel collect data on diagnosis, staging, and treatment from the medical records, including pathology and surgery reports. Vital status was established through linkage of cancer registry data with population registries of all municipalities in the Netherlands, last in 2005. In the Netherlands the municipal population registries contain information on the vital status of their inhabitants. Patients were staged according to the TNM system of the UICC.[2]

Guidelines

The Dutch Cancer guidelines (www.oncoline.nl) with respect to the standard surgical resection and pathological examination have not been changed during the study period. This implies that an en-bloc resection of the involved colon segment with wide margins and its mesocolon with draining lymph nodes should have been performed in all patients. Pathological examination of the resected specimens was performed according to these guidelines, which are followed by all Dutch pathologists. In brief, the lymph nodes were recovered with manual dissection after overnight fixation in 10% neutral buffered formalin and treated with conventional H&E staining at 5 mm intervals. According to the above mentioned guidelines at least 12 nodes have to be recovered to accurately predict nodal status. In the pathological report the histological type of tumour, the differentiation grade, the total number of lymph nodes as well as the number of positive nodes and their location have to be described. Adjuvant chemotherapy was indicated for node positive patients.

Statistical analysis

SPSS 12.0 (SPSS, Inc, Chicago, IL) and Stata 8.0 (Stata Corporation, College Station, Texas) for Windows were used for analyses. Differences in proportions between groups were assessed with the χ^2 test, the Mann-Whitney U test was used to compare continuous variables. The association of the number of examined nodes with patient and tumor characteristics was assessed in a log-linear regression analysis, binary logistic regression analysis was used to asses associations of these factors with the presence of nodal metastasis. Survival was calculated from the date of diagnosis until the date of death, the date of most recent linkage with the municipal population registries or the date of last contact (date of last hospital visit or last contact with the general practitioner), whichever came first. Follow-up was terminated at 31-12-2005. The Expected Survival (ES) probability was calculated using age and period matched mortality rates based on life expectancy tables for the Northern Netherlands.[19] The ES was estimated using the Ederer II method.[20] As we have no acces to cause of death data, we used relative survival and excess mortality ratio to estimate the mortality due to tumor. The relative survival, the ratio of the crude survival and the ES was analyzed using Stata and a relative survival function written by Dickman (www.pauldickman.com/teaching/tampere2004). The relative survival can be considered as an estimator of the excess risk of death or of the excess mortality ratio. The excess mortality rate was calculated by subtracting the expected number of deaths, as estimated from the expected survival probability, from the observed number of deaths and dividing this figure by the accumulated person-years. The excess mortality ratio (EMR) is derived from the ratio of the excess mortality rates. Excess mortality ratios were estimated in a generalized linear model with a Poisson error structure based on collapsed relative survival data, using exact survival times.[21] It estimates the excess hazard of death for a given covariate once the hazard for death of the general population has been taken into account. In this model the effect of the number of examined nodes was studied, while adjusting for the effect of various co-variables on the excess mortality. Follow-up time was stratified in annual intervals. Variables included in the model were the age at diagnosis, the tumor invasion depth, the number of positive lymph nodes and chemotherapy given. Model fit was evaluated with the model based Pearson Chi-square goodness-of-fit test statistic. Differences in 5-year overall survival were calculated using the Wilcoxon test. Differences in relative survival were calculated using the Wald test derived from Poisson regression analysis for relative survival. All reported p-values are two sided; the statistical significance level was set at a p-value of <0.05.

Results

Patients

A total of 2.751 patients fulfilled the selection criteria en were entered in this study. In 443 patients the exact number of examined nodes was not mentioned in the pathology report, in 18 patients the T-status was not mentioned in the pathology report and in 15 patients the tumor location was not described. Some of these missing data were in the same patients. The number of patients with an unknown number of examined nodes did not differ between age groups (p=0.76). The operative records were complete for all patients. Patients with missing data were excluded from the analysis. The remaining 2.281 patients were eligible for the final analysis. Patients and tumor characteristics are described in table 1.The mean age of the patients at the time of surgery was 69.9 (median 71, range 21-99) years.

Nodal status and adjuvant therapy

The proportion of node-positive patients increased with a larger number of examined nodes. In multivariate logistic regression analysis the odds ratio of having positive nodes was 25% higher for right-sided tumors and increased with invasion depth of the tumor (T-stage) and with a higher number of identified nodes. Patient age and tumor morphology were not associated with the nodal status (table 1). The effect of an increase in the number of examined nodes appeared to level off at 12-15 nodes. Adjuvant chemotherapy was given to 381 node-positive patients (51.6%). Younger patients more often received chemotherapy. While 82% of the node-positive patients younger than 60 years received adjuvant chemotherapy, this rate decreased to 71%, 42% and 3% for patients aged 60-69 years, 70-79 years and patients older than 80 years, respectively.

Number of examined nodes

The median number of examined nodes in the study period was 7 with an interquartile range of 4-11 (table 2). The number of examined nodes was significantly higher in 1998 compared to the other years. It increased with higher T-stage ($p<0.001$) and with a mucinous morphology ($p=0.002$), but decreased with older age with significantly more nodes being examined in the group aged younger than 60 years ($p<0.001$). The proportions of patients with <12 examined nodes were 75%, 83%, 85% and 85% for patients aged <60, 60-69, 70-79, and ≥80 years, respectively ($p<0.001$). In a multivariate log linear regression analysis, tumor location, T-stage and age were associated with the number of examined nodes (table 2).

Table 1. Patient and tumor characteristics and lymph node status

	N_0 (%)	N_1 (%)	N_2 (%)	Univariate (p)	Multivariate N_0 vs N_+		
				(N0 vs N+)*	p	OR	95% CI
Total	1543 (67,6)	556 (24,4)	182 (8,0)				
Tumor location				0.504	0.019		
Right	828 (68,3)	296 (24,4)	90 (7,3)	0.94		1.25	1.03-1.51
Left	715 (66,9)	260 (24,3)	92 (8,7)	1.00		1.00	
T-stage				<0.001	<0.001		
T1	146 (96,1)	5 (3,3)	1 (0,7)	1.00		1.00	
T2	262 (79,2)	63 (19,0)	6 (1,8)	6.41		5.77	2.43-13.65
T3	973 (63,6)	413 (27,0)	143 (9,4)	13.90		12.25	5.35-28.04
T4	162 (60,2)	75 (27,9)	32 (11,9)	16.07		14.35	6.08-33.85
Tumor type				0.892	0.337		
Mucinous	206 (68,0)	71 (23,4)	26 (8,6)	1.02			
Non-mucinous	1337 (67,6)	485 (24,5)	156 (7,9)	1.00			
Age (years)				0.127	0.285		
<60	299 (65,3)	109 (23,8)	50 (10,9)	1.00			
60-69	368 (64,9)	157 (27,7)	42 (7,4)	1.02			
70-79	560 (69,4)	191 (23,7)	55 (6,8)	0.83			
>80	316 (70,2)	99 (22,0)	35 (7,8)	0.80			
Age centered to mean (age-69.9)				0.045	0.313		
Nr lymph nodes				<0.001	<0.001		
0-5	713 (75,6)	207 (22,0)	23 (2,4)	1.00		1.00	
6-11	545 (63,7)	230 (26,9)	80 (9,4)	1.76		1.60	1.29-1.98
12-15	161 (58,8)	69 (25,2)	44 (16,1)	2.18		1.90	1.41-2.54
>16	124 (59,3)	50 (23,9)	35 (16,7)	2.13		1.85	1.34-2.56
Adjuvant chemotherapy	32 (2,1)	283 (50,9)	98 (53,8)	n.a.	n.a.		

*N_0 = node negative, N_1=1-3 positive nodes, N_2= 4 or more positive nodes, N_+=N_1or N_2

** The odds ratios for T-stadium are calculated compared to T1 as a reference.

Table 2. Log-linear model for number of examined nodes (>=1 nodes examined)

	Number	Median nr of nodes (interquartile range)	Univariate (p)	Multivariate P	RR	95% CI
Total	2180	7 (4-11)				
Tumor location			<0.001	<0.001		
Right	1137	6 (3-10)	0.72		0.74	0.69-0.79
Left	1043	8 (5-12)	1.00		1.00	
T-stage			<0.001	<0.001		
T1	114	4 (2-7)	1.00		1.00	
T2	317	6 (3-9)	1.42		1.40	1.20-1.64
T3	1492	8 (4-11)	1.83		1.69	1.47-1.94
T4	257	8 (4-12)	1.90		1.73	1.47-2.02
Tumor type			0.002	0.28		
Mucinous	293	9 (4-12)	1.00			
Non-mucinous	1887	6 (4-11)	0.88			
Age (years)			<0.001			
<60	446	9 (5-12)	1.00			
60-69	547	6 (4-11)	0.85			
70-79	764	6 (4-10)	0.80			
>80	423	6 (4-10)	0.79			
Age (continuous)			<0.001	<0.001*		
Age-69.9			0.99		0.991	0.988-0.994
Intercept**				<0.001	4.70	4.32-5.12

* For the multivariate analysis age centered to the mean was used

** the estimated median number of examined nodes for patients aged 69,9 years (mean age) with a T1 tumor located in the left colon

Model fit: Pearson X^2: 1075 (df=2174); p=1.00

Survival

The median follow up was 4.3 years with a range of 3.5 to 6,9 years. During follow up 872 patients died (32.5%). The 5-year overall survival rate was 51.3% (95% CI 47.4% -55.1%) for node positive patients and 68.2% (95% CI 65.6% -70.6%) for node negative patients, respectively. Table 3 shows the 5-year crude and relative survival proportions according to the number of examined nodes, stratified for the presence of positive nodes. The overall survival in node-negative patients was better in the group with more examined lymph nodes. In node positive patients there was a trend towards a better overall survival, although not statistically significant. Relative survival also improved among node-negative patients when more nodes were examined. However, for the node-positive group as well as for node-negative and node-positive patients combined, the relative survival was not associated with the number of examined nodes. In the latter group relative survival was only associated with the number of examined nodes after adjustment for the presence of positive nodes. In table 4 the observed and expected number of deaths and the EMR are groups shown according to age, invasion depth, number of lymph nodes examined, number of positive lymph nodes, chemotherapy received and year of diagnosis. In multivariate analysis, the EMR increased significantly with increasing depth of invasion and a higher number of positive lymph nodes. It decreased with a higher number of examined nodes and if treated with adjuvant chemotherapy. Age was also associated with excess mortality in this analysis. This implies that patients with tumors in higher T-stages and more positive lymph nodes experience higher excess mortality due to colon cancer, whereas patients with more examined lymph nodes or patients treated with adjuvant chemotherapy show lower excess mortality due to colon cancer.

Table 3 Five year overall survival (OS) and relative survival (RS) according to the number of nodes examined, stratified for the presence of positive nodes

	Pts	Deaths observed	5-yr OS	95% CI	P ¥	5-yr RS	95% CI	P #
Node-negative					0.0013			0.0323
<6 nodes	713	255	63.5%	59.5-67.2		82.1%	77.0-86.9	
6-11 nodes	545	177	70.2%	65.9-74.1		88.6%	83.2-93.4	
≥12 nodes	285	76	75.9%	70.0-80.8		91.6%	84.5-97.5	
Node-positive					0.0756			0.2927
<6 nodes	230	125	46.3%	39.4-52.9		59.6%	50.8-68.0	
6-11 nodes	310	145	53.9%	47.9-59.6		66.7%	59.2-73.7	
≥12 nodes	198	94	53.1%	45.5-60.1		62.1%	53.2-70.3	
All patients					0.0208$			0.3109$
					0.0002*			0.0288*
<6 nodes	943	380	59.3%	55.8-62.6		76.6%	72.2-80.8	
6-11 nodes	855	322	64.3%	60.8-67.6		80.6%	76.3-84.8	
≥12 nodes	483	170	66.5%	61.8-70.8		79.4%	73.7-84.5	

$ Overall test, unadjusted ; * Overall test, adjusted for the presence of positive lymph nodes (categorical); ¥ wilcoxon test ; # based on Wald test derived from Poisson regression analysis for relative survival. OS: Observed Survival; RS: Relative Survival; 95% CI: 95% confidence interval

Table 4 Estimated Excess Mortality Ratios (EMR) and 95% confidence intervals (95%CI)#

	Univariate statistics				Multivariate regression model		
	Patients	Deaths observed	Deaths expected	Person years	p-value	EMR	95%CI
Age					0.0353		
< 60 yrs*	458	101	11.9	2220.8		1.00	
60-69 yrs	567	161	45.1	2585.7		1.12	0.82-1.54
70-79 yrs	806	342	153.9	3163.6		1.35	0.99-1.83
80+ yrs	450	268	227.8	1541.2		0.78	0.48-1.26
Invasion depth					<0.0001		
T1/T2*	483	115	100.1	2289.3		1.00	
T3/T4	1798	757	338.7	7224.2		4.34	2.41-7.80
Positive nodes					<0.0001		
None*	1543	508	322.2	6750.3		1.00	
1-3	556	248	97.5	2186.4		3.20	2.39-4.29
4+	182	116	19.1	576.7		8.40	5.94-11.87
Nodes examined					<0.0001		
< 6*	943	380	194.9	3741.0		1.00	
6-11	855	322	167.7	3674.1		0.61	0.47-0.80
12-15	274	100	39.7	1190.4		0.50	0.34-0.74
16+	209	70	36.4	907.9		0.46	0.30-0.72
Chemotherapy					<0.0001		
No*	1868	720	404.6	7716.2		1.00	
Yes	413	152	34.1	1797.3		0.45	0.33-0.62

#Adjusted for time since follow-up

*Reference

Discussion

Radical surgical resection remains the most effective treatment for adenocarcinomas of the colon. It is known that the number of detected lymph nodes in a colectomy specimen varies widely. The difference in numbers of identified nodes may depend on variations in the pathological and/or surgical technique.

Because this study is retrospective there are several limitations. It was impossible to retrieve adequate information on the quality of the surgical resection other than the description of the surgical procedure in the operative record. This is also the case for the pathological reports. In The Netherlands, all pathologists are well organized and the specimens are usually examined according to the guidelines as provided by the Dutch Cancer Centers (www.oncoline.nl), which are based on the AJCC guidelines. [2] We assumed that the guidelines were followed properly.

This population-based study with a relatively large number of patients with a nearly complete follow up for vital status shows that tumor location, T-stage and patient age are associated with the number of nodes examined by the pathologist. It is well known that in sigmoidectomy and transversectomy specimens generally fewer lymph nodes are found than in a right or left hemicolectomy specimen. A clear-cut explanation for the association of age and T-stage with the number of retrieved nodes is difficult. It is possible that surgical resections are more limited in older patients or that the pathologists are less diligent in retrieving nodes in older patients. On the other hand fewer examined lymph nodes may reflect differences in the biological behavior of the tumor and/or host. The immune response against aggressive tumors may be different, or older patients and patients with more co-morbidity may have a diminished immune response leading to smaller lymph nodes in the draining lymphatic basin and thus fewer identified nodes.[22] It is known that mucinous tumors are of a different biological entity with a more aggressive behavior than other colorectal tumors.[23,24] In the univariate analysis in our study more lymph nodes were found for mucinous tumors, although this could not be confirmed in the multivariate analysis. A higher number of examined lymph nodes in T3 and T4 tumors might be explained by the fact that large tumors evoke a more intense inflammatory reaction than small tumors, leading to distension of lymphatic sinusoids with lymph node enlargement, and consequently to a higher number of nodes being identified by the pathologist.

T-stage and the retrieved number of nodes were associated with the nodal status in univariate as well as multivariate analysis. An increase in node-positivity with higher T-stages was expected, as these tumors usually represent more advanced disease. A higher number of examined nodes was associated with an increase in node-positivity, improving

the accuracy of the pathological status. This might be explained by the detection of small metastatic regional nodes with more diligent pathological sampling. Goldstein stated that there is no minimal number that reliably or accurately stages all patients and that as much lymph nodes as possible should be recovered, including those of 1 or 2 mm in diameter.[8] Furthermore, the studies of Haboubi et al. and Hida et al. showed that more than 70% of the metastatic lymph nodes are smaller than 5 mm in diameter.[9,10]

Numerous attempts have been made to estimate the minimum number of nodes necessary for correct staging, varying from 6 to 18 to an unlimited number of nodes.[8,12,17,25,26] There is currently consensus that at least 12 lymph nodes should be examined before considering a patient node-negative.[2] A review of over 100,000 patients from a National Cancer Institute registry showed that less than half of pathologic evaluations met these criteria during the period 1988 and 2001.[27] Joseph et al have estimated that more than 30 lymph nodes are needed to achieve a 85% probability of true N0 status at standard histology.[12] In our study we found a cut off point between 12 and 15 lymph nodes, which corresponds to the recommended amount of nodes to be examined by the AJCC [2] and the Dutch Oncological Society (www.oncoline.nl). However, compared to the study of Baxter et al, in the majority of patients in our study (79%) fewer than 12 nodes were examined, which reflects a rather poor pathological sampling in the study period. It is well possible that with more thorough pathological sampling more lymph nodes will be found and that the cut off point changes to a higher number of nodes.

There is substantial evidence in the literature that the number of lymph nodes examined has an important impact on survival in patients with colon cancer.[8,11,13,28] There are three potential explaining factors. Firstly, a more extensive lymphadenectomy may in itself convey a decreased risk of local and regional recurrence. Secondly, a surgeon who performs a more extensive lymphadenectomy may provide better cancer care in other respects. Thirdly, a pathologist who performs a more precise examination of the specimen will assure a more accurate staging, resulting in stage migration within patient populations. Until now, it has not been possible to identify a single mechanism for improved outcome with increasing nodal yield. In our study, node-negative patients showed a significantly higher 5-year crude and relative survival when more lymph nodes were examined. However, the relative survival in the node-positive group and the total group was not different when less than 6 or more than 12 nodes were examined. Only after adjustment for the presence of positive lymph nodes, the number of examined nodes was associated with a decreased survival in these groups. This implies stage migration to some extent in our study population: the improved staging accuracy leads to a better prognosis in all patient

strata, but will not affect the prognosis of the patient population as a whole.[29] There is probably a fourth reason why it is important to harvest more lymph nodes in a colectomy specimen. An increase in the number of nodes leads to more node positive patients with stage III colon cancer. These patients with stage III colon cancer are routinely offered adjuvant chemotherapy, as opposed to those with stage II colon cancer.[30,31] In our study only 51.6% of the patients were treated with adjuvant chemotherapy. This was mostly influenced by age, as >80 % of the younger stage III patients were treated with adjuvant therapy. It may be explained by the presence of more co-morbidity in older patients compared to younger patients.

This lack of treatment leads to insufficient power to calculate the exact survival benefit in our study. Looking at our study group in which the node-positive rate increased up to 12-15 examined nodes, the vast majority of patients had a less than optimum number of examined lymph nodes. This means that there is certainly potential for understaging and possibly, undertreatment with respect to adjuvant therapy. Following the recent ASCO guidelines for the use of adjuvant chemotherapy in high-risk stage II patients, about 80% of our N_0 patients are possible candidates for adjuvant therapy, because less than 12 nodes were detected at the pathological examination.[7] In summary, in our northern Dutch population with curable colon carcinoma there has been substantial pathological understaging from 1998 to 2002. At least 12 lymph nodes have to be examined to accurately predict nodal status. A higher number of examined nodes leads to stage migration. Through stage migration, more patients will be treated with adjuvant therapy. This may lead to a survival benefit for the entire group.

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Chapter 3

Effect of modified Davidson's fixative on examined number of lymph nodes and TNM-stage in colon carcinoma

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Abstract

Aims: We evaluated the effect of modified Davidson's Fixative (mDF) on the number of lymph nodes examined and staging in patients with colon carcinoma.

Methods: The results of two different fixation methods used in the pathological preparation of the resection specimens were analyzed. A traditional formalin preparation with manual dissection of all nodes was performed in 117 colon specimens between January 2003 and July 2004. After July 2004, the resected specimen of 125 patients were fixated in mDF. Differences in the retrieval and number of nodes and size of suspected nodal metastases were measured. All lymph nodes were stained with conventional H&E methods.

Results: The median number of examined nodes increased from 5 (0-17) to 13 (0-35) nodes after the introduction of mDF ($p<0.001$). The type of resection and the T-stage influenced the number of retrieved nodes significantly. The percentage of node positive cases increased from 30% to 41% ($p=0.077$) with mDF, the median size of the retrieved lymph nodes decreased from 9 mm before to 6 mm ($p<0.001$) and more micrometastases were found (6% vs 16%, $p=0.03$).

Conclusions: With mDF technique more lymph nodes were retrieved in the resected colon specimens. Smaller nodes and more micrometastases were found, leading to more node positive patients.

Introduction

The primary treatment for colon cancer is a radical surgical resection of the affected colon segment en-bloc with removal of related mesenteric lymph nodes. Adequate nodal staging is important for additional oncological treatment and to predict long-term survival based on the TNM classification.[1] In the assessment of nodal status the number of examined nodes is crucial. The impact of the surgeon and the surgical technique itself on quality and survival in patients with colorectal cancer have been described extensively.[2,3] However, the number of nodes detected in a surgical specimen also depends on the diligence of the pathologist and the extent of the pathological examination.[4-13] Numerous attempts have been made to estimate the minimal number of examined nodes for correct staging, varying from 6 to 18.[4,8,13-15]

Several methods have been developed to increase lymph node yield, including xylene fat clearance, alcohol treatment and ether based clearance. Most of these methods require special equipment and the use of noxious volatile compounds and are time consuming with a delay in outcome (up to 3 weeks). [5,6,11,16-20] Modified Davidson's fluid (mDF) is an acetic acid-alcohol-formalin based fixative that has been widely used for the preservation of different tissues for histological evaluation (www.histosearch.com, histonet archives, Davidson's fixative).[21,22] It is a rapid, simple to use substance that provides no additional safety hazards or disposal problems compared to routine formalin solutions. (http://members.aol.com/RSRICHMOND/histology.html).

This report compares traditional neutral buffered formalin fixation and manual identification of lymph nodes with the use of mDF on number, size, and presence of metastases of detected lymph nodes in surgical resection specimens of colon cancer in a routine daily practice.

Patients and methods

Patients

All patients were treated in a Dutch teaching hospital between January 2003 and January 2006. Patients with evidence of distant metastatic disease were excluded from the study as the presence of distant metastases might have led to an unusual surgical and pathological approach that differed from standard recommendations. Patients with adenomas or polyps were excluded for the same reason. Since the number of detected lymph nodes is influenced by pre-operative radiotherapy which is routinely applied in rectal cancer in the Netherlands, patients with rectal cancer were excluded from the study. Rectal cancer was defined as a tumor situated within 15 cm from the anal verge located beneath the

peritoneal reflection. Patients with previous colorectal surgery were also excluded from the study.

All patients underwent a potential radical surgical resection according to the standard rules, based on the location of the primary tumor. The performed procedure was deduced retrospectively from the surgical and pathological reports.

Pathology

All five pathologists employed at the Martini Hospital routinely examined the resected specimens. From January 2003 to July 2004, all 117 specimens were examined using the traditional technique of manual dissection after overnight fixation in 10% neutral buffered formalin. From July 2004, after overnight fixation of the 125 specimens in 10% neutral buffered formalin, the pericolic fat and mesentery was removed and immersed in mDF containing 500 ml of 37% formalin, 750 ml of absolute ethanol, 25 ml of 1.2% glacial acetic acid and 750 ml tap water. After mDF fixation, lymph nodes turn white in the mesenteric fat. During the whole study period, lymph nodes were examined with conventional H&E staining at 5 mm intervals. The size of the lymph nodes and nodal metastases of node positive patients was determined by one of the pathologists (A.T.) retrospectively by measuring, in millimeters, the largest diameter of the lymph node tissue on H&E stained cross-sections of the lymph nodes.

Equivalent to the description of nodal metastases in breast cancer, lymph node metastases <0,2mm were called isolated tumor cells, metastases between 0,2 and 2mm were called micrometastases, and metastases >2mm were called macrometastases.[1]

Statistical methods

SPSS 12.01 for Windows (SPSS, Inc, Chicago, IL) was the statistical software used for all the analyses. The level of significance was set to 0.05 for all tests. The χ^2 test was applied to test differences in proportions between groups. The Mann-Whitney U test was used to calculate the significance of differences in continuous variables.

Factors that were considered to be possible determinants of the number of examined lymph nodes and lymph node status were tested with an ANOVA analysis or regression analysis depending on the type of variable. The influence of possible determinants was also tested in multiple stepwise regression analysis for continuous variables and binary logistic regression analysis for nominal variables.

Results

Patients

Characteristics of the included patients and techniques before and after the introduction of mDF are listed in Table 1. Both groups did not differ significantly with respect to patient gender, age, T-stage, type of resection, length of specimen and the pathologist who examined the specimen. Due to changes in the surgical staff, there was a difference in the operating surgeons before and after the introduction of the mDF fixation.

Number of examined lymph nodes

All results for the number of examined nodes are shown in tables 1 and 2. The median number of examined nodes for the whole group was 10 (0-35). With traditional formalin fixation the median number of nodes was 5 (0-17). After the introduction of mDF the median number of nodes increased significantly to 13 (0-35). The ANOVA test showed that T-stage, the type of resection and the operating surgeon also might have an effect on the number of nodes. No effect was found for the pathologist and the length of the specimen. Linear stepwise regression analysis showed that the fixation technique was the most important predictor for the number of examined nodes, followed by the type of resection, T-stage and the operating surgeon. In this multivariate analysis the effect of the operating surgeon and T-stage were not significant. There was no significant difference in the mean number of nodes per surgeon when corrected for the type of resection. More nodes were removed with a right or left hemicolectomy compared to the other types of resection. In patients with a T1 tumor less nodes were removed compared to the other T-stages (mean 5 vs mean 10).

N-stage

Table 2 shows the results of the statistical analysis for nodal status. Table 3 shows the N-stage before and after the introduction of mDF. The percentage of node positive cases increases from 30% to 41% after the use of mDF. The mean number of nodes was 9.9 in the node-negative group and 10.4 in the node-positive group. The χ^2 test showed a possible effect of T-stage and fixation technique on N-stage. The type of resection, the operating surgeon, the pathologist, the length of the resected specimen removed and the number of examined nodes showed no effect. Both T-stage and fixation technique were tested in a binary logistic regression analysis. T-stage reached significance, while the fixation technique did not.

Using the cut off point of the required 12 examined nodes according to the Dutch Cancer Guidelines; the proportion of node positive patients was 34% if less than 12 nodes are examined versus 38% if 12 or more nodes were examined which is not significant. To determine the minimal number of nodes to be examined for an accurate prediction of the N-stage, we divided the patients in groups based on the number of nodes removed. However, no difference was noted in N-stage per group (table 4).

Table 1. Patient, surgical and pathological factors

	Total group	Traditional technique	mDF	P
Gender ratio (♂/ ♀)	137/105	64/53	73/52	NS
Mean Age	73 (35-95)	74 (42-91)	72 (35-95)	NS
T-stage				NS
Tis	4	2	2	
T1	13	7	6	
T2	34	16	18	
T3	167	84	83	
T4	24	8	16	
Median Nr of nodes	10(0-35)	5(0-17)	13(0-35)	P=0.000
Type of resection				NS
Right hemicolectomy	119	56	63	
Left hemicolectomy	25	8	17	
Transversectomy	8	4	4	
Sigmoïdectomy	86	46	40	
Ileocecal resection	4	3	1	
Surgeon				p=0.01
1	30	18	12	
2	13	5	8	
3	20	9	11	
4	22	8	14	
5	73	44	29	
6	30	9	21	
7	30	18	12	
8	24	6	18	
Mean length of specimen	25 (6-73)	25cm (7-70)	24cm (6-73)	NS
Pathologist				NS
1	23	12	11	
2	80	35	45	
3	34	19	15	
4	29	14	15	
5	76	37	39	

Table 2. Determinants of number of nodes and nodal status

	Univariate (p)	Multivariate (p)
Number of nodes	ANOVA	Linear regression
Fixation technique	0.000	0.000
T-stage	0.022	NS
Type of resection	0.042	0.010
Surgeon	0.034	NS
Pathologist	NS	
Length of specimen	NS	
Nodal status	χ^2	Logistic regression (p)
Fixation technique	0.077	NS
T-stage	0.009	0.004
Type of resection	NS	
Surgeon	NS	
Pathologist	NS	
Number of nodes	NS	
Length of specimen	NS (ANOVA)	

Table 3. N-stage before and after introduction of mDF

Stage	Total	Traditional technique	mDF
N0	156	82	74
N+[1]	86	35	51
N1	64	26	38
N2	21	9	13
Total	242	117	125

[1]All node-positive cases

Table 4. Percentage of N+ patients per nr of nodes

Nr of nodes (nr pts)	Node-positive patients (%)
≤ 6 (88)	34.1
≤ 8 (111)	35.1
≤ 10 (131)	32.8
≤ 12 (163)	33.7
≤ 14 (193)	35.2
≤ 16 (207)	35.7
≤ 18 (221)	35.3
≤ 20 (226)	35.4

Number of positive nodes and size of metastases

Before the introduction of mDF the total number of positive nodes was 84 with 5 micrometastases (5.9%) and 79 macrometastases (94%). After mDF fixation there were 126 positive nodes with 2 isolated tumor cells (1.6%), 18 micrometastases (14.2%) and 106 macrometastases (84%). This difference in the percentage of micro- and macrometastases is significant ($p=0.03$). The median size of the positive nodes found before introduction of the fixation technique was 9 mm. After changing the technique the size decreased to 6 mm. This difference is significant ($p<0.001$). The size of the negative lymph nodes found in the specimens with positive lymph nodes also decreased significantly from a median of 6 mm before the change of technique to 4 mm after ($p<0.001$).

Discussion

Methods

The serial study set up is not ideal for comparing two fixation methods. However, both study groups were comparable with respect to patient gender, age, T-stage, type of resection, length of specimen and the pathologist who examined the specimen. Although there was a difference in operating surgeons before and after the introduction of the mDF fixation, it was not a significant factor in the multivariate analysis in relation to the number of examined nodes. Moreover, there was no significant difference in the mean number of nodes per surgeon when corrected for the type of resection. Therefore, the study set up is applicable in this particular situation.

Number of examined lymph nodes

The principle of radical surgical resection of colon cancer includes removal of the affected colon segment with adequate margins en bloc with all draining lymph nodes in the corresponding mesocolon. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), in contrast to 45-50 % for patients with node positive tumors (stage III).[23] Adjuvant chemotherapy in patients with stage III colon cancer clearly improves survival.[24-26] The number of examined lymph nodes in a colectomy specimen varies widely. This may be due to variations in the surgical technique or the pathologist's attempt in retrieving the nodes from the resected specimen. There is substantial evidence that the number of lymph nodes examined has an important impact on survival in patients with colon cancer.[4,7,9,27] An oncological specialized surgeon probably performs a more extensive lymphadenectomy which yields more nodes in the specimen. In addition, a pathologist who performs a more precise examination of the specimen also provides more accurate staging. It has not been possible to identify a single mechanism for improved outcome with increased node count. In our study the type of resection and the fixation technique are significant factors in the number of recovered lymph nodes. It is known that generally less lymph nodes are found in a sigmoidectomy or transversectomy specimen than in a right or left hemicolectomy specimen. Regarding the fixation technique, comparisons of mDF with previously described methods are clearly in favor of mDF. It is neither time-consuming nor costly and does not involve the use of noxious substances like diethyl ether or xylene which are used in fat clearance techniques. (http://members.aol.com/RSRICHMOND/ histology.html). In addition, mDF can be used with conventional ventilation devices. After 24-48 hours of fixation specimens can be processed or transferred to alcohol or formalin for storage. Due to this rapid effect, safety and low costs it is ideal for use in a busy primary or tertiary care hospital. With mDF lymph nodes turn white in the yellow mesenteric fat, making it easier for the pathologist to identify even small lymph nodes, thereby reducing the operator dependence in lymph node retrieval.[21] Two studies showed that 72% of the metastatic lymph nodes are smaller than 5 mm in diameter.[5,6] In our study indeed more and smaller lymph nodes are found with mDF, which may lead to an increase in lymph node metastases.

N-stage

Not only found more lymph nodes were detected after the introduction of mDF, but we also found more and smaller positive nodes. This can be explained by the white color of regional nodes, which facilitates detection compared to conventional manual dissection

with non-white nodes. In addition, more micrometastases were noted with mDF. Both factors probably contributed to 11% more node positive patients after the introduction of this mDF. Although not significant with p=0.077, it does seem clinically relevant for nodal staging. It could be that our population is just too small to detect a significant difference. Therefore, larger studies are required to demonstrate the real impact of additional, smaller lymph nodes on prognosis and/or their therapeutic significance. It was not possible to find a cut off value in the number of lymph nodes to be examined to find more nodal metastases with this modified fixation method. Using the recommended cut off number of 12 nodes we did not find a significant difference in the percentage of node positive patients.[1,14] Even when we used cut off points of 6, 14 or 18 lymph nodes as mentioned in most studies[8,15,28], no significant difference in node-positivity was found. Again, insufficient patient numbers might play a role. As Goldstein stressed the importance to examine even lymph nodes of 1 or 2 mm in diameter[4] our study confirmed that the difference in N-stage seems to depends on the smaller metastases found after mDF fixation. Therefore, it is important to search also for smaller nodes and not only for the highest number of large nodes.[5]

In this single center study the number of nodes recovered, the surgeons involved in the operation and the pathologists were of no significant importance. The only important factors were T-stage and the use of mDF. The increase in node-positivity with higher T-stages is expected, as it represents a more advanced disease.

Effects of staging on adjuvant therapy

As the two patient groups are not related, we cannot state that there is any upstaging after mDF. We have only observed that with mDF 41% of the patients had lymph node metastases compared to 30% with formalin fixation. We have to wait for the survival data of both patient groups before we can draw any conclusions on the importance of this fixation technique for staging and prognosis. Hypothetically, it is interesting to calculate what could happen if 11% more patients would be offered adjuvant chemotherapy, keeping in mind that before July 2004 patients with less than twelve examined lymph node did not automatically receive chemotherapy in our region. In our hospital, we treat a part of the population covered by the Comprehensive Cancer Center North Netherlands (CCCNN). In this northern region, 625 colon resections are performed annually in colon cancer patients without proven metastases. An increase of 11% in lymph node metastases will lead to 69 more patients being referred for adjuvant chemotherapy. With the current chemotherapy regimens an increase in the 5-year survival rate of 15-20% can be expected compared to

no adjuvant therapy at all.[29] Considering this, about 10 to 14 people would benefit in overall survival, assuming that they all do receive adjuvant treatment.

Conclusion

After adequate surgical resection in patients with colon cancer, the pathologists may improve the staging procedure by using the mDF fixation technique which is simple, rapid and cheap. With this method more and smaller lymph nodes and smaller nodal metastases were detected. This may result in upstaging and a possible survival benefit as more patients will be offered adjuvant chemotherapy.

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Chapter 4

RT-PCR and immunohistochemical evaluation of sentinel lymph nodes after in vivo mapping with Patent Blue V in colon cancer patients

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Abstract:

Background: Lymph node status is the most important predictive factor in the treatment of colorectal cancer. As sentinel lymph node (SLN) biopsy might upstage stage II colon cancer it could have therapeutic consequences in the future. Therefore we studied the feasibility of in vivo SLN detection with Patent Blue V dye and evaluated nodal microstaging and ultrastaging using cytokeratin immunohistochemistry and RT-PCR methods.

Patients and Methods: In 30 consecutive patients operated for colon cancer, subserosal injection with Patent Blue dye was used in the SLN detection in 4 different hospitals under supervision of one regional coordinator. In searching for occult micrometastases each SLN was examined at three levels. In tumor-negative SLN's at routine hematoxylin-eosin (H&E) examination (pN0) we performed CK8/CK18 immunohistochemistry (IHC) and RT-PCR for CEA.

Results: The procedure was successful in 29 out of 30 patients (97%). The SLN was negative in 18 patients by HE and IHC. In 16 patients the non-SLN were also negative, leading to a negative predictive value of 89% and an accuracy of 93%. Upstaging occurred in 10 patients (33%); 7 by IHC and 3 by RT-PCR. Aberrant lymphatic drainage was seen in 3 patients (10%).

Conclusions: The SLN concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to an upstaging of nodal status in 33 % of patients when IHC and PCR techniques are combined. Therefore, the clinical value of SLN should be subject of further studies

Introduction

Colorectal carcinoma (CRC) is the most common gastro-intestinal malignancy and the second leading cause of cancer related deaths in the Western World. Lymph node status as the most important predictor of outcome indicates the use of adjuvant chemotherapy in these tumors. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but only 45-50 % for those with node positive tumors (stage III).[1] Adjuvant chemotherapy significantly improves the 5-year survival in patients with node positive CRC. Despite the favorable prognosis of patients with localized colon cancer without regional lymph node metastasis, 20-30% of these patients will develop recurrent disease, after apparently curative resection. This might be explained by abstinence of adjuvant treatment in case of pathological understaging at the time of resection. Understaging may be the result of inadequate numbers of examined lymph nodes, missing some metastases.[2,3] For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes harvested by pathological analysis is warranted according to the Dutch National Cancer Centre Guidelines and international guidelines.[4] Moreover, intensive pathologic examination of lymph nodes by immunohistochemical staining for cytokeratin or reverse transcriptase-polymerase chain reaction (RT-PCR) may reveal micrometastases that would be missed by routine hematoxylin & eosin (H&E) examination. Although these staging techniques are time consuming, labor intensive and costly, several authors have reported a decreased survival rate when nodal micrometastases are detected in CRC.[5,6] For optimal staging, examination of SLN's may therefore be helpful.

The technique of the sentinel node biopsy was first described and performed by Cabanas (1977) in penile carcinoma.[7] However, it was Morton et al. and Giuliano et al. who introduced the sentinel node biopsy for staging patients in general practice in melanoma and breast cancer.[8,9] In CRC the SLN's are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumor. They have the greatest potential to harbor metastatic disease when present, enabling focused examination with multilevel microsectioning of the SLN's to provide a more efficient and cost-effective detection of micrometastases. In addition, patterns of aberrant lymphatic drainage can be visualized with sentinel lymph node mapping, which may lead to a more extended resection. Most reported studies of the SLN concept in CRC showed good results with isosulfan blue (Lymphazurin). [10-22] However, Lymphazurin is not registered for clinical use in Europe. The results with Patent blue-V, that is commonly used in Western Europe, are variable with only one study showing comparable results to the isosulfan blue studies.[23-28]

Besides, only few sentinel node studies in colon carcinoma have been performed in a multi-center setup. We tested the utility of Patent Blue-V in vivo to identify SLN in colon cancer patients in four different hospitals and evaluated our experience with immunohistochemical and RT-PCR techniques in detecting occult micrometastases on routine H&E negative SLN's.

Patients and Methods

Patients

Only patients with histological proven primary colon carcinoma were included in the study. Patients with distant metastases or gross lymph node involvement as shown by pre-operative examinations or palpation during surgery were excluded. The local scientific ethics commission approved this study and all patients had given informed consent.

Surgical procedure

This feasibility study was performed by one surgeon in each of the four different hospitals. The first 5 procedures of each surgeon were supervised by one coordinating surgeon (J.T.P). Sentinel lymph node mapping was carried out through an open procedure by injection of 1-3 ml Patent Blue with a tuberculin syringe and 29 gauge needle subserosally in 4 quadrants around the tumor. The subserosal injection was carried out prior to vascular ligation. Within 5 to 10 minutes after the blue dye injection, the SLN's could be identified by following blue stained lymphatic vessels leading to the blue stained sentinel node. These nodes were tagged with a long suture. Sentinel nodes were defined as the first four blue-staining nodes seen within the regional basin. After marking of the SLN's, routine resection was performed.

Pathology

The tumor and all lymph nodes were examined according to standard guidelines.[2] If the SLN's were negative after routine hematoxylin-eosin (H&E) staining, they were sectioned at 150 µm intervals and examined at 3 levels with H&E as well as immunohistochemistry on cytokeratins (CK8/CK18). Metastases between 0,2 mm and 2 mm were referred to as micrometastases. Metastases smaller than 0,2 mm were described as isolated tumor cells.[2] From 12 out of 18 SLN-negative patients (of which 2 were false-negative), enough paraffin embedded material was available to perform real-time PCR for CEA on one level of the sentinel node. This method has been described earlier.[29] Total RNA was isolated from one 4

μm paraffin-embedded tissue section. In brief, tissue was incubated in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 2% SDS) and treated for 12 hours with 500 μg/ml proteinase K at 60˚C. Proteinase K was inactivated for 5 minutes at 95˚C, and RNA was extracted with 1/10 volume of 3M NaAc, 1/5 volume of chloroform, and 1 volume fenol. RNA was precipitated using an equal volume of isopropanol and 1 ul carrier glycogen (Roche). Total RNA was treated with DNAse I using the TURBO DNA-*free* kit™ according to manufacturer's instructions (Ambion,). RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Paisley, UK) in a volume of 20 μl using random hexamers (300 ng). An Assay-on-Demand Gene Expression Product™ (Applied Biosystems) was used for analysis of *CEA* (Hs 00237075_m1). Primers (Invitrogen) and probe (Eurogentec, Seraing, Belgium) for *GAPDH* were developed using primer design software (Applied Biosystems, Foster City, CA, USA). Primers used were: *GAPDH*F 5'-ccacatcgctcagacaccat-3', *GAPDH*R 5'-gcgccaatacgaccaaat-3'. Probe sequence labeled 5' with the FAM reporter dye and 3' with the TAMRA quencher dye molecules was: *GAPDH* 5'-cgttgactccgaccttcaccttccc-3'. Reactions were performed in 384-well plates (Applied Biosystems) in a volume of 20 μl containing real-time PCR mastermix (Eurogentec), 900 nM of each primer, 200 nM of an individual probe and 5 ng cDNA. PCR amplifications were performed using the ABI prism 7900HT sequence detection system (Applied Biosystems). Standard cycling conditions were used including a pre-amplification step of 50˚C for 2 min, 95˚C for 10 min, followed by amplification of 40 cycles of 95˚C for 15 s and 60˚C for 1 min. All samples were analyzed in triplicate. Mean cycle treshold values (Ct) and standard deviations (SD) were calculated.

Results

A total of 30 patients were included in the study, 14 women and 16 men. The mean age at the time of surgery was 69 years (48-85). The tumor characteristics are shown in table 1. A median number of 14 lymph nodes were harvested, with a mean number of 2,7 (range 1-4) sentinel nodes. The procedure was performed successfully in 29 patients (97%). The patient in whom the procedure failed, had a carcinoma of the sigmoid within an area of diverticulitis. Aberrant lymphatic drainage was seen in 3 patients (10%): to the splenic flexure in right-sided tumors (n=2) and a para-aortic node in a recto-sigmoid tumor (n=1). This resulted in a more extended resection. No patient developed toxicity associated with the use of Patent Blue.

SLN examination was negative for metastases by H&E and IHC in 18 patients (62%). In 16 of these patients, the non-sentinel nodes were also tumor-negative. This leads to a negative predictive value of 89%. One of the two patients with a false-negative SLN had extranodal

disease in the non-SLN's. The other failure occurred in a patient with a tumor in the ascending colon with H&E proven micrometastases in a small, peritumoral lymph node. Overall, the accuracy of the procedure in our study was 93% (27/29). In 11 patients (11/29, 38%) we found metastases in the SLN's. In 4 of these patients the SLN's were positive on H&E examination and in 7 patients the SLN's showed metastases after immunohistochemistry. In 5 of these 7 patients we only found isolated tumor cells. In 6 out of 29 patients (21%) the nodal stage could be identified by conventional H&E examination. In 13 patients the combination of H&E and IHC lead to a positive lymph node result (45%), leading to an upstaging of 25%. All sentinel nodes found by detection of aberrant drainage were negative in this study.

RT-PCR for CEA was performed on the paraffin embedded sentinel nodes for 12 out of 16 cases. At every run, we checked positive as well as negative controls. Every time, positive controls turned out positive, and negative controls turned out negative. The mean Ct-value for the housekeeping genes was 27,92 (26,3-29,7) with a mean standard deviation of 0,097 (0,034-0,27) indicating that the RNA quality and quantity was similar for all cases. This analysis revealed 3 patients with increased CEA levels indicating the presence of metastases in the SLN's. Taking this into account, we found a total of 16 out of 29 patients (55%) to be node positive: two patients had H&E positive non-SLN's and negative SLN's, 4 patients had H&E positive SLN's, 7 patients had IHC proven metastases, and 3 patients had metastases after RT-PCR examination. This leads to an upstaging of 33% by IHC and RT-PCR in our group.

We also looked at the stage of the primary tumor in relation to the occurrence of IHC or PCR detected metastases in H&E negative patients. In stage I patients ($T_{1/2}N_0$) we found micrometastases or a positive PCR result in 1/6 patients (17%). In stage II patients ($T_{3/4}N_{0)}$ there were 8/17 patients with IHC or PCR detected metastases (47%). The numbers are too small to obtain any significance from these results.

Table I Tumor Characteristics

Tumor location	
Caecum	6
Ascending colon	9
Transverse colon	0
Descending colon	1
Sigmoid colon	14
T-stage	
1	0
2	7
3	22
4	1
Median number of lymph nodes	14
Average number of sentinel nodes	2,7

Discussion

Isosulfan blue (Lymphazurin) and Patent blue dye are both used in sentinel lymphatic mapping. The chemical composition of both dyes is different. Patent Blue V has a calcium-ion instead of a sodium-ion, one extra hydroxy-group and one sulphonate-group in a different position. To our knowledge there is no study in which Lymphazurin and Patent Blue V were compared in SLN mapping for colon carcinoma. The indication or rationale for the SLN biopsy in colon cancer includes accurate staging to provide information regarding prognosis and regional control. Using the SLN procedure will also improve the diagnosis of micrometastases in the regional tumor-draining lymph nodes by providing a focused histopathological assessment of selected lymph nodes most likely to harbor occult disease. In addition, the SLN procedure results in blue stained nodes, sometimes very small, that could have been easily missed by the pathologist on routine examination, without the blue staining.

This study confirms the results of a previous study, showing that Patent Blue V is an adequate marker of the sentinel node in patients with colon cancer.[23] Based on this study we found that Patent Blue V can be used regularly in further studies on the sentinel node

concept in CRC. This study suggests that it may be possible to perform an in vivo SLN procedure in a multi-center study with adequate supervision during the learning curve. Recently, Bertagnolli performed the sentinel node procedure in 13 different hospitals with 25 different surgeons on 79 patients and concluded that the sentinel node was a poor predictor of lymph node status.[30] However, a mean number of three patients per surgeon may be insufficient to perform this procedure adequately and will probably lead to a relatively high number of technical failures. Read et al. also failed to obtain good results with this procedure. However, they included a relatively high number of stage III and IV patients (30%) which could have disturbed the normal lymphatic distribution resulting in a non-reliable SLN node procedure.[31] Other studies, including that of Bilchik and Saha performed at 3 different hospitals reported excellent results.[16,32] Paramo et al. found a stabilization of the learning curve of the SLN procedure in colon carcinoma after 5 operations.[13]. In our study the sentinel node procedure failed only once. The surgeons appreciated the presence of a supervising instructor at their first two to four procedures. The overall accuracy of 93 % is comparable to that found in larger, previous studies.[13,16,18,19,22,32]

The one failure in SLN detection can be explained by the presence of diverticulitis around the tumor. Diverticulitis could disturb the normal lymphatic distribution, thereby interrupting the movement of dye from the tumor to surrounding lymph nodes. In one of our two false negative SLN procedures the non-SLN's showed extra-nodal tumor invasion. It is well known that grossly involved lymph nodes or large bulky tumors with direct tumor invasion through the bowel wall can lead to obstruction of lymphatic channels and skip-metastases. These skip metastases (false negative SLN's) are reported in 18-25% depending on the use of ultrastaging methods.[13,18,20,22,24] Usually the dye-mapping affects the pericolic LN's directly around the bowel, assuming that they are first to be reached by metastatic disease. Sometimes intermediate and apical nodes just proximal of the main vessels are stained blue, suggesting that large bowel segments should be resected to obtain optimal regional control. In very rare cases direct lymphatic drainage to para-aortic nodes is seen, suggesting that an even more extended resection should be performed .

Aberrant lymphatic drainage was found in 10% of cases. This is according to the reported rate of 2-9% in the literature.[10,13,15,16,18,19,32] In all these cases we performed an extended resection. None of these sentinel nodes contained tumor cells. The importance of this aberrant drainage for staging can only be established in larger series with a long follow up.

Unlike the validated SLN concept in breast cancer and melanoma which affect the need for lymphatic dissection, the main reason for SLN mapping in CRC is to focus pathologic

examination on the SLN's, which will increase the accuracy of nodal staging, resulting in a higher percentage of node-positive patients, who may benefit from adjuvant chemotherapy.[4,5,19] Upstaging by H&E conventional examination is difficult to measure. It might be explained by the focused examination of blue stained nodes, because these blue nodes can be very small nodes and would otherwise not have been detected. The IHC in our study was performed on cytokeratins. The reason to test for cytokeratins, is the known and widely used protocol in Dutch hospitals for the SLN procedure in breast cancer using IHC for cytokeratins. Several studies described the PCR examination of lymph nodes in colon carcinoma using CEA or CK 20 as a marker.[6,33-37] All reported upstaging, and 4 studies reported an adverse effect of upstaging on prognosis.[6,35-37] We use CEA because it is a disease specific marker that is present in the majority of colon carcinomas.

The answer to the crucial question regarding the impact of occult nodal metastases detected by serial step sectioning combined with immunohistochemistry and RT-PCR examination remains unclear. We found an upstaging by immunohistochemical staining in 25% of patients, including micrometastases in two cases, and isolated tumor cells in five cases. Upstaging of the nodal status with multilevel pathologic sectioning and the use of immunohistochemistry has been described in 11-19 % of cases.[10,13-15,18-20,23] The higher percentage in this study can be explained by the difference in methods used for immunohistochemistry in previous studies. Most studies performed sectioning with intervals of 500µm or immunohistochemistry on 1-4 levels in total, while we used standard intervals of 150 µm at 3 levels standard in this study. Increasing the number of slices for immunohistochemistry probably improves the detection rate of micrometastases smaller than 2 mm, but the prognostic significance of these small deposits still has to be cleared in large studies. A variety of results on this subject have been described.[5,6,38-41] Some studies using immunohistochemistry on cytokeratins reported no effect of micrometastases on survival, while others described a worse survival in patients with micrometastases. [34,38-40] [5,41,42]

Liefers et al. examined lymph nodes in colorectal carcinoma using RT-PCR on CEA. They found a significant survival difference in patients with and without tumor cells in lymph nodes.[6] This could mean that our detection of isolated tumor cells in five patients and a positive PCR-result in another three with an upstaging to 35% is important.

The quantitation of gene expression in formalin-fixed, paraffin embedded tissue has been subject to serious limitations in the past. RNA isolated from paraffin embedded tissue blocks is of poor quality due to extensive degeneration during the formalin fixation process.

Moreover, formalin fixation causes cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methylol groups to the bases, making subsequent RNA extraction, reverse transcription and quantitation analysis problematic.[43]

The method we used to perform RT-PCR on formalin-fixed tissue, has been described and validated before.[29] For this method it is crucial to use an RNA extraction protocol that provides only minimally cross-linked RNA. In addition, small target sequences should be selected (60-100 basepairs) enabling the detection of fragmented and degraded RNA. The Ct values and standard deviations obtained for the housekeeping gene indicated that the quality and input amount of RNA is comparable for the different paraffin blocks.
We should wait for the results after follow up in a large group of patients before we can estimate the real impact. If future results confirm the importance of microstaging and ultrastaging in CRC, the sentinel node concept can help the pathologist to focus the examination on one or two sentinel nodes in H&E negative cases. The detection of micrometastases might then select a subgroup of patients who could benefit from adjuvant treatment.

Conclusion

The sentinel node concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to an upstaging of nodal status in 33 % of patients when IHC and PCR techniques are combined and may detect aberrant lymphatic drainage (10%). This procedure can be performed in a multi-center study under adequate supervision during the learning curve and may have diagnostic and therapeutic consequences in the future.

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Chapter 5

The sentinel node procedure in colon carcinoma: a multi-centre study in the Netherlands

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Abstract

Background: Lymph node status is the most important predictive factor in colorectal carcinoma. Recurrences occur in 20% of the patients without lymph node metastases. The sentinel lymph node (SLN) biopsy is a tool to facilitate identification of micro-metastatic disease and aberrant lymphatic drainage. We studied the feasibility of in vivo SLN detection in a multi-centre setting and evaluated nodal microstaging using immunohistochemistry (IHC).

Patients and Methods: Subserosal injection with Patent Blue dye was used in the SLN procedure in 69 patients operated for localized colon cancer in 6 Dutch hospitals. Each SLN was examined with routine haematoxylin-eosin (H&E) staining. In tumor-negative SLN's we performed CK7/8 or 18 IHC.

Results: The procedure was successful in 67 of 69 patients (97%). The SLN was negative in 43 patients. In 3 cases it was false negative, resulting in a negative predictive value of 93% and an accuracy of 96%. In 24 of 27 patients with lymph node metastases in a successful SLN procedure, the SLN was positive (sensitivity 89%). In 15 patients the SLN was the only positive node (21%). In 9 patients we only found micrometastases or isolated tumor cells, resulting in 18% upstaging. Aberrant lymphatic drainage was seen in 3 patients (4%).

Conclusion: The SLN procedure in localized colon carcinoma is reliable in a multi-centre setting. It is helpful to identify patients who would be classified as stage II with conventional staging (18%) and who might benefit from adjuvant treatment.

Introduction

Survival in patients with colon carcinoma is strongly correlated with lymph node status: the 5-year disease-free survival rate is 70-80% for patients with lymph node negative disease (stage I/II), but only 45-50 % for those with node positive disease (stage III).[1] The presence of lymph node metastases indicates the use of adjuvant chemotherapy in these patients, which increases the 5-year survival rate with about 10%.[2] Despite the favorable prognosis of patients with localized colon carcinoma without regional lymph node metastasis, 20-30% of these patients will develop recurrent disease after apparently curative resection.[3] It is possible that in this group of patients small lymph node metastases have been missed , resulting in understaging. This may be due to an inadequate surgical lymphadenectomy or insufficient pathological examination.[4] According to international guidelines meticulous pathological examination of at least 12 lymph nodes is warranted for adequate staging of patients with colon carcinoma.[5] However, several studies showed that the minimal number of lymph nodes necessary for correct staging varied considerably from 6 to 18 to as many as possible in the study of Goldstein et al.[4,6-9] In addition, in depth pathological examination of lymph nodes by immunohistochemical staining for cytokeratin or reverse transcriptase-polymerase chain reaction (RT-PCR) may reveal micrometastases that could have been missed by routine haematoxylin & eosin (H&E) examination. Several authors have reported a decreased survival rate when micrometastases are detected in colon carcinoma.[10,11] [12,13] The possible benefit of adjuvant therapy in this group of patients is not clear yet. These (ultra)staging techniques are time consuming, labour intensive and costly. For optimal staging, in depth examination of only the SLN could be helpful. In colon carcinoma the SLN's are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumour. They have the greatest potential to harbour metastatic disease when present, enabling focused examination with multilevel microsectioning of the SLN's to provide a more efficient and cost-effective detection of micrometastases. In addition, patterns of aberrant lymphatic drainage can be visualized with SLN mapping, which may lead to a more extended resection. Several studies have reported varying results of the SLN procedure in colon carcinoma.[14-20] This study presents the results of the SLN procedure in six Dutch hospitals. The primary aim of this study is to test the accuracy and sensitivity of the SLN procedure in a multi-center setting. Furthermore, we looked at upstaging and possible aberrant lymphatic drainage.

Patients and Methods

Patients

Only patients with histological proven primary colon carcinoma were included in the study. Patients with distant metastases or gross lymph node involvement as shown by pre-operative examinations or palpation during surgery were excluded.

Surgical procedure

This study was performed between May 2002 and May 2005 in five teaching hospitals and one university hospital. All procedures were supervised by one of the coordinating surgeons (JTMP, AEB). The procedure was only performed when one of the study coordinators was available for supervision (JTMP, AEB,WK). The study was approved by the local scientific ethics committee and all patients had given informed consent. Patients with rectal cancer were excluded from the study.

SLN mapping was carried out through an open procedure by injection of 1-3 ml Patent Blue with a tuberculin syringe and 29 gauge needle subserosally in 4 quadrants around the tumor. The subserosal injection was carried out prior to vascular ligation. Within 5 to 10 minutes after the blue dye injection, the SLN could be identified by following the blue stained lymphatic vessels leading to the blue stained SLN. These lymph nodes were tagged with a long suture. SLN's were defined as the first one to four blue-stained lymph nodes seen within the regional basin. After marking of the SLN's, routine resection was performed. If the SLN was found outside the normal lymphatic basin, we performed an extended resection.

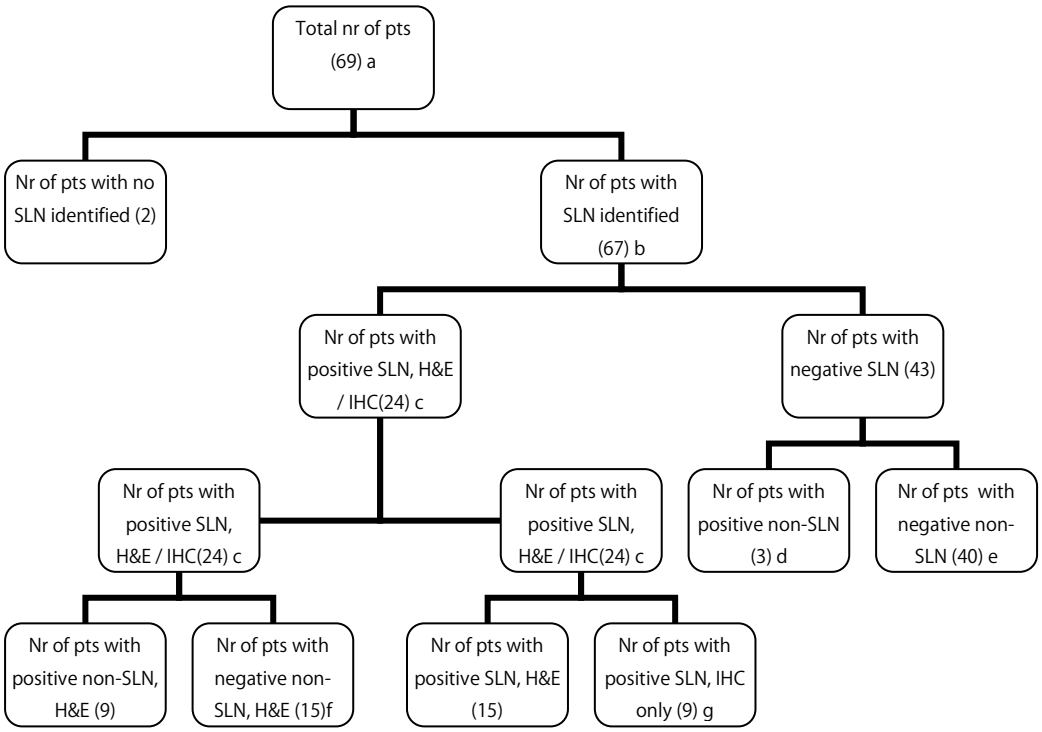
Pathology

The tumor and all lymph nodes were examined according to standard guidelines.[5] If the SLN's were negative after routine haematoxylin-eosin (H&E) staining, they were sectioned at 150 µm intervals and examined at 3 levels with H&E as well as immunohistochemistry on cytokeratins (CK7/8 or 18). Metastases between 0,2 mm and 2 mm were referred to as micrometastases. Metastases smaller than 0,2 mm were referred to as isolated tumor cells.[5] Upstaging was defined as the presence of micrometastases or isolated tumor cells after immunohistochemistry in patients with a negative lymph node status after H&E.

Definitions

See Figure 1. Identification rate is the number of patients with one or more SLN's identified (b) / the total number of procedures (a) x 100%. Negative SLN's were false-negative if one of the other regional lymph nodes (non-SLN's) were tumor-positive (d). The accuracy of the SLN procedure suggests a conformity of the SLN status and the regional nodal status i.e. the total number of patients with a positive SLN (c) + the number of patients with a true negative SLN (e) / the number of patients with an identified SLN (b) x 100%. Sensitivity is the number of patients with a positive SLN (c) / The total number of node positive patients (c+d) x 100%. Upstaging is the number of patients with positive SLN's by IHC (g) / the number of patients who were node negative by H&E examination (e+g) x 100%.

Fig. 1 Flowchart. *SLN* Sentinel lymph node; *pts* Patients



Results

The SLN procedure was performed in 69 patients. Tumor characteristics are shown in table 1. Figure 1 shows the total number of patients and SLN results. At pathological examination a mean of 11 nodes per specimen was found; per hospital this varied between 9 and 17 (9,10,12,14,17,17 respectively). The mean number of SLNs was 2,3 per patient. The SLN was identified in 67/69 patients (97%). One of the two failed procedures was in a patient with a carcinoma in the sigmoid colon surrounded by a concurrent diverticulitis. The other patient had extended lymph node metastases with angio-invasion at pathological examination. In 28 patients lymph node metastases were identified at the pathological examination, this includes the one case where the SLN procedure failed due to extensive lymph node metastases (28/69=41%). This one case with lymph node metastases and a failed procedure was excluded from further statistical analysis on the SN procedure, leaving 27 node positive patients in the final analysis. In 24 patients the SLN was positive, either with H&E staining or with IHC, resulting in a sensitivity of (24/27) 89% in the group of 67 patients with a successful SLN procedure. If we leave out the patients who had a positive sentinel node only after IHC, the sensitivity is 15/18= 83%. In 15 of 24 SLN positive patients, the SLN was the only involved lymph node (63%). In 9/27 lymph node positive patients metastases were found only after IHC. In 4 patients these were micrometastases, whereas in five cases isolated tumor cells were found. So without IHC the number of node positive cases would have been 27 minus 9 is 18. This corresponds to a total of 49 node negative cases by H&E in the group with a successful SLN procedure. With IHC the upstaging is 9/49 is 18% . The SLN was negative in 43 patients. In 40 patients the non-SLN's were also negative. This results in a negative predictive value of 93% (40/43). One of the three patients with a false-negative SLN had lymph node metastases with extra-nodal growth in the non-SLN. In another patient a small tumor deposit was found in the mesocolon right next to the primary tumor. This was classified as N1 according to the AJCC classification, although it is unclear whether this is a true lymph node metastasis or some kind of 'in transit' metastasis. The last patient with a positive non-SLN showed micrometastases at H&E examination in a small peritumoral lymph node. Aberrant lymphatic drainage was seen in three patients (4%). In two cases the SLN was found on the left side of the middle colic artery in patients with a tumor in the ascending colon. In both cases an extended right hemicolectomy was performed. The third patient had a tumor near the rectosigmoid junction with a high para-aortal SLN. Therefore we performed an extended left sided resection en-bloc with a partial para-aortal dissection. None of these lymph nodes contained metastases. All other SLNs were found in the mesocolon in close proximity to the tumor. In these cases, the central

lymph node as identified by the pathologist was always a non-SLN. The accuracy of the SLN procedure in this study was 96%, as the pathological status of the SLN corresponds with the definitive lymph node status in 64 of the 67 patients.

Table 1. Tumor characteristics

Tumor location	
Right colon	35
Left colon	2
Sigmoid colon	32
T-stage	
1	1
2	14
3	48
4	6
Mean nr of lymph nodes	11
Mean nr of SLN	2,3

Table 2. Results of multi-center studies of the SLN procedure in colon cancer

Study	Nr of patients	Nr of centers	Identification rate	Accuracy	Sensitivity	Upstaging
Bilchik[15]	40	3	100%	100%	100%	10%
Saha[18]	131	3	99%	97%	92%	16%
Bertagnolli[14]	72	13	92%	81%	42%	0%
Read[21]	38	2	79%	76%	25%	3%
Kelder/Braat	69	6	97%	96%	89%	13 or 18%

Discussion

With an identification rate of 97%, accuracy of 96%, sensitivity of 89% and negative predictive value of 93%, this study shows that it is possible to perform the SLN procedure properly in patients with localized colon carcinoma in a multi-center setting. Other multi-center studies showed varying results of this technique (table 2).[14-21] Our results correlate with those from other larger studies which show accuracy and sensitivity rates of 95-98% and 89-93% respectively.[15,18-20] Most smaller studies show worse results with low accuracy and success rates and corresponding low sensitivity rates and negative predictive values.[14,17,21] In one study the time between injection of the blue dye and identifying the SLN was too long, leading to a larger number of SLN's.[17] It is very likely that not all of these blue nodes were true SLN's. In the study by Bertagnolli et al 79 patients were operated on by 25 different surgeons in 13 different hospitals.[14] A mean of three procedures per surgeon seems insufficient to adequately learn this technique. It is known that the learning curve of the SLN in colon carcinoma stabilizes after about five procedures.[20] To minimize technical failures, the procedure in our study was performed by a few surgeons under direct supervision of one of the two surgeons coordinating this study (AEB, JTMP). Apart from too few procedures, the worse results in some studies might be explained by inclusion of patients with advanced disease. Some studies included patients with clinically apparent stage III or stage IV disease.[21] Widespread lymph node metastases could result in obstruction of lymphatic channels, and lymphatic drainage is bypassed to other (non-sentinel) lymph nodes. This phenomenon is called skip-metastasis. It was noted in one of the patients with a false-negative SLN in our study who had advanced lymphatic metastases with extranodal growth. Patient selection is therefore important for a reliable SLN procedure in colon carcinoma. In fact, the SLN procedure is not useful in patients with clinically apparent stage III or stage IV disease as false-negativity rates will be higher. Moreover, in these patients metastases will be easily found at routine pathological examination and the SLN procedure will not have any additional value. The SLN procedure could be useful in those patients with (micro)metastases that would not be identified with routine pathological examination. Furthermore, the failed procedure in one of our patients with concurrent diverticulitis, also suggests the importance of an undisturbed lymphatic drainage for a successful SLN procedure.

We saw aberrant lymphatic drainage in three patients (4%). This percentage correlates with the literature.[15,18,20] In this study, none of these aberrant SLN's showed metastases. However, potentially these aberrant SLN's are the only lymph nodes containing metastasis, as shown in a previous study.[22] In an experimental situation it seems justified to perform an extended

resection in these cases. Further study should be performed to justify an extended resection in the daily practice.

Literature not clearly indicates how many nodes should be examined to accurately predict lymph node status.[6-8,23,24] One study showed that a colon specimen usually contains about 50 lymph nodes, and that more than 70% of the lymph nodes containing metastases are smaller than 5 mm.[9] It is also known that the prognosis in node negative patients with colon carcinoma is better when more lymph nodes have been examined.[24] Taking this into account, the pathologist takes only a sample of the lymphatic basin of a resected colon specimen, even when international guidelines are followed which state that at least 12 lymph nodes are needed for adequate staging.[5] The mean number of 11 lymph nodes in our study is not enough to predict lymph node status according to the international guideline. This fact could theoretically lower the chance to detect metastases in non-SLN and thus could lower the false negative rate. However, we did not find any differences in false negative rates between the two hospitals with a mean number of 9 and 10 examined nodes (40 cases) and the hospitals with more than 12 examined nodes (29 cases). With regard to upstaging, most studies show an upstaging of 10-16%.[15,18-20] However, they calculated upstaging by dividing the number of IHC positive patients by the total number of patients (figure 1: g/a, 9/69, 13% in our group). We think it is better to consider upstaging solely in the H&E node negative group, as this is the group to be upstaged by IHC. Using this method we find 18% upstaging in our series. In addition to this true upstaging, patients with a SLN as the only site of metastases could have been 'possibly upstaged' as conventional pathological dissection of the mesentery might have missed this lymph node. The SLN procedure with patent blue might be able to improve adequacy of the lymph node examination by selecting the right lymph nodes , even small nodes <5mm, to be examined in depth by the pathologist. We found the SLN to be the single lymph node with metastasis in 15 (21%) of the patients (figure 1: f). 'Possible upstaging' might play a role here, but we cannot prove this.

As we believe that even isolated tumor cells are important for staging we assigned patients with micrometastases or isolated tumor cells to the group of node positive patients. It must be remarked however, that these cases were also used for the calculation of upstaging. Our idea of the biological importance of micrometastases and isolated tumor cells is based on a recent meta-analysis which showed that micrometastases detected retrospectively by RT-PCR correlated better with overall survival than IHC and carried significant prognostic value.[12] Regarding the detection of micrometastases, two studies showed a high reliability

of the SLN concept to predict micrometastases and/or isolated tumor cells also in non-SLN's. Therefore, it seems sufficient to perform IHC only on the SLN, while examining the non-SLN with H&E [25] [26] Prospective studies are needed to evaluate the potential benefit of systemic chemotherapy in patients with these micrometastases. A reliable SLN procedure might facilitate this intensive pathological examination by allowing focused examination of only the SLN and thereby aid in a better patient selection for adjuvant therapy in the future.

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Chapter 6

Additional value of RT-PCR analysis on sentinel nodes in determining the pathological nodal status in colon cancer

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Abstract

Background: Pathological examination on sentinel lymph node (SLN) and non-SLN's in colon cancer is frequently not performed identically. We examined whether non-SLN,s are truly negative in tumor-negative SLN's by reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods: RT-PCR with carcino-embryonic antigen (CEA) was performed in hematoxylin-eosin (H&E) and immunohistochemical (IHC) tumor-negative SLN's. In RT-PCR negative SLN's, we also performed RT-PCR on non-SLN's. Statistical analyses indicated a minimum of 72 accurate concur comparisons of non- SLN's and SLN's, which could be reached in12 patients.

Results: Negative and positive controls were performed. In nine of the 12 colon tumors, H&E and IHC negative SLN's were also negative with CEA-RT-PCR. A total of 105 lymph nodes, including 83 non-SLN's were retrieved in these nine specimens and none of the non-SLN's were CEA RT-PCR positive.

Conclusion: In this study, all CEA RT-PCR tumor-negative SLN's correctly represent tumor negative status of the non SLN's in primary colon tumors. The reliability of this method in colon cancer seems promising.

Introduction

In approximately 80% of all colon cancer patients, the tumor is in a stage that a curative treatment will be possible. Lymph node status still is the most important predictor of outcome after a radical resection of the tumor. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but is only 45-50 % for those with node positive tumors (stage III).[1] Adjuvant chemotherapy significantly improves the 5-year survival with 10-15% in patients with node positive colon cancer.[2] Despite a favorable pathological outcome, 20-30% of the patients with localized colon cancer without regional lymph node metastases will develop recurrent disease after an apparently curative resection.[1] It is possible that small tumor metastases are missed or not detectable, so called occult lesions, leading to understaging in these tumors.[3] For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes harvested at pathological examination is warranted according to international guidelines.[4] Immunohistochemical (IHC) staining or reverse transcriptase-polymerase chain reaction (RT-PCR) for carcinoembryonic antigen (CEA) or cytokeratin may reveal micrometastases missed by routine haematoxylin and eosin (H&E) examination. Several authors reported a decreased survival rate in colon cancer patients with nodal micrometastases.[5,6] However, ultrastaging techniques are time consuming, labor intensive and costly. For optimal and efficient staging focused examination of only the sentinel lymph nodes (SLN's) may be helpful in detecting the presence of micrometastases. In colon cancer, the SLN(s) are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumor, after peritumoral injection with Patent Blue.[7] They are the most likely to harbor metastatic disease when present, enabling focused examination with multilevel microsectioning to provide a more efficient and cost-effective detection of micrometastases.

To validate a procedure in which it would be sufficient to examine only the SLN's with ultrastaging methods in stead of all H&E negative lymph nodes, we performed a highly sensitive RT-PCR method for CEA on H&E and IHC negative SLN's as well as the other, so-called non-SLN's.

Patients and Methods

Patient selection

Only patients with histological proven primary colon carcinoma, without pre-or intra-operative visible distant metastases or gross lymph node involvement were included. This

study was approved by the local scientific ethics committee and all patients gave informed consent. Based on statistical power measurements we had to perform a total of 72 accurate comparisons of non-SLN status to SLN status for a reliable pathological examination of the SLN's (95% confidence interval of the concordance rate 0.95 to 1.7). If one comparison would be inaccurate, for example: a negative sentinel node with a positive non-sentinel node,109 comparisons should be performed. Assuming a mean of at least 12 lymph nodes per specimen, the analyses could be performed in nine to ten patients, if one sentinel node was false-negative.

Sentinel lymph node technique

SLN mapping was carried out through an open procedure. With a tuberculin syringe and 29 gauge needle 1-3 ml Patent Blue was injected subserosally in 4 quadrants around the tumor prior to any vascular ligation in the mesocolon. Within 5 to 10 minutes after the blue dye injection, the blue stained SLN's were identified by following the blue stained lymphatic vessels. After tagging these nodes with a long suture routine resection was performed. The tumor and all lymph nodes were examined histologically according to standard guidelines.[8] If the SLN's were negative after routine H&E staining, they were sectioned at 150 µm intervals and examined at 3 levels with H&E as well as immunohistochemistry on cytokeratins (CK8/CK18). Metastases between 0.2 mm and 2 mm were described as micrometastases and those smaller than 0.2 mm were referred to as isolated tumor cells.

Quantitative RT-PCR-analysis

As a positive control, we used tumor tissue samples from lymph nodes containing metastatic tumor. As a negative control tissue samples were obtained from lymph nodes of histologically benign resected colon specimens.

Sentinel nodes that were negative after H&E and IHC staining were examined by quantitative (q) RT-PCR. Real-time, quantitative PCR applications include gene expression and are able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process. qRT-PCR can detect their accumulation and quantify the number of substrates present in the initial PCR mixture before amplification began. Before the RT-PCR procedure, all lymph nodes were carefully dissected from the surrounding tissue to prevent false positive results due to admixture of non-lymph node tissue. All SLN's of the 12 patients were tested for the presence of (micro)-metastases by RT-

PCR and subsequently the non-SLN were analyzed in case of a negative SLN. In nine patients the RT-PCR analysis of the sentinel node was negative and in these patients all non-SLN's were tested to determine the reliability of our concept. We chose glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) as the housekeeping gene because the Cycle threshold (Ct) value was comparable to, or slightly less than the number of cycles needed to get a positive result from positive CEA controls in a previous study.[9] This indicates that the expression level of CEA is higher than or similar to the expression level of GAPDH. Total RNA was isolated from one 4 μm paraffin-embedded tissue section using the Specht method.[10] In brief, tissue was incubated in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 2% SDS) and treated for 12 hours with 500 μg/ml proteinase K at 60˚C followed by Proteinase K inactivation for 5 minutes at 95˚C. RNA was purified by extraction with 1/5 volume of chloroform and 1 volume phenol. RNA was precipitated using 1/10 volume of 2 M NaAc, an equal volume of isopropanol and 1 ul carrier glycogen 10 mg/ml (Roche). Total RNA was treated with DNAse I using the TURBO DNA-*free* kit™ according to manufacturer's instructions (Ambion, Inc., Austin TX, USA). RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Paisley, UK) in a volume of 20 μl using random hexamers (300 ng). An Assay-on-Demand Gene Expression Product™ (Applied Biosystems) was used for analysis of *CEA* (Hs 00237075_m1). Primers (Invitrogen) and probe (Eurogentec, Seraing, Belgium) for *GAPDH* were developed using primer design software (Applied Biosystems, Foster City, CA, USA). Primers used were: *GAPDH*F 5'-ccacatcgctcagacaccat-3', *GAPDH*R 5'-gcgccaatacgaccaaat-3'. Probe sequence labeled 5' with the FAM reporter dye and 3' with the TAMRA quencher dye molecules was: *GAPDH* 5'-cgttgactccgaccttcaccttccc-3'. Reactions were performed in 384-well plates (Applied Biosystems) in a volume of 20 μl containing real-time PCR mastermix (Eurogentec), 900 nM of each primer, 200 nM of an individual probe and 5 ng cDNA. PCR amplifications were performed using the ABI prism 7900HT sequence detection system (Applied Biosystems). Standard cycling conditions were used including a pre-amplification step of 50˚C for 2 min, 95˚C for 10 min, followed by amplification of 40 cycles of 95˚C for 15 s and 60˚C for 1 min. All samples were analyzed in triplicate. Mean cycle threshold values (Ct) and standard deviations (SD) were calculated. The amount of target gene was normalized relative to the amount of GAPDH ($\Delta Ct = Ct_{(CEA)} - Ct_{(GAPDH)}$) and the SD of the ΔCt (SD(ΔCt)) was calculated ($SD(\Delta Ct) = \sqrt{((SD_{CEA})^2 + (SD_{GAPDH})^2)}$). The factor difference is calculated ($2^{-\Delta Ct}$). At each run, positive and negative controls were included. A Ct value of >30 for the housekeeping gene indicates that the RNA input and/or quality was poor, these lymph nodes were excluded from further analysis. Only good quality samples were used to calculate the relative

expression levels of CEA. Lymph nodes were considered negative when Ct value of GAPDH was less than 30 and the ΔCt ≥ 10, indicating a relative expression level of 0.001 or less as compared to GAPDH. Lymph nodes were considered positive when ΔCt was <5 indicating a relative expression level of 0.03 or more as compared to GAPDH.

Results

As a control, all five tissue samples obtained from metastatic lymph nodes were positive with Ct values varying from 23-31. The ten negative control lymph nodes stayed negative, even after 40 cycles. The relative expression levels for the positive controls varied from 0.084 to 0.330.

The SLN's of the 12 patients without lymph node metastases from our aforementioned study were examined. Tumor characteristics and RT-PCR results are shown in Table 1. These 12 patients had a total of 29 sentinel nodes, with a mean of 2.2 (range 1-4). Three out of the 12 patients with negative SLN's on H&E and IHC examination showed positive results in at least one of the SLN after CEA RT-PCR. The relative expression levels for these SLN's were 0.084, 0.470 and 0.004. According to our definitions the last sentinel node was actually neither positive nor negative. Therefore, this patient was not included in the non-sentinel node analysis. All positive PCR results were found in T3 tumors. None of the three T2 tumors showed lymph node metastases after PCR.

The remaining nine patients had a negative SLN status after H&E, IHC and RT-PCR examination. The resected specimens in these nine patients had a total of 102 lymph nodes, with a mean number of 11.3 examined lymph nodes per patient. In each run, positive controls turned out positive with Ct values varying from 23.95 to 29.59. Negative controls turned out negative with Ct values of 40 or occasionally with 1 out of 3 Ct values of more than 37. The mean Ct-value for the housekeeping genes was 27.92 with a mean standard deviation of 0.0707 indicating that the RNA quality and quantity was similar for all cases. Three lymph nodes had GAPDH Ct values of 31 or 32, indicating that the RNA quality and input for these samples was poor. However, Ct values for CEA for these three samples were >40, suggesting a negative result. None of the other 99 non-sentinel nodes showed positive results after RT-PCR.

Table 1. Overview of lymph node status

Nr	Tumor site	T-stage	Nr of LN	Nr of SLN	PCR SLN	ΔCt (relative expression level of CEA)	PCR non-SLN
1	Right colon	3	13	3	positive	3,57 (0,084)	-
2	Sigmoïd colon	3	23	3	positive	1,09 (0,470)	-
3	Sigmoïd colon	2	5	1	positive	8,14(0,004)	-
4	Sigmoïd colon	3	10	3	negative	>10 (<0,001)	negative
5	Right colon	2	4	1	negative	>10 (<0,001)	negative
6	Right colon	2	11	2	negative	>10 (<0,001)	negative
7	Left colon	3	10	1	negative	>10 (<0,001)	negative
8	Right colon	3	19	3	negative	>10 (<0,001)	negative
9	Right colon	3	14	4	negative	>10 (<0,001)	negative
10	Right colon	3	15	1	negative	>10 (<0,001)	negative
11	Sigmoïd colon	3	14	4	negative	>10 (<0,001)	negative
12	Sigmoïd colon	3	8	3	negative	>10 (<0,001)	negative

Discussion

Contrary to the SLN in breast cancer and melanoma aiming to limit the surgical procedure, the rationale for SLN in colon cancer patients is to upstage tumors by identifying micro-metastatic nodal disease. If the SLN does not contain (micro)metastatic disease it is unlikely to detect metastatic disease in the other regional nodes. Using the SLN method for proper pathological staging, a proportion of node-negative tumors at conventional pathological examination will be upstaged and this subset of patients may benefit from adjuvant treatment. The SLN procedure will not alter the surgical resection in colon cancer patients. This concept is clinically relevant if identification of nodal micro-metastasis affects the prognosis. Studies on the SLN concept in colorectal carcinoma demonstrated varying results usually depending on different used techniques. [11-21] [22-28] Most studies performed cytokeratin IHC on the SLN's, whereas the non-sentinel nodes were only examined by conventional H&E staining. In these cases, enhanced detection of metastatic tumor in the sentinel lymph node may only reflect the more intensive histopathological technique rather than the biologic significance of the sentinel node. One study validated the procedure by examining both the sentinel nodes and non-sentinel nodes by IHC.[29] They found a false-negative rate of the sentinel node procedure of 13% with IHC on all lymph nodes, in an unselected population that represented the early experience with dye-directed lymphatic mapping in colon cancer. The authors also considered cases with single cytokeratin-positive cells node-negative because these may lack specificity in the setting of colorectal neoplasms. In our study we used CEA RT-PCR on lymph nodes in a selected population, that was part of a larger study on the sentinel node biopsy.[9] Patient material was selected based on node-negative status after H&E examination of sentinel and non-sentinel nodes. In addition, the sentinel nodes were negative by IHC. We used qRT-PCR to detect CEA transcript levels because it is a disease specific marker that is present in the majority of colon carcinomas.[30]

Several studies described the PCR examination of lymph nodes in colon carcinoma using CEA or CK 20 as a marker.[6,31-35] A disadvantage with RT-PCR is the false-positivity that may occur.[35-37] On the other hand the consequences of RT-PCR node positivity is still not clear. RT-PCR nodal positivity may occur because of a very small tumor burden which has the ability to metastasize or a single mRNA copy in a cell without metastatic potential. In addition, some non-tumor cells bear a few copies of CEA and might result in a positive RT-PCR result when enough cycles are performed. Because the aim of our study was to determine whether the sentinel node is truly the lymph node most likely to harbor

metastatic tumor and to assess the true histological false-negative rate of the SLN-procedure, we were interested in the most sensitive technique to detect tumor cells. As RT-PCR is more sensitive than IHC, it appeared to be the best technique to use. In our study design, false-positive results are not really a problem because we macro-dissected all lymph nodes from surrounding tissue. We only saw three positive RT-PCR results in sentinel nodes, and these patients were excluded from the non-sentinel lymph node analysis. The sentinel nodes that were negative after H&E, IHC and CEA RT-PCR examinations in our study, indeed represented a node-negative status of the lymphatic basin of the primary tumor in all 102 examined non-sentinel nodes. This shows that the sentinel node procedure is indeed a reliable concept in colon cancer and seems to be useful in selecting high-risk groups. We would like to mention that the RNA quality of our samples was good enough to perform RT-PCR in most cases with only three out of 102 non-sentinel nodes having Ct values for GAPDH of >30 indicating poor quality RNA. Therefore, it is indeed possible to perform RT-PCR on paraffin embedded lymph nodes as demonstrated previously.[10]

This study does not present any evidence in terms of prognosis for the routine use of qRT-PCR examination of (sentinel) lymph nodes in colorectal cancer. However, some reports do suggest that micrometastatic and/or molecular evidence of tumor in lymph nodes does influence survival.[5,6,33-35,38,39] Two RT-PCR studies confirmed the negative influence on survival of RT-PCR proven metastases in colon cancer.[6,35] Recently, a meta-analysis was presented in which micrometastases detected retrospectively by RT-PCR correlated with overall survival more than IHC and thus carried significant prognostic value.[40] Prospective studies are needed to evaluate the potential benefit of systemic chemotherapy in patients with these micrometastases. A reliable sentinel node procedure might facilitate intensive pathological examination by allowing a focused qRT-PCR/IHC examination of only the sentinel node(s), with routine H&E examination of the non-sentinel nodes.

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Chapter 7

Effects of 5-fluorouracil adjuvant treatment of colon cancer

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Abstract

Since the late eighties and early nineties, 5-fluorouracil (5-FU) based chemotherapy is the standard adjuvant treatment for stage III colon cancer. After the initial introduction of 5-FU in standard treatment protocols, several changes have been made based on results of randomized studies on various treatment regimens, including new cytotoxic agents. In stage II patients, the role of adjuvant chemotherapy is debatable. However, there might be a role for adjuvant treatment in certain high-risk patients. Following a search of the Medline database, the authors review the results of randomized studies on 5-FU-based adjuvant therapy and discuss future therapeutic options.

Introduction

Overview of the disease

Colorectal carcinoma (CRC) is the most common gastro-intestinal malignancy and the second leading cause of cancer related deaths in the world. Each year, worldwide, 500 000 people die of the disease and nearly one million cases are newly diagnosed. The disease is relatively more common in the Western World. Both genetic factors and non-genetic factors, mostly related to the Western lifestyle, contribute to the pathogenesis of colon carcinoma. Genetic predisposition may have a very strong effect in the dominantly inherited cancer syndromes, including familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). The cumulative risk in developed countries is about 5% by the age of 75 years. The number of patients suffering from the disease will probably increase in the future because of aging of the population in developed countries. [1] There is no established way of preventing colon cancer and there is no cost-effective screening method at the moment.[2,3] In general, symptomatic patients are treated as they present themselves. In the end, for half of these patients cure won't be possible. However, approximately 80% of the patients are presented in a stage that is considered to be curable. Lymph node status is still the most important predictor of outcome. However, several molecular biological factors, including *TP53* mutation, the microsatellite instability (MSI) phenotype and TS and DPD mRNA expression seem to play an important role in the success of adjuvant treatment.[4-6] *TP53* has a negative impact on disease-free survival. In patients with high-frequency microsatellite instability (MSI-H) tumors, adjuvant chemotherapy will not significantly improve survival. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but only 45-50 % for those with node positive tumors (stage III).[7] Adenocarcinomas are by far the most common malignant tumors of the large bowel. Other bowel tumors include carcinoid tumors, lymphomas, gastrointestinal stromal cell tumors and metastases of primary tumors elsewhere. All of these tumors are rare. Sixty percent of colorectal cancers arise in the distal part of the large bowel, which is defined as distal to the splenic flexure. In recent years, however, more proximally located tumors have been diagnosed.[8] For the pathologist, colorectal cancer is heterogeneous, both macroscopically and microscopically. The lesions can be exophytic, polypoid or endophytic. Although endophytic lesions may present as a small intraluminal tumor, they usually show an extensive infiltration of the bowel wall. Under the microscope, most cancers are moderately differentiated, gland-forming adenocarcinomas. Mucinous or colloid cancers, which produce extensive amounts of mucin, and signet-ring cell adenocarcinomas, occur much less frequently. The primary treatment for colon cancer is a radical surgical resection.

However, even in radical resections of stage III tumors, small tumor deposits not detected with currently available techniques (micrometastases), are present. Until the 1980's the consensus was, that surgery was the best and only standard treatment. In the early nineties, Moertel et al. showed a clear benefit from multimodality treatment, including adjuvant fluorouracil and levamisole for patients with node-positive disease.[9] Since then, numerous new agents and combinations of therapy have evolved for palliative and adjuvant therapy.[10-20] Despite the favorable prognosis of patients with localized stage II colon cancer without regional lymph node metastasis, 20-30% of these patients will develop recurrent disease, even after apparently curative resection. Generally there are high and low risk groups within stage II colon cancer. One of the therapeutic challenges now and in the future is to find a better way to select these patients and to treat them appropriately with an individualized adjuvant regimen.

Pathology and carcinogenesis

The great majority of colon cancers develop from colon adenomas or adenomatous polyps. Adenomas are benign neoplastic lesions that arise from the colon epithelium. The origin of adenomas and thus carcinomas is genetic. Colon cancer develops through a multistep process with an accumulation of multiple genetic alterations that are often the cause of a form of genomic instability. The two best known mechanisms of genomic instability are chromosomal instability (CIN) and microsatellite instability (MSI).[5] The CIN pathway is characterized by changes in the cellular genome, such as aneuploidy, multiple chromosomal rearrangements and an accumulation of somatic mutations in several known oncogenes. The loss of function of two tumor suppressor genes, the adenomatous polyposis coli (APC) and the *TP*53 gene, is considered to be essential for the initiation and progression of colorectal carcinogenesis in this pathway.[21-24] The CIN phenotype is found in approximately 85% of sporadic colon cancers.[5] The remaining 15% of colorectal cancers display a phenotype with small insertions and deletions mainly in repetitive sequences (microsatellites). This form of genetic destabilization is most commonly caused by the loss of the DNA mismatch-repair function and is referred to as the microsatellite-instability pathway. The phenotype of tumors with this defect is termed the high-frequency-microsatellite-instability phenotype (MSI-H).[5,25-29] Distinct clinical and pathological features of colorectal tumors arising from these two separate mutational pathways have been identified. Mutations in the *TP*53 gene are associated with an aggressive tumor growth and

subsequent reduced survival.[5] MSI-H is observed more frequently in colon cancers that occur proximal to the splenic flexure. These MSI-H tumors mostly exhibit poor differentiation, mucinous cell type and peritumoral lymphocytic infiltration.[27,29] They have also been associated with a larger size of the primary tumor and a more favorable stage distribution.[30] Patients with the MSI-H phenotype have longer survival than stage-matched patients with chromosomal instability (CIN) tumors.[29-31] There are two well-known dominantly inherited cancer syndromes named familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is the most common hereditary cancer syndrome. It is inherited in an autosomal dominant manner. The prevalence of HNPCC in newly diagnosed colon cancer patients is 2-4%. Most tumors in HNPCC are characterized by microsatellite instability of tumor DNA (MIN-pathway).[28] A diagnosis of HNPCC should be suspected when patients with colon cancers have a positive family history of HNPCC related cancers, especially if the diagnosis was made before the age of 50 years. FAP is an autosomal dominantly inherited disorder characterized by the development of hundreds or thousands of adenomatous colorectal polyps during adolescence and early adulthood. Malignant transformation will occur in one or more of these polyps and invasive cancer will develop in most patients before the age of 40. The disease is due to mutations in the APC-gene (CIN-pathway). [23] Less than 1% of colon cancers are due to FAP.

Disease staging

Accurate staging of colon cancer is essential to clinical decision making and to prognosis. The American Joint Committee on Cancer (AJCC) has designated staging of cancer by the Tumor Node Metastasis (TNM) classification (Box 1).[32] T indicates the progressive degree (1-4) of invasion of the tumor into the bowel wall. N represents the nodal involvement and M indicates distant metastasis. Disease prognosis (without chemotherapy) derived from this staging process is shown in Box 2.

Box 1. TNM Staging

T	Primary tumor
T_0	No evidence of primary tumor
T_{is}	Carcinoma in situ
T_1	Tumor invasion of submucosa
T_2	Tumor invasion of muscularis propria
T_3	Tumor invasion of subserosal fat
T4	Tumor invasion of other organs or structures and/or perforation of the visceral peritoneum
N_0	No lymph node metastases
N_1	Metastases in 1 to 3 regional lymph nodes
N_2	Metastases in 4 or more regional lymph nodes
M_0	No distant metastases
M_1	Distant metastases

Box 2. Stage grouping for colon cancer and prognosis

pTNM	Stage (AJCC)	5-year survival rate (%)
T_{1-2}, N_0, M_0	I	90
T_{3-4}, N_0, M_0	II	80
T_{1-4}, N_{1-2}, M_0	III	50
T_{1-4}, N_{1-2}, M_1	IV	5

THERAPEUTIC APPROACHES

Overall patient management

The prognosis of colon cancer is related to the degree of penetration of the tumor through the bowel wall and the presence or absence of lymph node involvement and distant metastases. Curative treatment involves a multi-modality approach, in which surgery is an essential part. Cure can be achieved in approximately 50% of resected patients without adjuvant chemotherapy.[33] Adjuvant chemotherapy is routinely administrated when lymph node metastases are present. In case of severe co-morbidity or extensive metastatic

disease, local palliative treatment modalities are frequently applied. This includes stenting for bowel obstruction and radiotherapy for bleeding and pain.[34,35] Following curative intended treatment of colon cancer, periodic evaluations in selected groups may lead to the early identification and management of asymptomatic recurrent disease.

Role of surgery and pathology

As already mentioned surgery remains the principal treatment modality for stage I to III colon tumors. The extent of the resection is determined by its location within the regional lymph drainage area. For tumors in the right colon (coecum/ascendening colon) a right hemicolectomy is performed. This includes resection of the last 10 cm of the terminal ileum and part of the transverse colon with ligation of the ileocolic artery, right colic artery and the right branch of the middle colonic artery. For tumors in or just distal of the hepatic flexure, a right extended hemicolectomy should be performed. This includes ligation of both branches of the middle colic artery. The whole transverse colon is resected when a tumor is located in the transverse colon. Tumors in the splenic flexure or the descending colon are usually treated by a left hemicolectomy, including the area supplied by the left colic artery and left branch of the middle colic artery. For sigmoid tumors a sigmoid resection is performed during which the superior rectal artery and its branching sigmoidal arteries are ligated. To prevent spill of tumor cells due to mobilization of the tumor, the 'no-touch' technique was developed. It should be noted that this is not a standard procedure in various countries. It involves early ligation (before mobilization) of the feeding artery and central vein. In a prospective study no significant survival benefit of this technique was shown, although it did show a decreased incidence of liver metastases.[36] Treatment of rectal tumors is beyond the scope of this review. Regional lymph nodes are removed en bloc with the resected colon. For adequate staging and treatment of patients with colon cancer, a meticulous histological examination of at least 12 nodes harvested by pathological analysis is warranted according international guidelines.[32]

Role and biological basis of fluorouracil based adjuvant chemotherapy

Thymidylate synthase (TS) has been used as a target for cancer chemotherapy in the development of fluoropyrimidines such as 5-fluorouracil (5-FU). However, the precise

mechanism by which TS inhibition leads to cell death is still not completely resolved.[37] TS inhibition results in depletion of dTTP, an essential precursor for DNA, and an increase in dUTP. This results in the so-called thymine-less death due to misincorporation of dUTP into DNA. Its excision, catalyzed by uracil-DNA glycosylase, results in DNA damage. Both this imbalance in dTTP/dUTP and DNA damage can result in induction of downstream events, leading to apoptosis.[37] On the other hand a specific interaction exists between oncogenes like *TP53* and *TS*. These complex indirect and direct interactions between oncogenes and *TS* may have as yet unclear clinical implications, since most data are based on in vitro or in vivo studies and some results are contradictive. Randomized trials in the 1980s demonstrated that fluorouracil (FU)-based adjuvant therapy could decrease the chance of death by approximately 30%.[9,38,39] Since then, FU-based adjuvant therapy is recommended for all medically fit patients with completely resected stage III colon cancer. For stage I patients, there are no relevant studies on the use of adjuvant therapy. The benefit of adjuvant therapy for patients with stage II colon cancer has long been an area of controversy. However, consensus guidelines on this subject have been published recently.[40] We will further discuss the use of adjuvant therapy in the subsequent stages of disease in the next paragraphs.

Stage III

In 1990, as mentioned before, Moertel and coworkers showed that fluorouracil (FU)-based therapy decreased the chance of death in stage III patients by approximately 30%, with a greater than 10% absolute benefit in 5-year survival.[9] They added levamisole, an antihelminthic immunomodulater, to the FU regimen. Later studies showed that the inclusion of levamisole in chemotherapy regimens for colorectal cancer does not delay recurrence or improve survival compared to the combination of 5-FU with leucovorin (Mayo regimen).[10,41] The addition of folinic acid or leucovorin (LV) seems to potentate the effects of FU. The supplementation of the intracellular reduced folate pool by folinic acid prolongs the competitive inhibition of TS by FU. [42]The benefits of the combination FU and leucovorin were supported by several studies.[15,43,44] The IMPACT trial published in 1995 demonstrated a disease free survival of 71% with the FU/leucovorin combination with an overall survival of 83%.[43] The Quick and Simple and Reliable (Quasar) study in 3239 stage II and III colon and rectal cancer patients, randomizing to either observation or bolus 5-FU plus leucovorin at low or high dose, or 5-FU plus levamisole, showed that higher dose

folinic acid produced no extra benefit over low-dose folinic acid with a significant survival benefit of 3% in the treatment group (p=0.02).[10] Survival rates were 70% with 3-year recurrence rates around 36%. These results were confirmed by Link et al who showed a 5-year overall survival rate of 60,5% for stage III patients treated with FU and levamisole versus 72 % if folinic acid was added to this schedule.[15] Furthermore, in the Intergroup 0089 study on 3759 colon cancer patients, 80% of whom were in stage III and 20% in high risk stage II, the 5-FU/low-dose LV (Mayo scheme) proved to be equivalent to 5-FU/ high-dose LV (Roswell Park scheme). Based on these studies and the relative high neurotoxicity of levamisole, which was standard treatment in the early 90's, this drug was abandoned in favor of leucovorin.[45] The oral fluoropyrimidines capecitabine and tegafur-uracil (UFT)/LV generate fluorouracil preferentially in tumor tissue with an equal activity as 5-FU/LV. The final stage of conversion to fluorouracil is catalyzed by thymidine phosphorylase, which is appreciably more active in tumor than healthy tissue. Twelves et al found that oral capecitabine (Xeloda) is an effective and at least equivalent alternative to intravenous FU plus leucovorin in the adjuvant treatment of stage III colon cancer. In addition, it was associated with significantly fewer adverse events than FU plus leucovorin.[20] An equal effect was found for UFT/LV compared to FU/LV.[46] Treatment of advanced CRC has dramatically been improved in the last decade due to the development of new treatment options, including irinotecan (CPT11) and oxaliplatin (L-OHP). Irinotecan is a semisynthetic camptothecin which inhibits topoisomerase I, impeding DNA uncoiling which leads to double-stranded DNA breaks. Oxaliplatin is a platinum-based drug, forming cross-linking adducts, which blocks DNA replication and transcription. Combination therapy of FU/leucovorin with irinotecan or oxaliplatin are effective in stage IV colon cancer, which will be discussed later.[47] Based on the results in advanced disease these new strategies have been used in an adjuvant setting in stage II/III colon cancer patients. However, in stage III colon cancer, the combination of irinotecan with an IV-bolus scheme of FU/leucovorin showed no survival benefit to FU/leucovorin alone.[48] From studies in stage IV patients treated with this combination scheme, it is known that the FU should be administered in a continuous infusion.[49] The results of this FOLFIRI scheme in stage III patients as an adjuvant therapy (Pan European Trial in Adjuvant Colon Cancer: PETACC-3 study; n=2111 pts) have not been published yet. Preliminary data show insufficient effect of the FOLFIRI schedule in stage III colon cancer, with a 3-year disease-free survival of 59,9% and 62,9% in FU/LV and FU/LV/Irinotecan, respectively.[50] Irinotecan seems to have a more additive effect with FU/LV, while oxaliplatin has a more synergistic effect. The Multicenter International Study of Oxaliplatin/5-FU/LV in Adjuvant Treatment of Colon Cancer (MOSAIC) trial included 2246

patients with stage II (40%) and III (60%) colon cancer. It evaluated the efficacy of adjuvant treatment with FU/leucovorin plus oxaliplatin (FOLFOX 4 schedule) versus FU/leucovorin alone. The DFS in the FOLFOX 4 schedule was 85,1% vs 81,3 in the FU/LV schedule. The absolute survival benefit for the FOLFOX schedule was 6,6%.[51] These results were confirmed by the National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 study which randomly assigned for LV/5-FU alone or combined with oxaliplatin (FLOX) in which 5-FU was given as bolus.[52] This study showed a significant better DFS at 3 years in the FLOX-group (76.5% vs 71.6%, p=0,04) and a 23% reduction in recurrence risk. Based on the above mentioned results FOLFOX became the treatment of choice in the adjuvant setting, although the incidence of myelosupression and neurotoxicity is relatively higher compared to 5-FU/LV alone. These studies also suggest oxaliplatin to be of benefit independent of 5-FU/LV administration. Whether the substitution of 5-FU/LV by oral alternative equivalents including capecitabine will be beneficial is currently under investigation as first-line therapy.[53] Results of the major trials are summarized in table 1.

Table 1. Results of FU based adjuvant therapy in stage II and III colon cancer

Treatment	Disease stage	Number of patients	Disease free survival rate (%)*	p-value	Ref
None vs FU/levamisole(Lev)	II and III	1269	47 vs 63	<0,0001	9
None vs FU/leucovorin(LV)	II and III	1526	62 vs 71	<0,0001	38
FU/Lev vs FU/Lev/LV	II and III	891	60 vs 70	<0,01	39
FU/Lev/LV vs FU/LV	I,II,III	4927	63 vs 65	0,06	10
FU/Lev vs FU/Lev/LV	III	855	57 vs 66	0,0004	15
FU/LV vs capecitabine	III	1987	60 vs 64	0,12	20
FU/LV vs FU/LV/oxaliplatin	II and III	2246	72,9 vs 78,2	0,002	11

*3- or 5-year survival

Stage II

As mentioned in the introduction, the benefit of adjuvant therapy for patients with stage II colon cancer has long been an area of controversy. Too few stage II patients have been included in most trials to determine whether they derive a small benefit from FU-based postoperative therapy. The trials that include higher numbers of patients show conflicting results.[54-58] The results of the trials are summarized in table 2. The varying results between the trials might be explained by differences in the study populations. It is possible that there is a difference between the studies in the proportion of patients with poor prognostic indicators such as bowel obstruction, perforation, adhesion to adjacent organs and in those cases with <12 lymph nodes examined in the pathology report. Patients with these characteristics are classified as high risk patients. Because the number of high risk stage II patients is usually low in the study population, it is unlikely that significant survival differences are found when administering chemotherapy. In the NSABP pooled analysis a 5% disease free survival difference was found after adjuvant therapy between high and low risk stage II patients.[56] Andre et al, using the FOLFOX schedule, also found a 5% disease free survival difference. Both studies did not reach significance, probably because of insufficient numbers. The American Society of Clinical Oncology (ASCO) recently published guidelines on this subject, in which the routine use of adjuvant chemotherapy is not supported in node-negative patients. However, they did find indirect evidence of benefit for patients with high-risk disease. [40] We have to refine the category of patients with stage II colon cancer who may benefit from adjuvant treatment. At least 12 lymph nodes have to be examined to consider the tumor as node negative. Whether the sentinel node procedure will be valuable is still a matter of debate

Table 2. Results of FU based adjuvant therapy in stage II colon cancer

Number of patients	survival rate (%)*	p-value	Ref
1116	82 vs 80	NS	54
1565			56
-26% high risk*	75 vs 70	NS	
-74% low risk	87 vs 82	0,01	
318	72 vs 72	NS	57
1029	78 vs 70	0,007	58
3238	80 vs 77	0,06	55
576	84,9 vs 79,8	NS	11

*high risk: obstruction, perforation, T4

Table 3. Results of FU based therapy in stage IV colon cancer

Schedule	Number of patients	Median survival	Ref
None vs FU/LV or FU/Lev	1365	8 vs 11-12	59
FOLFIRI/FOLFOX vs FOLFOX/FOLFIRI	220	20,6 vs 21,5	49
FU/LV/irinotecan vs FOLFOX	531	15,0 vs 19,5	13
FU/LV/irinotecan vs FU/LV/irinotecan/bevacizumab	1029	15,6 vs 20,3	61
FU/LV vs FU/LV/bevacizumab	500	17,9 vs 14,6	62
Cetuximab vs cetuximab/irinotecan	329	6,9 vs 8,6	12

Stage IV

Although the treatment of stage IV patients is beyond the scope of this review on adjuvant 5-FU based chemotherapy, we would like to discuss it shortly. Palliative chemotherapy commonly increases the median survival of stage IV colon cancer patients from around 8 months without treatment to 12 months with FU/leucovorin therapy.[59] Better results could be achieved with the new generation of chemotherapeutic agents like irinotecan and oxaliplatin showing median survival periods of 20- 21 months.[13,47,49] Recently, the Dutch Colorectal Cancer Group finished inclusion of 820 patients in the CAIRO study, in which a sequential approach of 5-FU/LV, CPT-11 and oxaliplatin in phase III trials showed promising results in patients with metastatic disease, with a prolonged survival of more than 20 months. In a prospective study, Adam et al used a combination of FOLFOX/chronomodulated chemotherapy to obtain reduction of tumor load in patients with non-resectable liver metastases. After chemotherapy, 13.5% of patients were found to be resectable on re-evaluation and underwent a potentially curative resection. The 5-year survival was 35% in this group.[60] The development of drugs that inhibit signal transduction pathways have provided new opportunities in treating metastatic colon cancer. The concept of targeting tumor vasculature as a therapeutic strategy in human cancer was based on the observation that rapid growth of transplanted tumors was often preceded by a local increase in vascular density. This is called angiogenesis. One of the most potent mediators of angiogenesis is vascular endothelial growth factor (VEGF). VEGF mediates its effects by interacting with the membrane-bound tyrosine kinase receptors, thereby influencing angiogenesis. Bevacizumab is the recombinant humanized version of a murine antihuman VEGF monoclonal antibody. Several studies showed a beneficial effect of bevacizumab in combination with FU-based chemotherapy in patients with stage IV colon cancer.[61,62] In patients with metastatic disease the combination of bevacizumab and CPT-11/5-FU/LV should be considered as first-line treatment. Another example of anti-tumor therapy by inhibiting signal transduction is the inhibition of the epidermal growth factor receptor (EGFR). The EGFR signaling pathway regulates cell differentiation, proliferation, migration, angiogenesis and apoptosis, all of which become deregulated in cancer cells. Cetuximab is a chimeric IgG1 monoclonal antibody that binds to EGFR with high specificity and with a higher affinity than EGF, thereby blocking ligand-induced phosphorylation of EGFR. Cunningham et al showed that cetuximab has clinically significant activity when given alone or in combination with irinotecan in patients with irinotecan-refractory colorectal cancer.[12] This study suggests that cetuximab may circumvent irinotecan

resistance. Studies on palliative treatment in stage IV colon cancer are summarized in table 3.

Treatment toxicity

Dihydropyrimidine dehydrogenase (DPD), the first and rate-limiting enzyme in the three-step pathway of uracil and thymine catabolism, is also important in the degradation and inactivation of 5-FU.[63] DPD converts over 85% of the clinically administered 5-FU into the inactive metabolite dihydrofluorouracil, a process which takes place mainly in the liver. [64] Patients with a complete or near-complete deficiency of the enzyme do suffer from severe toxicity following the administration of standard doses of fluoropyrimidines, due to significantly increased and prolonged plasma levels of 5-FU.[63-65] The incidence of this pharmacogenetic syndrome in the general population is estimated to be as high as 3% and may be much more common than originally thought.[66] The use of fluoropyrimidines in patients with this metabolic defect is associated with a very high mortality.[64] The most common toxic reactions in patients without a known DPD deficiency to FU are nausea, vomiting, diarrhea, stomatitis, dermatitis and leucopenia. These reactions were rarely in the range of grade 3-4 toxicity in the study of Moertel et al. [9] This same study showed that 22% of patients experienced some degree of alopecia. A variety of neurological symptoms was reported by 18% of patients. These ranged from vague lightheadedness and emotional changes to disabling cerebellar ataxia. They usually abated when therapy was discontinued. 3% of patients developed severe leukopenia (WBC< 1000). The combination of FU with leucovorin increases some toxicity effects of FU, especially nausea; diarrhea and stomatitis are more often seen. The International Multicenter pooled Analysis of Colon Cancer (IMPACT) trial showed that severe toxic effects (WHO grade 4) occurred in fewer than 3% of cases with a combined FU/LV treatment.[43] Capecitabin and UFT, which are chemically related to FU showed significantly fewer side effects than FU.[20,46] One of the most frequent side effects of Capecitabin is the hand-foot syndrome in which the skin of hands and feet is affected. The FOLFOX regimen leads to a significantly higher rate of grade 3-4 leukopenia and neuropathy compared to FU/leucovorin alone (41 vs 5% and 12 vs 0,2%).[11] This neurotoxicity is a typical side effect of oxaliplatin and is usually reversible. The total prevalence of neurotoxicity in the FOLFOX study was 82%, of which 12 % consisted of a grade 3 toxicity. After 12 months follow up this rate decreased to 1,1% for grade 3 toxicity and 4,8% for grade 2 toxicity. The signal transduction inhibitors (bevacizumab and

cetuximab) do not cause major side effects in combination with the other agents. Bevacizumab caused grade 3 hypertension in 16% of FU/leucovorin/bevacizumab treated patients. In addition, proteinuria was seen more often with bevacizumab. There was also a disbalance in the incidence of arterial thrombotic events with an increased occurrence in the group with bevacizumab (10 vs 5%). No increase in grade 3 or 4 bleeding or venous thrombotic events was seen in bevacizumab treated patients. Two patients (2%) developed gastrointestinal perforation. [62] Cetuximab caused severe anaphylactic reactions in 1,2% of patients. An acne-like rash developed in about 80% of the patients, but grade 3 or 4 toxic effects on the skin were observed in only 9% of patients. [12]

Summary of current available strategies of adjuvant chemotherapy treatment

Approximately 80% of the patients present with colon cancer in a stage that is considered to be potentially curable. Lymph node status is the most important predictor of outcome. The principal treatment for stage I, II and III tumors is surgery. In patients with stage II colon cancer, the use of adjuvant chemotherapy is debatable. The routine use of adjuvant chemotherapy is not supported by the ASCO in node-negative patients. However, there is indirect evidence of benefit for patients with high-risk stage II disease including bowel obstruction, perforation, T4 stage and less than 12 examined lymph nodes in the pathology report.[40] Systemic adjuvant therapy is standard treatment for stage III colon cancer and should consist of FU, leucovorin and oxaliplatin when possible.[11] Individual exceptions based on age and coexisting diseases are possible. Capecitabin and UFT are effective alternatives to FU/leucovorin in Stage III patients.[20,46] Fluoropyrimidines, irinotecan and oxaliplatin have efficacy in the management of metastatic colorectal cancer. Combinations of therapy can increase the median survival time from 8 to 20 months.[49,67] Neo-adjuvant treatment with the FOLFOX regimen is possible in patients with non-resectable stage IV disease, and can downsize non-resectable metastases to resectable metastases.[60] Targeting of the EGF-Receptor and VEGF-receptor is currently the most promising biological approach.

Future directions

In adjuvant treatments, the group that does not benefit from therapy consists of patients who were qualified for adjuvant therapy but in spite of this still developed metastases and

patients who would never develop metastases in the spontaneous course of their disease (with or without this adjuvant treatment). The ratio between patients subjected to the side effects of therapy without any benefit and the patients who do benefit from chemotherapy is 5:1.[68] The great challenge for the future is to develop better selection criteria for adjuvant treatment, thereby reducing the group of patients who do not benefit at all from the treatment.

Better staging detects more lymph node positive patients and immunohistochemistry might help to detect stage II patients at higher risk. It is well known that the 5-year survival in node-negative patients is significantly higher when more lymph nodes have been examined.[69] Therefore, surgeons and pathologists should be stimulated to harvest and examine as much lymph nodes as possible. For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes by pathological analysis is warranted.[32] However, with a fat-clearance technique a mean number of 50 lymph nodes per specimen can be found.[70] In addition, intensive pathological examination of lymph nodes may reveal micrometastases that would be missed by routine hematoxylin & eosin (H&E) examination. Several authors have reported a decreased survival rate when nodal micrometastases are detected in CRC.[71,72] Based on the above mentioned studies, we can readily assume that the pathologist only samples a small part of the regional lymph nodes, and will certainly miss some lymph node metastases. The intensive staging techniques are time consuming, labor intensive and costly. The sentinel node procedure might select the right lymph nodes for intensive pathological examination, which will save time and money. The procedure has been validated in large studies.[73-75] It is a relatively simple procedure which only takes 5-10 minutes during surgery.

Besides ultrastaging, we should probably exploit our knowledge of tumor genetics and biology. A start in this area has already been made by treating stage IV colon cancer patients with signal transduction inhibitors like bevacizumab (anti-VEGF) and cetuximab (anti-EGFR).[12,61] These drugs still need to be tested in stage II and III patients.

Molecular biological factors might help to better select stage II + III patients at risk and those who are sensitive to and benefit from 5-FU based adjuvant therapy. This has already been done in breast cancer. [76-78] Wang et al found a combination of gene expression in colon cancer that predicted a 13-fold increased risk of relapse in stage II patients. These patients were selected for adjuvant therapy.[79] The results of this study still need to be evaluated in a larger study.

Genetically determined variability of the function of certain key enzymes has been shown to influence toxicity and response to certain types of chemotherapy and survival.[80] For example, patients with high thymidylate synthase gene expression profit from adjuvant therapy.[6;81] Patients with low thymidylate synthase expression and adjuvant therapy seem to have a worse prognosis than surgery alone.[81] This seems to be different from the situation in palliative 5-FU based chemotherapy.[82] Patients with colon tumors exhibiting high-frequency microsatellite instability do not benefit from FU-based chemotherapy.[4] Volk et al published a case report on an alternative chemotherapy regimen which was very well tolerated in a DPD-deficient patient.[83] More future trials should incorporate these genetic tumor variations in the choice of therapy.

In summary, better selection of patients should lead to a more targeted therapy in the future.

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Chapter 8

Summary, Conclusions and Future Perspectives

Summary

The prognosis of patients with colon cancer is generally related to the degree of invasion of the tumor through the bowel wall and the presence or absence of lymph node involvement and distant metastases. Adjuvant chemotherapy is given to patients with lymph node metastases (stage III) and some patients without nodal metastases but with certain unfavorable tumour characteristics. Despite the good prognosis of patients without lymph node metastases (stage II colon cancer), 20-30% of these patients will develop recurrent disease, even after apparently curative resection.[1]

In this thesis an attempt was made to improve current staging and to identify those patients in the current stage II group who have an increased risk of developing recurrent disease in the future and who might benefit from adjuvant treatment. This was based on the assumption that some patients in the stage II group actually belong to the stage III groep (patients with lymph node metastases). In other words, with the current techniques of lymph node analysis some nodal metastases may be missed, leading to a false node-negative classification in some stage II patients. There are two explanations for this nodal 'understaging'. It is possible that not enough nodes are identified from the colon specimen, leaving some positive nodes unidentified. In addition, it might be that the identified nodes are insufficiently examined, thereby missing the smaller metastases. Both hypotheses are examined in this thesis.

Chapter 2 starts with an evaluation of the quality of lymph node sampling in colon carcinoma in the Northern part of the Netherlands. The main goal was to study the impact of the reported number of lymph nodes at pathological examination on survival. Data of 2,281 patients with localized colon cancer were analyzed for factors associated with the number of examined lymph nodes. The effect of tumor characteristics and examined lymph node numbers on nodal status and survival were analyzed. From these data we can conclude that in the majority of cases less than the recommended number of twelve nodes in the guideline are examined.[2] T-stage, tumor localization and patient age were related to the number of nodes examined. A higher number of examined nodes was associated with an increase in node-positivity. The survival benefit with more examined lymph nodes in N_0 patients can be explained by stage migration. This means that with a higher number of examined nodes, more metastases are found, leading to less patients with occult nodal metastases who are unjustly assigned to the stage II group. These patients now belong to the stage II group, where they are a subgroup with a relatively good prognosis. This stage

migration may eventually lead to a survival benefit, as more patients will receive adjuvant therapy when more lymph node metastases can be detected. In **chapter 3**, we evaluated the effect of a different fixation method (modified Davidson's Fixative (mDF)) on the number of lymph nodes examined and staging in patients with colon carcinoma. Traditional formalin preparation with manual dissection of all nodes was performed in 117 colon specimens, while the specimens of 125 patients were fixated in mDF. Differences in the retrieval and number of nodes and size of suspected nodal metastases were measured. All lymph nodes were stained with conventional H&E methods. With the mDF technique the median number of examined nodes increases from five to thirteen. Smaller nodes and more micrometastases (6% vs 16%) were found. The percentage of node positive patients increased from 30 to 41%, leading to more patients being eligible for adjuvant chemotherapy.

In the next 3 chapters we report on the results of the sentinel lymph node biopsy (SLN) in colon carcinoma. In **chapter 4**, a short pilot study of 30 patients is described in which the feasibility of in vivo SLN detection with Patent Blue V dye is tested. In addition, we evaluated nodal microstaging and ultrastaging using cytokeratin immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR) methods. Subserosal injection with Patent Blue dye was used. In searching for occult micrometastases each SLN was examined at three levels. In tumor-negative SLN's at routine haematoxylin-eosin (H&E), IHC analyses and RT-PCR were performed. The procedure was successful in 29 out of 30 patients (97%). Upstaging occurred in 10 patients (33%); 7 by IHC and 3 by RT-PCR. Aberrant lymphatic drainage was seen in 3 patients (10%). From this pilot study, we conclude that the SLN concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to an upstaging of nodal status in 33 % of patients when IHC and PCR techniques are combined. The results of this study were confirmed in a larger multi-center setting in **chapter 5**. Without RT-PCR, we found 18% upstaging. It might be that these patients belong to the high risk stage II patients that we are looking for in our selection of patients for adjuvant therapy. However, long follow up results of these patients have to be awaited in order to interpretate the real significance of this upstaging. In addition to this upstaging, the SLN procedure might be helpful in selecting the right nodes that should to be examined in any case by the pathologist. With this procedure, small, blue sentinel lymph nodes might be detected that would have been missed with routine pathological analysis. In **chapter 6** we examined the validity of the SLN concept by performing reverse transcriptase-polymerase chain reaction (RT-PCR) with carcino-embryonic antigen (CEA) on tumor negative SLN's as well as non-SLN's. In nine colon tumors, H&E and IHC negative

SLN's were also negative with CEA-RT-PCR. A total of 105 lymph nodes, including 83 non-SLN's were retrieved in these nine specimens and none of the non-SLN's were CEA-RT-PCR positive. From these data we conclude that in this study, all tumor-negative SLN's correctly represent the tumor-negative status of the non SLN's in primary colon tumors. The reliability of this method in colon cancer seems promising.

In **Chapter 7,** the results of a review on adjuvant chemotherapy in colon carcinoma are presented, with a special focus on chemotherapy in high risk stage II patients. Since the late eighties and early nineties, 5-fluorouracil (5-FU) based chemotherapy is the standard adjuvant treatment for stage III colon cancer. In stage II patients, the role of adjuvant chemotherapy is still debatable. However, there is indirect evidence of benefit for patients with high-risk stage II disease including bowel obstruction, perforation, T4 stage and identification of less than 12 examined lymph nodes in the pathology report.

Conclusion and future perspectives

In this thesis we present some tools that might be used for the improvement in the nodal staging of colon cancer and thereby the selection of patients eligible for adjuvant chemotherapy. This can be accomplished by the examination of more lymph nodes and a better selection of these nodes, or by the use of more sensitive techniques in the detection of metastases. Both possibilities are outlined separately in the following paragraphs. In addition, alternative options for improvement in staging are discussed.

Although the international guidelines warrant examination of at least 12 nodes for adequate staging and treatment of patients with colon cancer, a retrieval of more nodes might be better.[2] With a fat-clearance technique a mean number of 50 lymph nodes per specimen can be found.[3] More than 70% of the metastatic lymph nodes were smaller than 5 mm in diameter and more than 30 lymph nodes were needed to achieve a 85% probability of true N0 status at standard histology.[3,4] Based on the above, we can readily assume that the pathologist only samples a small part of the regional lymph nodes, even when the minimum number of twelve nodes is examined. The chances of missing some lymph node metastases seem considerable, especially when we take into account that in a considerable amount of patients less than twelve nodes are examined as shown in this and other studies.[5] This assumption is supported by our finding of stage migration with more lymph nodes examined, as shown in chapter 2. Several studies have tried to find a cut off point for the minimal number of lymph nodes necessary for correct staging. This number varied considerably from 6 to 18 to as many as possible in the study of Goldstein et al.[3,6-9] Based on these studies and our study, it is not possible to make an evidence based statement on the amount of lymph nodes to be examined. Until there is evicence, the effort should indeed be to examine as much nodes as possible.

Next to the assumption that 'more is better' in lymph node staging, there is also the option of a more intensive pathological examination of the detected lymph nodes, as described in the introduction. Several authors have reported a decreased survival rate when nodal micrometastases are detected in CRC.[10-12] Liefers et al found a clear distinction in 5-year disease free survival in a group of stage II patients, based on the presence or absence of tumor RNA in lymph nodes.[12]

These intensive staging techniques are time consuming, labor intensive and costly. The technique with mDF as described in chapter 3 is a cheap and simple alternative to increase the number of nodes. As shown in our study in the chapters 4-6 in which the SLN procedure

was validated with RT-PCR examination of sentinel and non-sentinel lymph nodes, the SLN concept showed to be reliable in predicting micrometastases and/or isolated tumor cells or tumor RNA also in non-SLN's. This is confirmed with IHC in two other studies.[13,14] It seems sufficient to perform ultrastaging only on the SLN, while examining the non-SLN with H&E, which will save time and money. As shown in our data and other studies, the sentinel node procedure reveals aberrant lymphatic drainage in 2-9% of the cases.[15-17] Aberrant lymphatic drainage might be especially interesting in tumors situated at the rectosigmoid junction, as these tumors might behave either as sigmoid tumors or as rectal tumors. We will start a study on lymphatic mapping in these tumors in the near future.

As the ultimate goal is to improve the survival in patients with colon cancer, we have to consider whether it might be possible to improve the surgical technique, next to the pathological technique. From the introduction of the total mesorectal excision (TME) in rectal cancer it is known, that adequate resection of an intact rectal specimen leads to a better patient survival compared to survival when the specimen has been damaged during surgery.[18] In addition, it is known that the long-term survival following colorectal cancer surgery in general, improves significantly with increasing hospital caseload and surgeon's education.[19-21] Next to the harvest of a sufficient number of mesenteric lymph nodes, a diligent operative technique is probably essential to prevent intra-abdominal spill of tumor cells through manipulation of the tumor. Here the 'no-touch' technique seems important. It involves early ligation (before mobilization) of the feeding artery and central vein before manipulation of the tumor and associated mesentery. An important part of the no-touch principle is the preparation in existing anatomical plains and the avoidance of manipulation of the tumor and disruption of lymphatic channels. The early ligation of vessels at the base of the mesentery forces to perform an adequate dissection of the mesentery with the harvest of a sufficient number of lymph nodes in it. Turnbull et al found an increase in disease-free survival after the introduction of this technique.[22] In a prospective study no significant survival benefit of this technique was shown, although it did show a decreased incidence of liver metastases.[23] Although it is not clear which factor in the surgical technique is most important for survival, we should certainly not neglect the surgeon's effect on prognosis in colon cancer. We recently started a study to analyze the influence of the individual surgeon and pathologist on the number of examined lymph nodes and survival.

Regarding lymph node staging, it may be possible to improve the pre-operative knowledge of the tumor status by the use of new imaging techniques in the near future. In rectal

cancer, magnetic resonance imaging (MRI) is the gold standard for optimal pre-operative imaging of the distance of the tumor to the mesorectal fascia. It can predict the circumferential resection margin with a high accuracy and consistency, allowing preoperative identification of patients with a small margin to the fascia who have an risk of recurrence. These patients will benefit from preoperative chemo- and/or radiotherapy.[24] Until recently, there was no accepted, ideal imaging modality or technique for diagnosis of lymph node metastases. However, in the last few years, MRI with ultra small super paramagnetic iron-oxide nanoparticle (USPIO) as a contrast agent is used for diagnosis of lymph node metastases. It offers higher diagnostic precision than unenhanced MRI for detection of lymph node metastases, and allows functional and anatomical definition when used as an imaging modality.[25] USPIO-MRI has been tested in several solid cancers and seems useful in identifying benign and malignant lymph nodes, which may greatly improve the pre-operative planning.[26-33] In colon cancer MRI might also be useful, although it has no role in the planning of pre-operative chemo- or radiotherapy, as this is not indicated in colon cancer. Compared to rectal cancer, the local situation in colon cancer is much more permissive to do an extended resection when necessary. In addition, the local recurrence rate is much lower in colon cancer compared to rectal cancer. Until there is evidence of a benefit of pre-operative chemotherapy in stage II colon cancer, there is no indication for USPIO-MRI. One study showed that MRI lymphangiography is a useful technique for the detection of sentinel lymph nodes.[34] However, we do think that intra-operative sentinel node detection with patent blue is a much cheaper, quicker and easier technique, which should not yet be replaced with expensive pre-operative MRI scans in patients with colon cancer.

Besides the need for enough lymph nodes in the surgical and pathological process, ultrastaging and pre-operative imaging, we should probably exploit our knowledge of tumor genetics and biology to select the appropriate patients for adjuvant treatment. Molecular biological factors might help to select stage II + III patients at risk and those who are sensitive to and benefit from 5-FU based adjuvant therapy. This has already been done in breast cancer.[35-37] In colon cancer, several studies showed that it was possible to predict stage II cancer prognosis by tumor gene expression profiling.[38,39] It is not known yet, if the patients identified with this gene expression profiling benefit from adjuvant therapy. Further study is needed on this subject. Apart from identifying high risk patients, gene expression might help in identifying patients who benefit from adjuvant therapy. For example, patients with high thymidylate synthase (TS) expression levels benefit from

chemotherapy, whereas patients with low TS expression levels have a worse outcome when treated with FU-based chemotherapy. [40] Another option is to treat patients with a therapy based on biological characteristics of the tumour. A start in this area has already been made by treating stage IV colon cancer patients with signal transduction inhibitors like bevacizumab (anti-VEGF) and cetuximab (anti-EGFR).[41,42] These drugs still need to be tested in large trials in stage II and III patients.

Not all patients receiving adjuvant treatment benefit from the therapy. In spite of the treatment with adjuvant therapy some patients will develop metastases still, while others will never develop metastases in the course of their disease, with or without this adjuvant treatment. [43] On the other hand, some of the 20-30% of the patients with a stage II tumor who develop recurrent disease might have had a benefit from the adjuvant treatment for which there was no strong indication according to the current guidelines. The great challenge for the future is to develop better selection criteria for adjuvant treatment, thereby reducing the group of stage III patients who do not benefit at all from the treatment and maybe add an extra group of patients in the current stage II high risk group. At the moment, lymph node status is the best criterion we have to predict the course of the disease. But with the current advances in genomics and proteomics, it is likely that within the coming years it is possible to genotype and phenotype tumors to determine prognosis based only on analysis of the primary tumor.[44] This analysis might be much more informative than lymph node status and adjuvant therapy could probably be based on the results of this genetic mapping. However, nowadays in colon cancer, genomics has not been fully developed yet. And until it is, surgeons as well as pathologists should concentrate on accurate lymph node staging in which as much lymph nodes as possible are examined in a diligent way.

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Chapter 9

Samenvatting, conclusies en toekomstperspectieven

Samenvatting

De prognose van patiënten met coloncarcinoom hangt samen met de mate van tumorinvasie in de darmwand en de aan- of afwezigheid van lymfekliermetastasen en afstandsmetastasen. Adjuvante chemotherapie is voorbehouden aan patiënten met kliermetastasen (stadium III) en enkele patiënten met ongunstige tumorkenmerken uit de stadium II groep (zonder kliermetastasen). Derhalve is het van groot belang om een goede stagering van de lymfeklierstatus te verrichten. Ondanks de gunstige prognose van patiënten zonder kliermetastasen, zullen toch 20 tot 30% van deze patiënten een recidief ontwikkelen, zelfs na een schijnbaar curatieve resectie.[1] In dit proefschrift wordt een poging gedaan om de huidige lymfeklierstagering bij het coloncarcinoom te verbeteren en de patiënten met een hoog risico in de huidige stadium II groep te identificeren, die baat zouden kunnen hebben bij adjuvante behandeling. Dit wordt gedaan op basis van de veronderstelling dat er zich een aantal patiënten in de stadium II groep bevinden, die in geval van nauwkeuriger onderzoek eigenlijk tot de stadium III groep (patiënten met kliermetastasen) zouden behoren. Met andere woorden, waarschijnlijk wordt een deel van de kliermetastasen met de huidige onderzoekstechnieken gemist, waardoor patiënten onterecht als kliernegatief worden geclassificeerd. Enerzijds zou het kunnen zijn dat er te weinig klieren uit een preparaat worden geïdentificeerd en onderzocht waardoor juist de klieren met metastasen worden gemist. Anderzijds is het mogelijk dat de klieren onvoldoende nauwkeurig worden onderzocht en dat de kleinere metastasen over het hoofd gezien worden. Beide hypothesen worden onderzocht in dit proefschrift.

Hoofdstuk 2 start met een onderzoek naar de kwaliteit van de lymfeklierstagering bij patiënten met een colon carcinoom in Noord-Nederland. Het belangrijkste doel was om de invloed van het aantal onderzochte klieren op overleving in kaart te brengen. Hiervoor werden gegevens van 2.281 patiënten met een stadium I, II of III coloncarcinoom geanalyseerd, waarbij speciale aandacht was voor factoren die gerelateerd waren aan het aantal onderzochte klieren. Tevens werd gekeken naar het effect van kenmerken van de primaire tumor en klieraantallen op de klierstatus en overleving. De onderzoeksdata toonden dat in het merendeel van de de gevallen minder dan de twaalf vereiste klieren (zoals beschreven in de richtlijn) werden onderzocht.[2] Het aantal onderzochte klieren was gerelateerd aan het T-stadium van de tumor, de tumorlokalisatie en de leeftijd van de patiënt. In geval van een toename van het aantal onderzochte klieren nam ook het percentage patiënten met een klierpositieve status toe. Het overlevingsvoordeel dat ontstond bij een toename van het aantal onderzochte klieren kon verklaard worden door

migratie van het tumorstadium. Bij een groter aantal onderzochte klieren worden meer kliermetastasen gevonden, waardoor de groep patiënten die onterecht als kliernegatief (stadium II) is afgegeven, kleiner wordt. Deze patiënten gaan naar de stadium III groep, waar ze een subgroep vormen met een relatief gunstige prognose. Op deze manier verbetert de prognose in zowel de stadium II als de stadium III groep. Bovendien kan dit uiteindelijk tot een overlevingsvoordeel voor de gehele groep patiënten leiden, aangezien meer patiënten in aanmerking komen voor adjuvante chemotherapie.

In **hoofdstuk 3** werd het effect onderzocht van een andere fixatiemethode, modified Davidson's fixatief (mDF) op het aantal onderzochte klieren en klierstatus bij patiënten met een coloncarcinoom. Hiervoor werden resultaten van 125 patiënten bij wie het colonpreparaat gefixeerd werd met mDF, vergeleken met een groep van 117 patiënten bij wie fixatie op conventionele wijze werd uitgevoerd. Er was speciale aandacht voor het aantal gedetecteerde klieren en de grootte van de gevonden kliermetastasen. Alle klieren werden gekleurd met haematoxyline en eosine (H&E). Met mDF werd een mediaan aantal klieren van 13 gevonden versus 5 bij de conventionele techniek. Er werden kleinere metastasen gevonden en meer micrometastasen (16% vs 6 %). Het percentage patiënten met kliermetastasen nam toe van 30 naar 41%. Dit betekent dat er een toename was van 11% in het aantal patiënten dat in aanmerking kwam voor adjuvante chemotherapie.

In de volgende drie hoofdstukken worden de resultaten gepresenteerd van het toepassen van de schildwachtklier (SWK) procedure bij het coloncarcinoom. In **hoofdstuk 4** wordt een kleine pilot studie van 30 patiënten beschreven waarin de toepasbaarheid van het SWK-concept in vivo wordt getest met de kleurstof Patent Blue. Naast de toepasbaarheid op zich werd met behulp van immunohistochemie (IHC) en reverse transcriptase-polymerase chain reaction (RT-PCR) gezocht naar het voorkomen van micrometastasen en tumor RNA in lymfeklieren, in dit geval 'upstaging' genoemd. Hiervoor werd Patent Blue subserosaal rondom de tumor geïnjecteerd. Van elke SWK werden op 3 niveaus coupes onderzocht. Wanneer bij routine H&E onderzoek geen metastasen werden gevonden, werd IHC op cytokeratinen en RT-PCR toegepast. De procedure gelukte bij 29 van de 30 patiënten (97%). Bij tien patiënten werd opwaardering van het tumorstadium gezien: in zeven gevallen door IHC; drie keer door RT-PCR. Bij 3 patiënten werd aberrante lymfeklierdrainage gezien. Concluderend kan gesteld worden dat de SWK procedure bij het coloncarcinoom toepasbaar is en accuraat. De procedure leidt tot upstaging bij 33% van de patiënten wanneer IHC en RT-PCR gecombineerd worden. De resultaten van deze studie werden

bevestigd in een grotere multi-center studie in **hoofdstuk 5**. Zonder RT-PCR, maar met IHC werd 18% opwaardering van het tumorstadium gezien. Het zou kunnen zijn dat deze patiënten behoren tot de hoog-risico patiënten in de huidige kliernegatieve groep (stadium II). Dit zouden patiënten kunnen zijn die misschien baat hebben bij adjuvante chemotherapie. Echter, de werkelijke betekenis van de opwaardering van tumorstadium door IHC en RT-PCR uit deze studie kan pas worden vastgesteld, nadat de resultaten van de follow-up bekend zijn. In **hoofdstuk 6** werd de validiteit van het SWK concept onderzocht door de toepassing van RT-PCR op het carcino-embryonic antigen (CEA) op tumornegatieve SWK en non-SWK. Bij 9 patiënten met kliernegatieve colontumoren na H&E en IHC kleuring waren de SWK ook negatief na RT-PCR. Alle bijbehorende non-SWK waren ook negatief na RT-PCR. Hieruit kon geconcludeerd worden dat kliernegatieve SWK de tumornegatieve status van de non-SWK goed voorspellen, hetgeen voor de betrouwbaarheid van het SWK concept bij coloncarcinoom pleit.

Hoofdstuk 7 bestaat uit een review over de toepassing van adjuvante chemotherapie bij het coloncarcinoom met speciale aandacht voor chemotherapie bij patiënten met een stadium II colon tumor. Sinds de eind tachtiger jaren is chemotherapie met daarbij in ieder geval 5-fluorouracil (5-FU) de standaard adjuvante behandeling bij patiënten met een stadium III colon tumor. De rol van deze adjuvante therapie bij patiënten zonder kliermetastasen is controversieel. Er is echter indirect bewijs voor een gunstig effect van chemotherapie bij patiënten met een hoog-risico stadium II colon tumor. Dit hoge risico kan bestaan uit een T4 tumor, tumorperforatie, darmobstructie en/of een te laag aantal gerapporteerde klieren in het pathologie verslag.

Conclusie en toekomstperspectieven

Dit proefschrift laat zien dat er ruimte is voor verbetering in de stagering van het coloncarcinoom en daarmee de selectie van patiënten die in aanmerking komen voor adjuvante chemotherapie. Dit zou kunnen worden bereikt door het onderzoeken van een groter aantal lymfeklieren en een betere selectie hiervan of door een nauwkeuriger onderzoek van de al gedetecteerde klieren door de patholoog. In deze paragraaf zullen beide onderdelen separaat worden belicht. Daarnaast worden alternatieve opties voor verbetering beschreven.

Hoewel in internationale richtlijnen een minimum aantal van 12 klieren wordt beschreven om adequaat te kunnen stageren en behandelen bij het coloncarcinoom, zou een groter aantal klieren wel eens beter kunnen zijn.[2] Met een speciale techniek voor 'fat-clearance' werd een mediaan aantal van 50 klieren per preparaat gevonden.[3] Ook is bekend dat meer dan 70% van de metastatische klieren kleiner dan 5 mm in diameter zijn en dat meer dan 30 klieren moeten worden onderzocht om met een 85% waarschijnlijkheid de werkelijke kliernegatieve status te kunnen voorspellen bij het standaard histologisch onderzoek.[3,4] Bovenstaande gegevens suggereren dat de patholoog slechts een steekproef neemt van de regionale lymfeklieren bij een coloncarcinoom, zelfs wanneer de vereiste twaalf klieren worden onderzocht.

De kans dat er enkele kliermetastasen worden gemist, lijkt niet gering. Dit geldt zeker indien bij een substantieel aantal patiënten minder dan twaalf klieren worden gedetecteerd, zoals beschreven in hoofdstuk 2 van dit proefschrift en andere studies.[5] Verschillende studies hebben getracht een aantal klieren te vinden dat het mimimum aangeeft dat onderzocht moet worden om een adequate stagering uit te voeren. Echter, dit aantal varieerde van zes tot achtien tot een zo hoog mogelijk aantal klieren in een studie van Goldstein et al.[3,6-9] Op basis van deze en onze gegevens is het onmogelijk om een bewijs te vinden voor een minimum aantal klieren dat onderzocht moet worden. Totdat dit bewijs er is, zal er gestreefd moeten worden om met de huidige technieken zoveel klieren als mogelijk te onderzoeken.

Naast het idee 'meer is beter' bij klierstagering, moet zoals in de inleiding geschreven is, aandacht gegeven worden aan intensief pathologisch onderzoek van de gedetecteerde klieren. Enkele auteurs hebben een negatief effect op overleving aangetoond bij patiënten met nodale micrometastasen bij het coloncarcinoom.[10-12] Liefers et al vonden een duidelijk onderscheid in overleving in een groep patiënten met stadium II tumoren, gebaseerd op de aan- of afwezigheid van tumor RNA in lymfeklieren.[12]

De technieken om een groot aantal lymfeklieren te detecteren en micrometastasen aan te tonen zijn tijdrovend, arbeidsintensief en kostbaar. Het gebruik van mDF, zoals beschreven in hoofdstuk 3, is een goedkoop en eenvoudig alternatief om de klieropbrengst te verhogen. De SWK procedure met de validatie door middel van RT-PCR zoals beschreven in de hoofdstukken 4-6, is betrouwbaar in het voorspellen van de aanwezigheid van micrometastasen, geïsoleerde tumorcellen of tumor RNA in de andere, non-SWK-en. Een tweetal andere studies toonden identieke resultaten met IHC.[13,14] Het lijkt voldoende om arbeidsintensieve, dure 'ultrastagering' alleen op de SWK toe te passen, hetgeen ook veel

tijd bespaart in het pathologisch onderzoek. Een andere functie van de SWK procedure is het detecteren van aberrante lymfedrainage, hetgeen voorkomt bij 2-9% van de patiënten.[15-17] Deze aberrante drainage zou vooral bij tumoren op de rectosigmoïdale overgang interessant kunnen zijn, omdat deze tumoren zich qua metastasering zowel als sigmoïd- en/of als rectumcarcinoom kunnen gedragen. Binnenkort zullen we een studie opstarten, waarbij we de SWK procedure juist bij deze tumoren zullen toepassen.

In ogenschouw nemend dat ons uiteindelijke doel is de overleving van patiënten met coloncarcinoom te verbeteren, zal naast verbetering op pathologisch gebied ook gedacht moeten worden aan een verbetering van de chirurgische techniek. Hier zou een parallel kunnen worden getrokken aan de invoering van de totale mesorectale excisie bij het rectumcarcinoom. Het uitvoeren van een adequate resectie met een intact preparaat leidt tot een significant betere overleving bij het rectumcarcinoom.[18] Als ondersteuning voor het belang van een goede chirurg bij colonresecties kan ook aangevoerd worden, dat de lange termijn resultaten significant beter zijn, wanneer een gespecialiseerde chirurg de operatie uitvoert in een ziekenhuis met een hoger volume voor colonchirurgie.[19-21] Los van de klieropbrengst is een nette operatietechniek waarschijnlijk van essentieel belang om intra-abdominale tumorspill door mobilisatie van de tumor te voorkomen. In het kader hiervan is het van belang om de 'no-touch' techniek te bespreken. Bij deze techniek worden eerst de aanvoerende arterie en afvoerende vene geligeerd, alvorens het te reseceren colondeel te mobiliseren. Onderdeel van deze techniek is het werken in bestaande anatomische vlakken en het vermijden van manipulatie van de tumor en bijbehorende lymfebanen. Ook dwingt het in een vroeg stadium ligeren van aanvoerende arterie en afvoerende vene aan de basis van het mesenterium tot het verrichten van een ruime klierdissectie. Turnbull et al vonden een duidelijke verbetering van de ziektevrije overleving na introductie van deze techniek.[22] In een prospectief onderzoek werd geen significant overlevingsvoordeel door deze techniek aangetoond.[23] Wel werd een lagere incidentie van levermetastasering gevonden. Hoewel niet duidelijk is welk onderdeel van de chirurgische techniek nu van belang is, mag het effect van de chirurg zeker niet verwaarloosd worden wat betreft de prognose van patiënten met een coloncarcinoom. Inmiddels is door ons een studie opgestart, waarin de invloed van de individuele chirurg en patholoog op het aantal gerapporteerde lymfeklieren en overleving wordt onderzocht.

In de nabije toekomst is het waarschijnlijk mogelijk om de pre-operatieve kennis van het tumorstadium te verbeteren door gebruik te maken van nieuwe beeldvormende

technieken. Tegenwoordig is bij het rectumcarcinoom MRI, magnetic resonance imaging, de gouden standaard voor het optimaal in beeld brengen van de relatie van de tumor tot de endopelviene fascie. MRI kan de circumferentiele resectie marge met een grote betrouwbaarheid voorspellen, hetgeen de mogelijkheid biedt om pre-operatief de patiënten te identificeren met een grotere lokale tumor uitbreiding en daarmee de grotere kans op een lokaal recidief.[24] Deze patiënten hebben voordeel bij pre-operatieve (chemo)radiotherapie. Tot voor kort was er geen geschikte beeldvorming voor de diagnostiek van lymfekliermetastasering. Echter, de laatste jaren wordt MRI met ultra kleine paramagnetische ijzeroxide deeltjes (USPIO) als contrastmiddel gebruikt voor diagnostiek van kliermetastasen. Het biedt op dit gebied een grotere diagnostische precisie dan de conventionele MRI-scan en maakt een combinatie van anatomische en functionele beeldvormende diagnostiek mogelijk.[25] USPIO-MRI is toegepast bij verschillende solide tumoren en lijkt nuttig in het maken van onderscheid tussen benigne en maligne lymfeklieren, hetgeen de pre-operatieve kennis wat betreft het tumorstadium kan verbeteren.[26-33] Bij colontumoren zou MRI nuttig kunnen zijn, maar het is vooralsnog niet essentieel wat betreft de pre-operatieve planning van chemo- en radiotherapie. Dit geldt mede, omdat de lokale situatie bij colontumoren vaak meer ruimte biedt voor een uitgebreide resectie (indien nodig). Ook is de kans op een lokaal recidief bij het coloncarcinoom beduidend kleiner dan bij het rectumcarcinoom. Er zijn vooralsnog geen studies verschenen die aantonen dat er een indicatie is voor pre-operatieve chemotherapie bij het coloncarcinoom. Totdat die er zijn, lijkt er geen indicatie voor USPIO-MRI bij het coloncarcinoom. Er is een studie verschenen die gebruik maakte van MRI lymfangiografie voor de detectie van de SWK.[34] Echter, naar onze mening is de intra-operatieve SWK detectie met patent blauw veel goedkoper, sneller en eenvoudiger dan deze techniek en is er vooralsnog geen plaats voor deze MRI scan bij patiënten met een coloncarcinoom.

Naast de behoefte aan voldoende lymfeklieren voor een betrouwbare TNM stagering en technieken voor ultrastagering, zullen we in de toekomst bij de selectie van patiënten voor adjuvante therapie waarschijnlijk meer gebruik gaan maken van de genetische en biologische eigenschappen van tumoren. Moleculair biologische factoren kunnen helpen bij de selectie van patiënten met een stadium II of III coloncarcinoom die kans hebben op een tumorrecidief na operatie en die baat hebben bij adjuvante chemotherapie gebaseerd op 5-fluorouracil. Dit is uitgebreid in kaart gebracht bij het mammacarcinoom.[35-37] Ook bij het coloncarcinoom hebben enkele studies laten zien dat het mogelijk is om de prognose

van stadium II patiënten te voorspellen door middel van gen-expressie profielen van de primaire tumor.[38,39] Het is nog niet bekend of de patiënten die met deze techniek herkend worden als potentiële hoog-risico patiënt, baat hebben bij adjuvante therapie. De resultaten van follow-up studies moeten hiervoor afgewacht worden. Naast het identificeren van patiënten met een risico op recidief tumor, kan gen-expressie helpen bij het identificeren van patiënten die voordeel hebben bij het toepassen van adjuvante chemotherapie. Een voorbeeld hiervan zijn patiënten met een hoog thymidylate synthase expressieniveau die baat hebben bij 5-fluorouracil, terwijl patiënten met lage expressieniveaus een slechtere uitkomst hebben wanneer ze behandeld worden met adjuvante therapie.[40] Een andere mogelijkheid betreft het geven van een therapie gebaseerd op biologische eigenschappen van een tumor. Hierbij kan gedacht worden aan signaal transductie inhibitie, zoals toegepast bij patiënten met een stadium IV coloncarcinoom met middelen als bevacizumab (anti-VEGF) en cetuximab (anti-EGFR).[41,42] Deze therapie moet nog onderzocht worden in een klinische setting bij patiënten met stadium II of III coloncarcinoom.

Niet alle patiënten die adjuvant behandeld worden, hebben baat bij de therapie. Sommige patiënten ontwikkelen ondanks de adjuvante therapie hoe dan ook metastasen, terwijl anderen, zowel met als zonder therapie, ziektevrij zullen blijven.[43] Aan de andere kant is het mogelijk dat de 20-30% patiënten met een stadium II tumor die een recidief ontwikkelen, baat hebben bij adjuvante chemotherapie, terwijl daar volgens de huidige richtlijnen geen strikte indicatie voor is. Het ontwikkelen van betere selectiecriteria voor adjuvante therapie bij het coloncarcinoom is de uitdaging voor de toekomst. Door betere selectiecriteria moet zowel de groep patiënten met kliermetastasen die geen baat heeft bij chemotherapie en dus onterecht adjuvant behandeld wordt, als de groep patiënten zonder kliermetastasen die wel voordeel heeft bij chemotherapie, geïdentificeerd worden. Hiermee kan veel onnodige behandeling worden voorkomen. Vooralsnog is klierstatus het best beschikbare criterium voor het voorspellen van het verloop van de ziekte postoperatief. Met de huidige vooruitgang in genetica en proteomics is het waarschijnlijk dat binnen enkele jaren de mogelijkheid bestaat om met behulp van het geno- en fenotype van de primaire tumor de prognose van patiënten te voorspellen.[44] Deze gegevens zijn mogelijk veel informatiever dan lymfeklierstatus, en adjuvante therapie kan misschien wel gebaseerd worden op de resultaten van moleculaire tumor diagnostiek. Echter, vooralsnog is het moment nog niet aangebroken voor enkel en alleen moleculaire diagnostiek bij het coloncarcinoom. En tot

die tijd is het voor zowel chirurgen als pathologen van het grootste belang om een adequate klierstagering uit te voeren waarin zoveel mogelijk klieren op een nauwkeurige wijze worden onderzocht.

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Dankwoord

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When you look at yourself from a universal standpoint, something inside always reminds or informs you that there are bigger and better things to worry about.

Albert Einstein