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Detailed examination of lymph nodes improves prognostication in colorectal cancer

Fania S. Doekhie[1,2], Wilma E. Mesker[1], Peter J. Kuppen[1], Gijs A. van Leeuwen[3], Hans Morreau[4], Geertruida H. de Bock[5], Hein Putter[6], Hans J. Tanke[7], Cornelis J. van de Velde[1] and Rob A. Tollenaar[1]

[1] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[2] Department of Emergency Medicine, Flevo Hospital, Almere, The Netherlands
[3] Department of Pathology, Rijnland Hospital, Leiderdorp, The Netherlands
[4] Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[5] Department of Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[6] Department of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands
[7] Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

Up to 30% of stage II patients with curatively resected colorectal cancer (CRC) will develop disease recurrence. We evaluated whether examination of lymph nodes by multilevel sectioning and immunohistochemical staining can improve prognostication. Lymph nodes (n = 780) from 36 CRC patients who had developed disease recurrence (cases) and 72 patients who showed no recurrence of disease for at least 5 years (controls) were analyzed. Sections of 4 levels at 200-μm interval were immunohistochemically stained for cytokeratin expression. The first level was analyzed by conventional and automated microscopy, and the 3 following levels were analyzed by automated microscopy for the presence of tumor cells. Overall, cases showed more micrometastases (3 patients) than controls (1 patient). Analysis of a second level led to the additional detection of 1 patient with micrometastases (case) and 1 patient with macrometastasis (case). Examining more levels only led to additional isolated tumor cells, which were equally divided between cases and controls. Likewise, automated microscopy resulted only in detection of additional isolated tumor cells when compared with conventional microscopy. In multivariate analysis, micrometastases [odds ratio (OR) 26.3, 95% confidence interval (CI) 1.9–364.8, p = 0.015], T4 stage (OR 4.8, 95% CI 1.4–16.7, p = 0.013) and number of lymph nodes (OR 0.9, 95% CI 0.8–1.0, p = 0.028) were independent predictors for disease recurrence. Lymph node analysis of 2 levels and immunohistochemical staining add to the detection of macrometastases and micrometastases in CRC. Micrometastases were found to be an independent predictor of disease recurrence. Isolated tumor cells were of no prognostic significance.

The presence of lymph node metastases is 1 of the most important prognostic factors in colorectal cancer (CRC) for which adjuvant systemic chemotherapy is generally recommended.[1–4] Patients with curatively resected stage I and II CRC without nodal tumor involvement do not receive adjuvant systemic therapy since only small improvements in survival have been shown.[4–6] However, 10–30% of these node-negative patients will develop locoregional recurrence or distant metastases.[1,7,8] Adjuvant systemic treatment of all node-negative CRC patients is not recommended by the American Society of Clinical Oncology as it would lead to overtreatment and unnecessary complications.[9] Identification of node-negative CRC patients with a high risk of disease recurrence may lead to a more appropriate selection for adjuvant treatment.

Conventional histopathology has a limited sensitivity to detect occult tumor cells in lymph nodes, described as micrometastases (>0.2 mm and ≤2 mm) and isolated tumor cells (≤0.2 mm or single-tumor cells).[10–12] Alternative approaches to detect occult tumor cells in lymph nodes have been reported such as: polymerase chain reaction,[13] reverse transcriptase polymerase chain reaction (RT-PCR),[14] multilevel sectioning,[15] immunohistochemical staining[16] and automated microscopy.[17] In a study published by Liefers *et al.*[14] was shown that stage II patients with carcinoembryonic antigen (CEA) RT-PCR negative lymph nodes have a significantly better 5-year disease-related survival than patients with CEA

Key words: colorectal cancer, lymph nodes, multilevel sectioning, immunohistochemistry, micrometastases, isolated tumor cells
Abbreviations: CEA: carcinoembryonic antigen; CI: confidence interval; CRC: colorectal cancer; HE: hematoxylin and eosin; OR: odds ratio; PBS: phosphate-buffered saline; RT-PCR: reverse transcriptase polymerase chain reaction; TNM: tumor-node-metastasis.
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Correspondence to: Rob A. Tollenaar, Department of Surgery, K6-R, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands, Fax +31-71-5266750.,
E-mail: r.a.e.m.tollenaar@lumc.nl

RT-PCR positive lymph nodes (91 *vs.* 50%, $p = 0.02$). These results were confirmed by several other groups.[18–22]

The detection of immunohistochemically stained tumor cells has the advantage of visual examination of the detected cells using microscopy. Multilevel sectioning and immunohistochemistry have been shown to increase the detection rate of lymph node metastases in CRC patients.[13] Because screening of multiple immunohistochemically stained sections is time consuming and difficult to reproduce, automated microscopy has been implemented.[17,23]

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

Material and Methods

Patient selection

Between January 1981 and December 2001, 1,044 patients underwent surgery for a primary CRC at the Leiden University Medical Center. For this study, a selection was made for patients with negative lymph nodes (N0) and no metastases (M0) at the time of surgery ($n = 506$). Patients who were operated on their first CRC in another hospital or who were diagnosed with another invasive malignancy before or within 5 years after the date of diagnosis of the primary colorectal carcinoma, and patients who developed a local recurrence were excluded for this study. The latter group was excluded to rule out the factor of inadequate surgery. Cases ($n = 40$) were defined as patients who had regional or distant recurrent disease at least 3 months after but within 5 years after the date of diagnosis of primary CRC. Regional metastases were considered intra-abdominal or intrapelvic metastases in lymph nodes or in connective tissue. Thirty-six cases could be included in this study, because lymph nodes from 4 cases could not be retrieved from the archive. Controls ($n = 189$) were patients who did not develop locoregional or distant disease within 5 years after diagnosis of primary CRC. For each case, 2 controls were matched for tumor-node-metastasis (TNM) stage, date of incidence and date of birth, leading to a total number of 72 controls. The median follow-up of the case group was 2.5 years (range, 5.3 months–6.3 years) and the median follow-up of the control patient group was 10.8 years (range, 5.1–21.4 years).

Of 19 patients with rectal carcinoma, 2 patients had received preoperative radiotherapy and 3 patients had received postoperative radiotherapy. None of the patients had received adjuvant chemotherapy.

Tissue specimens

After resection, the lymph nodes were fixed in formalin, processed through graded ethanol and embedded in paraffin as part of a routine procedure for histopathological investigation. From 108 patients, a total of 780 lymph nodes were harvested (median 6; range, 1–26 lymph nodes). The lymph nodes were embedded in a total of 225 paraffin blocks.

Immunohistochemistry

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

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

Analysis of the slides

All sections were analyzed using the ARIOL system® (Applied Imaging a Genetix company). The features of the ARIOL system® have been published previously.[17] Sections of the first level were also analyzed by a pathologist (AML) using conventional microscopy at a total magnification of 100 times. Selected candidate tumor cells were verified by the operator and also visually confirmed by an independent pathologist (HM). Nonspecifically stained cells such as hematopoietic cells were visually recognized and excluded from the analysis. Macrometastases were defined as groups of cells larger than 2 mm. Deposits of tumor cells of 2 mm or less but larger than 0.2 mm were considered as micrometastases and single-tumor cells or clusters of tumor cells of 0.2 mm or less were classified as isolated tumor cells.[10–12]

Statistical analysis

Statistical analysis was carried out using SPSS software, version 12.0.1 (SPSS, Chicago, IL). Numerical data are presented as mean ± standard deviation or median and range in case

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

Characteristics	Cases (n = 36)		Controls (n = 72)		p^1
	n	%	N	%	
Sex					
Female	16	44	37	51	0.496
Male	20	56	35	49	
Age (years)[2]	67 ± 12		67 ± 12		0.911[3]
TNM stage[4]					
I	2	6	4	6	1.000[5]
II	34	94	68	94	
T stage[6]					
T2	2	6	4	6	0.126
T3	26	72	62	86	
T4	8	22	6	8	
Tumor size (cm) (n = 104)[2,7]	4.9 ± 1.9 (n = 34)		5.2 ± 2.1 (n = 70)		0.539[3]
Tumor location					
Colon (coecum–sigmoid)	30	83	59	82	0.858
Rectum (rectosigmoid–rectum)	6	17	13	18	
Differentiation					
Good	9	25	18	25	0.912
Moderate	22	61	46	64	
Poor	5	14	8	11	
Mucinous[8]					
No	32	89	66	92	0.728[5]
Yes	4	11	6	8	
Preoperative serum CEA level (n = 43)[9]					
<6 μg/l	5	56	25	74	0.417[5]
≥6 μg/l	4	44	9	26	
Number of examined lymph nodes[2]	6.1 ± 4.5		7.8 ± 5.7		0.096[10]

CEA, carcinoembryonic antigen.
[1]Chi-square test of cases versus controls, unless mentioned otherwise. [2]Presented as mean ± standard deviation. [3]Student t-test. [4]According to the 6th edition of the TNM classification.[11] [5]Fisher's exact test. [6]If T2 and T3 stage were combined, the P-value was 0.043; this comparison was therefore used in the logistic regression. [7]Tumor size could not be found in pathology reports from 4 patients. [8]A tumor was considered mucinous when more than 50% of its volume existed of mucinous component. [9]Serum CEA had been determined in only 43 of 108 patients as it was not a standard procedure. [10]Mann–Whitney test.

of skewness. The clinicopathologic features of cases and controls were compared either by a Chi-square or Fisher's exact for categorical variables or by a Mann–Whitney or Student's t-test for numerical variables. Differences between screening results by automated microscopy and conventional microscopy were analyzed by using the McNemar's test. Univariate and multivariate odds ratio's (OR), 95% confidence intervals (95% CI) and p values (p) were calculated by applying logistic regression analysis. A p value of less than 0.05 was considered an indication of statistical significance. Variables with a p value lower than 0.10 in the univariate analysis were entered in the multivariate analysis. Because the number of examined lymph nodes has been shown to be prognostically relevant in several studies,[24] this variable was also entered in the multivariate analysis.

Results

Clinicopathological features

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

Table 2. Detection of tumor cells in paraffin-embedded lymph nodes from colorectal cancer patients (n = 105) after multilevel sectioning[1] and immunohistochemical staining combined with automated microscopy[2]

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

[1]Four micron sections were cut at 4 levels of each paraffin block with intervals of 200 μm. [2]Patients with macrometastases in the first lymph node level that had previously not been recognized as such, were excluded.

number of lymph nodes showed the following: OR 0.9, 95% CI 0.9–1.0, $p = 0.132$.

Multilevel sectioning, immunohistochemical staining and automated microscopy

Analysis of the first lymph node level identified micrometastases in 3 (3%) patients (2 cases and 1 control) and isolated tumor cells in 39 (37%) patients (13 cases and 26 controls) (Table 2). One micrometastasis found in an immunohistochemically stained section was also present on the original Hematoxylin and eosin (HE) stained slide but was not recognized by the pathologist. Macrometastases in 3 (3%) patients (2 cases and 1 control) that had not been recognized as lymph node metastases on the original HE stained slides were also seen. They had been described as vascular invasion or tumor deposits without lymphoid tissue in the pathology reports. On the immunohistochemically stained slides of all levels, lymphoid tissue was present around the tumor cells. These macrometastases were excluded from further analysis.

All macrometastases and micrometastases were detected by both conventional and automated microscopy. Automated microscopy led to the detection of additional isolated tumor cells (McNemar's test, 39 *vs.* 10 patients, respectively, $p < 0.001$) (Table 3). All but 1 isolated tumor cell found by conventional microscopy was also detected by automated microscopy. The missed isolated tumor cell was overlooked during visual inspection of ARIOL system results by the operator.

Analysis of a second level, resulted in additional detection of 1 patient with macrometastasis (a case), 1 patient with micrometastases (a case) and 9 patients with isolated tumor cells (2 cases and 7 controls) (Table 2). These patients had no tumor cells detected in the first level or in the original HE-stained slide.

When analyzing a third level, 11 patients (3 cases and 8 controls) were additionally identified with isolated tumor cells and assessment of a 4th level, identified 2 patients (2 controls) with isolated tumor cells. This led to a total number of 61 patients with isolated tumor cells (Table 2). No additional macrometastases or micrometastases were found when analyzing the 3rd and 4th level. In Figure 1, examples of micrometastases (Figs. 1*a* and 1*b*) and isolated tumor cells (Figs. 1*c*–1*f*) are shown.

Concluding, after analysis of 2 lymph node levels, macrometastases were observed in 1 patient (1 case) and micrometastases in 4 patients (3 cases and 1 control). Analysis of 2 additional lymph node levels solely identified the presence of isolated tumor cells.

Prognostic significance of lymph node metastases

More patients with micrometastases were seen in the case group than in the control group (Fisher's exact test, 3 of 34 (9%) *vs.* 1 of 71 (1%), $p = 0.099$). There was no difference in the presence of lymph nodes harboring isolated tumor cells, between the case and control group (Chi-square test, 18 of 34 (53%) *vs.* 43 of 71 (61%), $p = 0.459$) (Table 4). Separate analysis of patients with colon carcinoma, rectal carcinoma, right-sided or left-sided carcinoma did not result in significant difference in the presence of isolated tumor cells or percentage of lymph nodes harboring isolated tumor cells for the case and control group. Neither did excluding patients with T4 tumors or disregarding single-tumor cells as isolated tumor cells.

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

Table 3. Detection of tumor cells in lymph nodes from colorectal cancer patients after immunohistochemical staining of the first lymph node level: conventional microscopy versus automated microscopy[1]

	Conventional microscopy		Automated microscopy		Combined microscopy	
	n	%	*n*	%	*n*	%
Lymph node status ($n = 772$)						
Macrometastases	–	–	–	–	–	–
Micrometastases	6	0.8	6	0.8	6	0.8
Isolated tumor cells	15	1.9	99	12.8	100[2]	13.0
Patient status ($n = 105$)						
Macrometastases	–		–		–	–
Micrometastases	3	2.9	3	2.9	3	2.9
Isolated tumor cells	10	9.5	39	37.1	39	37.1

[1]Patients with macrometastases in the first lymph node level that had previously not been recognized as such, were excluded. [2]One lymph node with isolated tumor cells was missed by ARIOL automated microscopy.

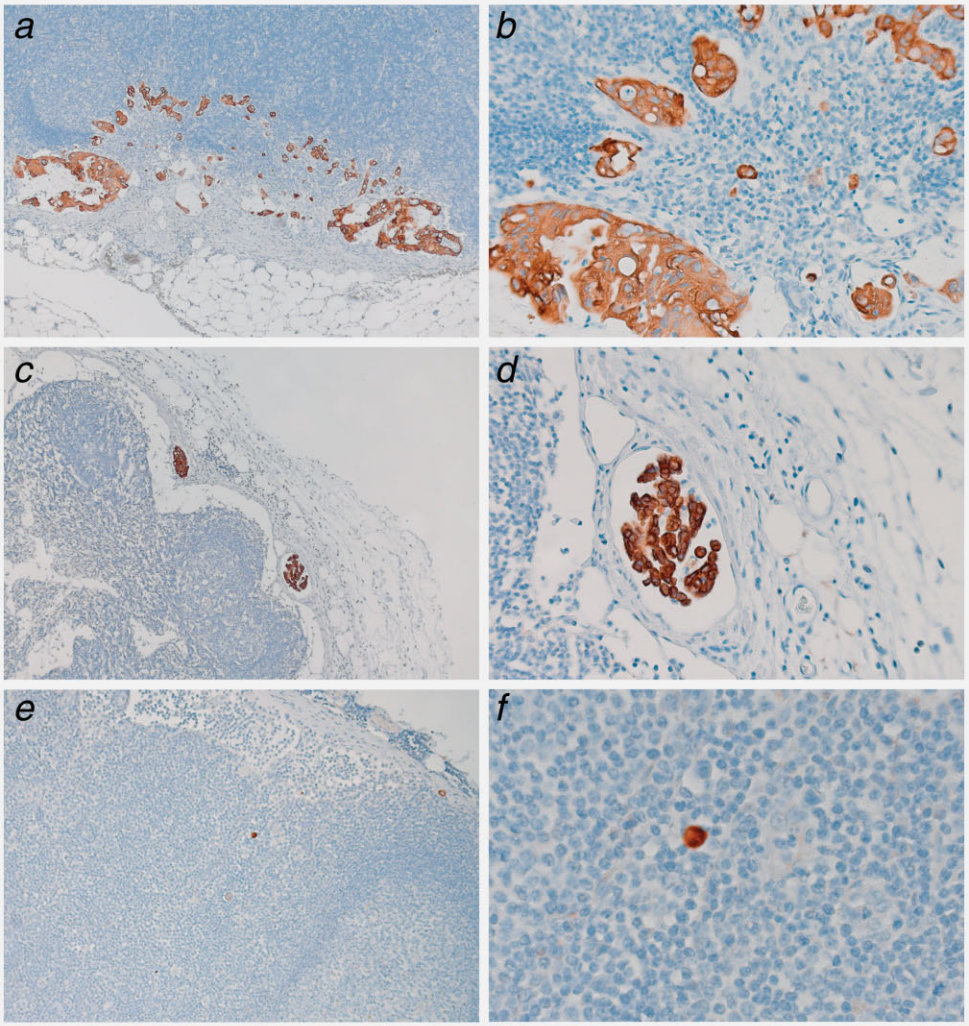

Figure 1. Lymph node sections stained for cytokeratin by AE1/AE3 antibodies resulting in brown cells and counterstained using hematoxylin and eosin. Micrometastases (>0.2 mm and ≤2 mm) at a magnification of 125× (*a*) and 250× (*b*); isolated tumor cells (tumor cell clusters ≤ 0.2 mm) at a magnification of 125× (*c*) and 500× (*d*); isolated tumor cells (single cells) at a magnification of 250× (*e*) and 1,000× (*f*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 4. Clinical relevance of occult tumor cells in lymph nodes from patients with colorectal cancer (n = 105)[1]

	Cases (n = 34)		Controls (n = 71)		
Lymph node status	n	%	n	%	p^2
Tumor-positive	22	65	44	62	0.786
Macrometastases	1	3	0	0	0.324[3]
Micrometastases	3	9	1	1	0.099[3]
Isolated tumor cells	18	53	43	61	0.459

Cases were defined as patients who had regional or distant disease recurrence within 5 years after the date of diagnosis of primary colorectal cancer; Controls were patients who did not develop locoregional or distant disease within 5 years after diagnosis of primary colorectal cancer. [1]Patients with macrometastases in the first lymph node level that had not been previously recognized as such, were excluded. [2]Chi-square test of cases versus controls, unless mentioned otherwise. [3]Fisher's exact test.

micrometastases (OR 34.5, 95% CI 2.7–440, $p = 0.006$), a T4 stage (OR 2.9, 95% CI 0.4–23.5, $p = 0.040$) and the number of examined lymph nodes (OR 0.9, 95% CI 0.8–1.0, $p = 0.025$) to be independent predictors for disease recurrence.

Relation between lymph node metastases and clinicopathological features

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

Discussion

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

We performed this study, because the prognostic significance of occult tumor cells in lymph nodes in CRC is still a matter of debate. A meta-analysis showed that immunohistochemical detection of occult tumor cells combined with serial or multilevel sectioning led to upstaging of 32% of previously considered node-negative patients, but the presence of occult tumor cells did not lead to a statistically significant adverse clinical outcome.[25] The meta-analysis reported an upstaging of 37% by RT-PCR and in contrast to immunohistochemistry studies, most RT-PCR studies found the presence of occult tumor cells in lymph nodes to predict a worse clinical outcome (overall 3-year survival of 78% *vs.* 97%, $p < 0.001$).[13,14,18-22,25] However, although several alternative techniques are evaluated for improving RNA yield,[26,27] optimal results with RT-PCR are mainly achieved when using fresh frozen tissue.[28,29] This is more laborious and lacks morphological assessment. Therefore, serial or multilevel sectioning combined with immunohistochemical staining is preferred, because it can be reliably used on paraffin-embedded tissue and the morphology of stained cells can be examined.

The fact that most immunohistochemistry studies show no difference in clinical outcome between patients with lymph nodes containing occult tumor cells and patients with tumor-negative lymph nodes, we ascribe to the lack of making a distinction between micrometastases and isolated tumor cells.[13,30–37] The last 6th TNM edition[11,12] recommends classifying occult tumor cells in lymph nodes into micrometastases and isolated tumor cells. According to this TNM edition, isolated tumor cells in lymph nodes, which are less than 0.2 mm in diameter, are insignificant and should be classified as pN0(i+). Lymph-node deposits with a diameter between 0.2 and 2 mm should be classified as micrometastases (mi) and staged as node-positive pN1(mi). Even so, this recommendation, published in 2002, was not based on evidence but more on logical reasoning. In our study, isolated tumor cells were found of no clinical relevance and do confirm the guidelines of the TNM classification system. Only 2 other research groups[29,38] have previously published study results regarding CRC patients in which they differentiated between micrometastases and isolated tumor cells, emphasizing the importance of our study. Messerini *et al.*[38] examined lymph nodes from 395 stage IIA CRC patients by immunohistochemical staining of 12 serial sections with antibodies directed against cytokeratin 20. Micrometastases were detected in lymph nodes from 9.9% of the patients, and isolated tumor cells were seen in 28.4% of patients. Similar to our findings, they did not find prognostic relevance of isolated tumor cells and showed a lower survival rate in patients with micrometastases compared to patients with isolated tumor cell-positive and tumor-negative lymph nodes. We suggest that the lack of prognostic significance of isolated tumor cells might be explained by assuming that these cells are shed from the primary tumor

and transported through lymphatic vessels to the lymph nodes, but do not have the potential of independent outgrow and, therefore, do not form established metastases. The second research group[29] who made a distinction between micrometastases and isolated tumor cells in CRC, examined 2 lymph node levels at a 200-μm interval with antibodies AE1/AE3. They detected micrometastases in 7 of 234 patients (3%), which correspond to the detection rate in our patient group. This group did not evaluate the prognostic relevance of micrometastases in CRC leaving only 2 studies by Messerini *et al.*[38] and our study who did.

Although we choose for a case–control design, because this was more cost-effective than a retrospective cohort study, our study has advantages such as reliable follow-up of the patients by our Department of Oncological Documentation and the matching of 2 controls for each case. A limitation of our study, which is known when evaluating archival material for research purposes, was the infeasibility to select for patients with at least 12 lymph nodes examined for accurate staging as recommended by the American Joint Committee on Cancer Staging[12] and the International Union against Cancer.[11] Twenty percent of our patient group underwent an adequate lymph node harvest of at least 12 lymph nodes. Other studies examining large archival material also demonstrated this limitation. Only 22% of 569 CRC specimens studied by Johnson *et al.*[39] and 37% of 116,995 CRC patients in a study by Baxter *et al.*[40] received adequate evaluation of 12 or more lymph nodes. Factors that have been reported to affect the number of lymph nodes retrieved in CRC specimens were the effect of different pathology assistants, older age, rectal cancer and the T stage.[40–42] We also saw less patients with a harvest of at least 12 lymph nodes in rectal carcinoma than in colon carcinoma, and patients with at least 12 lymph nodes harvested were significantly younger than patients with less than 12 lymph nodes harvested. Even so, evidence exists that the number of harvested lymph nodes has increased during the last 2 decades. In our group, an RT-PCR study regarding detection of occult tumor cells in lymph nodes from 26 CRC patients who were included from January 1990 and February 1992 has been previously published.[14] In this study, 246 lymph nodes were freshly isolated leading to an average of 9.5 compared to an average of 7.2 in our present study in which lymph nodes were isolated after fixation in formalin. Experience in our pathology laboratory has shown that more lymph nodes are harvested when isolated freshly than when harvested after formalin fixation (data not published). Also, in a prospective sentinel lymph node study, in CRC patients[29] between 1996 and 2001, more lymph nodes were harvesting in the study group (average 14) than in the control group (average 10), suggesting that more lymph nodes are harvesting within a trial design. Fat clearance techniques increases the number of harvested lymph nodes, especially small lymph nodes less than 5 mm.[43,44] However, these methods are time consuming, expensive and impractical as they involve noxious volatile agents.

Nevertheless, in our study, the number of examined lymph nodes was included in the multivariate analysis and in line with the literature, we found that a lower number of examined lymph nodes was an independent risk factor[24] for disease recurrence together with T4 stage and the presence of micrometastases. Because detailed analysis of lymph nodes using serial or multilevel sectioning and immunohistochemistry is costly and time- and labor-consuming, we should take into account restriction of lymph node sampling or ultrastaging of sentinel lymph node(s), which have the highest risk for harboring metastases. In a recent study by Pusztaszeri *et al.*,[42] the value of sampling lymph nodes located at distance sidelong CRC specimens was assessed. Mesocolic and perirectal fat were divided into 2 fractions: close to (<5 cm) and distant from (>5 cm) from the primary tumor. They found that in the colon, lymph node location is more important than lymph node number, because metastatic lymph nodes were present mostly in the peritumoral area. This suggested that lymph nodes should be initially recovered from the pericolic fat close to the tumor. If there are less than 4 tumor-positive lymph nodes and less than 12 tumor-negative lymph nodes examined in total, only then additional lymph nodes should be retrieved from the distal fraction for potential upstaging. In the rectum, systematic sampling of close and distant lymph nodes seemed mandatory, because, in some cases, metastases were detected only in distant lymph nodes, particularly in patients who had undergone neoadjuvant radiotherapy.

Additionally, interim results from Bilchik *et al.*[45] assessing sentinel lymph nodes for the presence of occult tumor cells, suggests the presence of micrometastases in sentinel lymph nodes to increase the risk for disease recurrence. Importantly, quantitative restriction of lymph node sampling and sentinel lymph node mapping in CRC does not substitute a complete oncologic resection and thus far all lymph nodes will continued to be examined with conventional HE staining.

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

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