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Dissemination and clinical impact of minimal metastatic disease in gastrointestinal cancer

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CHAPTER TWO

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

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ABSTRACT

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

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

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

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

INTRODUCTION

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

PATIENTS AND METHODS

Patient source and selection

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

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

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

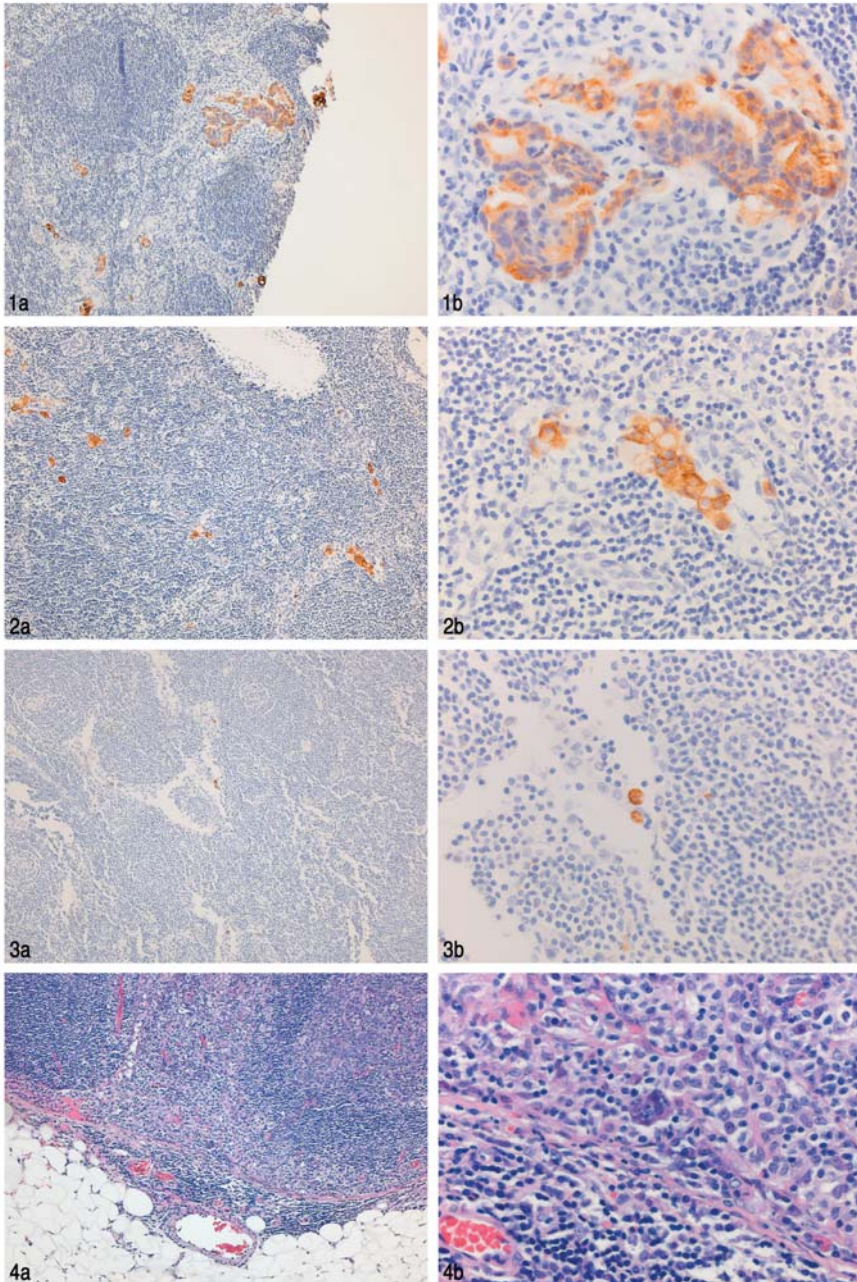


Figure 1-4. Occult tumor cells in lymph nodes from gastric cancer patients. Micrometastases (> 0.2 mm and ≤ 2 mm) at original magnifications of x100 (**1a**) and x400 (**1b**). Isolated tumor cells (tumor cell clusters ≤ 0.2 mm) at original magnifications of x100 (**2a**) and x400 (**2b**). Isolated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

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

Immunohistochemistry

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

Screening of the slides

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

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

Statistical analysis

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

RESULTS

Clinical relevance of occult tumor cells

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

Table 1. Patient characteristics

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

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

- continued

Table 1. Patient characteristics

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

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

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

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

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

Table 2. Results from conventional microscopy and automated microscopy.

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

HE, hematoxylin and eosin staining; IHC, immunohistochemistry; MM, micrometastases; ITC, isolated tumor cells; ^a Seven lymph nodes from three patients were found tumor-positive by HE staining only and scored negative on the IHC slides because these sections did not contain tumor cells anymore; ^b Four lymph nodes from two positive patients were recorded as negative in IHC evaluated by automated microscopy screening because the objects found by the system were considered as doubtful.

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

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

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

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

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

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

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

Screening of the slides

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

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

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

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

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

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

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

OR, odds ratio; CI, confidence interval.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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