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ORIGINAL ARTICLE

Gastric carcinoma: stage migration by immunohistochemically detected lymph node micrometastases

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Abstract
Background Immunohistochemically detected micrometastases of the regional lymph nodes in previously pN0-classified gastric cancer have been incorporated in the TNM staging system. This study aims to determine the incidence of such micrometastases in gastric carcinoma and to investigate their impact on stage grouping and prognosis.
Methods Ninety-five patients with gastric carcinoma classified as pN0 by conventional histological examination were enrolled. All patients underwent gastric resection with regional lymphadenectomy between 2006 and 2010. A total of 2018 lymph nodes was obtained (median, 20 Lymph nodes) and immunohistostained with anti-pan cytokeratin antibody (KL1).
Results Micrometastases were detected in regional lymph nodes by immunohistostaining in 16 out of all 95 patients. Fourteen patients were upstaged by micrometastasis-positive regional lymph nodes. Three patients demonstrated lymph nodes with isolated tumor cells alone. A significantly higher incidence of micrometastases was observed in patients with diffuse histologic type ($p = 0.007$) and total gastrectomy ($p = 0.007$). When isolated tumor cells were also regarded as lymph node involvement, the recurrence rate was significantly higher for node-positive than for node-negative patients and for those younger than 70 years (33.3 and 6.7 %, respectively; $p = 0.026$; $n = 39$). Overall survival analysis revealed no significant difference between micrometastasis-positive and micrometastasis-negative patients.
Conclusion Immunohistostaining of regional lymph nodes in node-negative gastric carcinoma patients leads to an increased detection of micrometastases with significant implications for the staging system. Although no impact on survival time was shown, the higher recurrence rate for node-positive patients younger than 70 years indicates a prognostic value of immunohistochemically detectable micrometastases.

Keywords Micrometastasis · Lymph nodes · Gastric cancer · Immunohistochemistry · Prognosis

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Introduction

In gastric carcinoma metastasis of the regional lymph nodes (LN) is considered to be the strongest prognostic indicator [1]. Improved diagnostic techniques have resulted in earlier detection of gastric carcinoma in Japan and many European centers [2, 3]. Consequently, the proportion of node-negative cases has accordingly increased. Despite curative R0 resection and histologically diagnosed tumor free LN (pN0) recurrence has been observed in gastric cancer patients on a regular basis [4, 5]. A possible explanation might be the existence of undetected micrometastatic foci in the regional LN representing an early stage of metastatic spread [6]. Recently, these tumor cells have been designated as micrometastasis (mi) and isolated tumor cells (ITC) according to the extent of metastasis [7]. If such micrometastatic lesions had proliferative activity, cancer relapse could originate from them [8].

Immunohistochemistry is a frequently described method to detect ITC and mi not visible in routinely performed haematoxylin and eosin (HE) stain [6, 8–23]. Besides immunohistostaining serial sectioning and molecular methods

are options to improve the accuracy of pathologic examination. Serial sectioning of regional LN combined with HE staining leads to an increased detection of mi [24]. However, it is costly in terms of labor and might still miss mi and ITC only identifiable by immunohistostain. With the employment of reverse transcriptase-polymerase chain reaction, detection rates of LN involvement even higher than the ones in immunohistochemistry have been reported [25]. Yet, this new approach to LN evaluation has been limited by the detection of false-positive results as the presence of DNA of non viable tumor cells had also been noted as LN involvement [26, 27]. Hence, immunohistochemistry was applied in this trial as it remains to be the most precise and implementable method.

This study has been designed to further clarify the prognostic significance of immunohistochemically detectable regional LN mi in gastric carcinoma patients, who have previously been classified as pN0 by conventional HE stain. In the current staging system mi have the same value as macrometastases, while ITC are not taken into account. With the aim to examine the importance of the extent of nodal metastases, the incidence of mi and ITC, the correlation between mi and other clinicopathologic parameters, and their influence on staging and survival have been evaluated.

Materials and methods

Patients

Ninety-five patients with primary gastric carcinoma, who had received gastric resection with regional LN dissection between 2006 and 2010, were enrolled. The LN of all patients were classified as pN0 by routine histopathologic examination. No patient had distant metastasis. Patients received surgery with curative R0 resection defined as no local residual tumor (61 total gastrectomy and 34 distal gastrectomy). D1 or D2 lymphadenectomy was performed, and in 77 cases the claimed number of 16 regional LN was obtained. Patients with less than 16 LN dissected ($n = 18$) were mainly those who had developed gastric stump carcinoma after prior gastric surgery ($n = 8$) or had received only partial gastric resection. Multiple attempts to find at least 16 LN were made by repeated examination of perigastric soft tissue. A total of 2018 LN was obtained (median 20 LN per person; range 1–50). No patient received postoperative chemotherapy, while preoperative chemotherapy was applied in eight cases. Tumors were classified according to the histological classification of the WHO and Lauren's criteria [28, 29]. Further clinicopathological details of patients are given in Table 2. Patients with carcinoma of the oesophagogastric junction were excluded from the study. Surgery was performed in different hospitals, while the gastric specimens and the LN of all patients were histopathologically examined at the Institute of Pathology at University Hospital Leipzig. Follow-up information was drawn from the data bases of the regional cancer centers ("Tumorzentrum Leipzig" and "Südwestsächsisches Tumorzentrum Zwickau e.V."). In cases of insufficient data, the treating doctor was contacted to complete information on disease recurrence and survival time. The median time of follow-up was 33 months (range 0–77 months).

Histopathology and Immunohistochemistry

Primary tumors and LN were fixed in formalin and embedded in paraffin for conventional histopathologic examination by the time of surgery. All LN were routinely stained with HE and classified as pN0. For the present study LN were re-evaluated by immunohistochemistry using the monoclonal anti-pan cytokeratin antibody KL1 (Clone KL1, Beckmann and Coulter Company, Marseille, France). One representative slice of 3 μm thickness was taken, then LN sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. For antigen retrieval LN sections were pressure cooked in citrate buffer pH 6. LN sections were incubated with primary antibody KL1 at room temperature for 1 h (dilution 1:80). Subsequently, HRP-Polymer Kit was used (Zytomed Systems, Berlin, Germany), and AEC was then applied as chromogen substrate (DCS Innovative Diagnostik-Systeme, Hamburg, Germany). A section of ileum mucosa was utilized as positive control.

Pathological classification of node-positive findings

Immunohistochemically stained sections were explored by two independent investigators without knowledge of clinical outcome. Cells were considered as tumor cells if their cytoplasmic membranes were mainly immunoreactive, they were found within the LN capsule at the same level with lymphocytes, and their morphology conformed to that of a tumor cell (Fig. 1). Node-positive findings were further subdivided into mi and ITC. Metastatic lesions of a size between 0.2 and 2 mm were classified as mi. ITC were defined as single tumor cells or clusters of tumor cells with a diameter smaller than 0.2 mm. Immunohistochemically detected mi led to a revision of pN category and tumor stage based on the 7th edition of the International Union against Cancer (UICC) staging system [7]. ITC did not require a change of pN category or UICC stage, but their occurrence was further reviewed in correlation with histopathologic parameters and survival time. Tumors

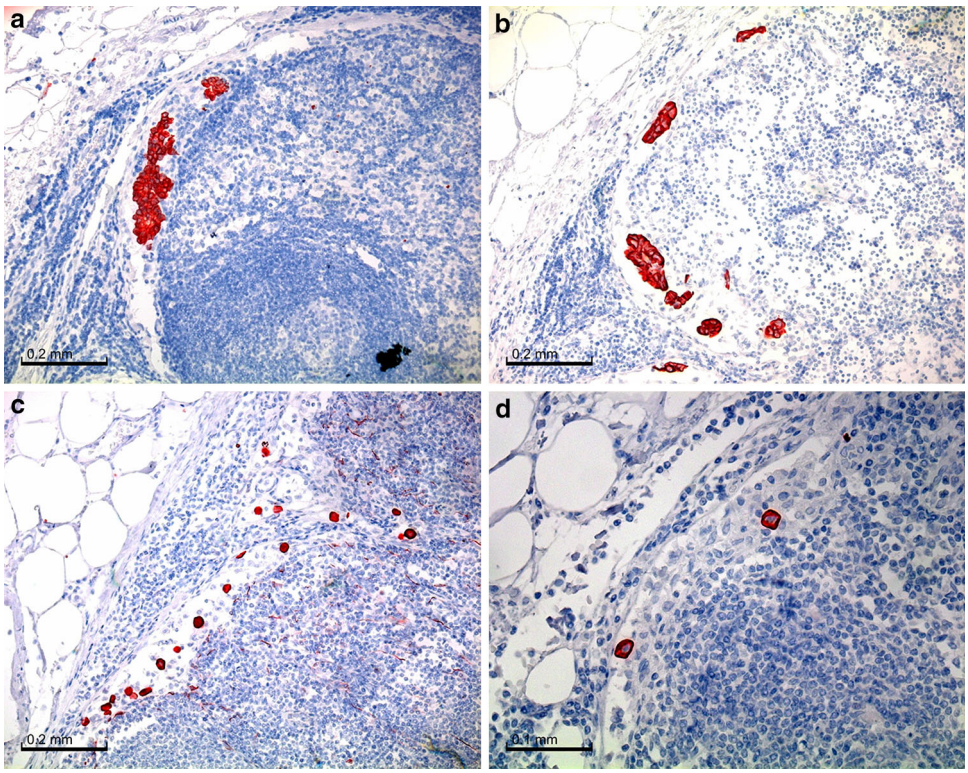

Fig. 1 Sections of regional lymph nodes presenting metastasis by immunohistochemical staining with anti-pan cytokeratin antibody KL1. **a**, **b** Micrometastasis (0.2–2 mm, original magnification ×200), **c** micrometastasis with scattered single tumor cells (original magnification ×200), **d** two isolated tumor cells (≤0.2 mm, original magnification ×400)

previously categorized by the 6th edition of the TNM classification were reclassified [30].

Statistical analyses

The Chi square test and Fisher's exact test were adopted for correlation analysis between immunohistochemically detected mi and clinicopathologic parameters. The relation between mi and age was tested by the Mann–Whitney *U* test. Survival curves were estimated by Kaplan–Meier methods, and the differences between subgroups were analyzed using the log-rank test. The Cox proportional hazards model was used to test the independent effects of potentially important factors obtained by univariate analysis ($p < 0.05$) on survival time. All p values <0.05 were considered statistically significant. IBM SPSS Statistics 20.0 was used for all analyses [31].

Results

mi and ITC

Node-positive findings were identified by immunohistochemical LN examination in 19 out of all 95 cases (20.0 %, Table 1), respectively in 47 out of all 2018 LN (2.3 %). Of these 19 cases, LN of 16 patients showed mi and in some cases additionally ITC. LN of three patients solely had

Table 1 Frequency of node-positive findings and micrometastasis

Variable	Node-positive[a]		mi[b]		Total no.
	Absolute	%	Absolute	%	
Patients	19	20.0	16	16.8	95
No. of LN[c]	47	2.3	34	1.7	2018

[a] Micrometastasis or isolated tumor cells

[b] Micrometastasis

[c] Lymph nodes

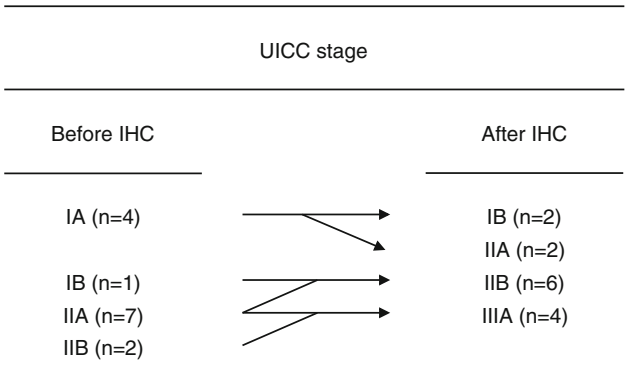

Fig. 2 Stage migration after immunohistochemical lymph node examination (IHC) according to the International Union against Cancer (UICC) staging system ($n = 14$)

Table 2 Correlation between the type of lymph node involvement and clinicopathologic features

Characteristics	No. of patients				
	Node-positive[a] (%)	*p*	mi[b] (%)	*p*	Total
Age in years[c]	66.0 (±10.0)		66.3 (±9.0)		69.9 (±9.8)
Sex					
Male	15 (23.4)	0.229	13 (20.3)	0.194	64
Female	4 (12.9)		3 (9.7)		31
Preoperative chemotherapy					
Negative	16 (18.4)	0.196	13 (14.9)	0.129	87
Positive	3 (37.5)		3 (37.5)		8
Operative procedure					
Total gastrectomy	17 (27.9)	0.010*	15 (24.6)	0.007*	61
Distal gastrectomy	2 (5.9)		1 (2.9)		34
Tumor localization					
Proximal	0 (0.0)	1.000	0 (0.0)	0.857	3
Middle	5 (20.0)		5 (20.0)		25
Distal	11 (21.2)		8 (15.4)		52
Multiple	2 (28.6)		2 (28.6)		7
Anastomosis	1 (12.5)		1 (12.5)		8
Greatest tumor dimension in mm					
≤30	9 (17.3)	0.471	8 (15.4)	0.676	52
>30	10 (23.3)		8 (18.6)		43
Median	35		30		30
Tumor invasion depth					
No evidence of tumor	1 (50.0)	0.014*	1 (50.0)	0.056	2
Mucosa ± submucosa	4 (10.3)		4 (10.3)		39
Muscularis propria	1 (5.9)		1 (5.9)		17
Subserosa	10 (33.3)		7 (23.3)		30
Serosa ± adjacent structures	3 (42.9)		3 (42.9)		7
No. of LN evaluated					
<16	1 (5.6)	0.048*	1 (5.6)	0.080	18
16–25	10 (18.2)		8 (14.5)		55
>25	8 (36.4)		7 (31.8)		22
Median	25		25		20
Lymphatic vessel invasion					
Negative	6 (14.0)	0.180	5 (11.6)	0.217	43
Positive	13 (25.0)		11 (21.2)		52
Venous invasion					
Negative	18 (21.4)	0.454	15 (17.9)	0.684	84
Positive	1 (9.1)		1 (9.1)		11
Perineural invasion					
Negative	10 (15.2)	0.075	9 (13.6)	0.240	66
Positive	9 (31.0)		7 (24.1)		29
Laurén classification					
Intestinal	5 (9.4)	0.004*	4 (7.5)	0.007*	53
Diffuse	14 (33.3)		12 (28.6)		42

* *p* value was statistically significant

[a] Micrometastasis or isolated tumor cells

[b] Micrometastasis

[c] Mean (±standard deviation)

ITC. mi led to an increase in pN category: In 11 cases pN category changed from pN0 to pN1(mi) and in five cases to pN2(mi). None of the patients showed more than six LN with mi. In consequence of pN category changes, 14 of all patients were upstaged as presented in Fig. 2. Out of the patients with mi, two did not undergo stage migration: One

of them had stage IIIB disease, which holds both pN0 and pN1 nodal status, and the other one showed a complete regression of the primary tumor (pT0) after preoperative chemotherapy, and therefore was not staged by the UICC system.

Correlation of mi and clinicopathologic features

The incidence of immunohistochemically detected mi for different clinicopathologic parameters is shown in Table 2. Patients with carcinoma of Lauren's diffuse histologic type showed a significantly higher incidence of mi: Twelve out of 42 patients with diffuse type featured mi (28.6 %), while only four out of 53 with intestinal type were mi-positive (7.5 %, $p = 0.007$). Significant correlation was also observed between immunohistochemically detected mi and total gastrectomy (24.6 % with total gastrectomy showed mi versus 2.9 % of the patients with distal gastric resection, $p = 0.007$). Of patients with total gastrectomy, 88.5 % had more than 15 LN removed whereas only 67.6 % of patients with distal gastric resection exceeded that number (Chi square test, $p = 0.013$). When analyzing the distribution pattern of node-positive findings (both mi-positive and ITC-positive LN), the frequency of LN involvement increased significantly with advancing depth of tumor invasion and higher numbers of dissected LN (Table 2). Several LN featured immunohistochemically stained dendritic cells and macrophages that had to be distinguished from tumor cells. Their presence was not associated with LN involvement.

Survival analysis

Of 95 patients, 27 died during the postoperative follow-up (28.4 %). Within the mi-positive group, the mortality of 37.5 % (6 out of 16 patients) within that period was slightly higher than the one of mi-negative patients (26.6 %, 21 out of 79 patients). This difference was not statistically significant. With a median follow-up of 33 months, a significant difference in overall survival could neither be calculated between mi-positive and mi-negative nor between node-positive and node-negative patients (Fig. 3). Multivariate analysis revealed only venous invasion as an independent risk factor for death. Cancer relapse was observed in 10 of all 95 patients. Within this study population, younger patients were more likely to be mi-positive or node-positive (Mann–Whitney test; $p = 0.046$ and $p = 0.040$, respectively). Considering this finding patients were distributed into two groups according to their age, with the mean age of 70 years as benchmark (mean age in years: 69.9 SD $\pm$ 9.8). When patients younger than 70 years were analyzed separately, a significant difference in the rates of cancer relapse for node-positive and node-negative subgroups was observed (33.3 and 6.7 %, respectively; $p = 0.026$, Fig. 4). Though, only five out of all 39 patients younger than 70 years presented with recurrence. In patients older than 70 years, LN involvement was not related to cancer relapse.

Discussion

LN metastasis is recognized as one of the strongest independent prognostic factors in gastric carcinoma. Together with depth of tumor invasion, a positive metastatic status of regional LN significantly worsens prognosis and reduces time to recurrence [1, 32–35]. Histopathological LN examination in gastric cancer patients is routinely performed by HE staining method. Beyond that, immunohistochemistry with anti-cytokeratin antibodies is a widely accepted method to detect tumor cells in lymphatic tissue not visible in conventional HE staining [6, 8–23]. Anti-pan cytokeratin antibody (KL1), known to react with a broad spectrum of human cytokeratins that are expressed by regular as well as by neoplastic epithelial cells, was employed in this study [36]. The 7th TNM classification states that LN mi detected by morphological techniques such as HE staining or immunohistochemistry should be included in the staging of disease [7]. It defines mi as metastatic lesions between 0.2 and 2 mm and clearly distinguishes them from ITC, which do not influence the UICC stage. Many previous studies regarding the prognostic influence of immunohistochemically detected mi lack a consistent definition of the term mi. Frequently, all tumor cells detected by immunohistochemistry were defined as mi [6, 9, 10, 12, 14, 16, 21, 23]. In contrast, this study discriminates between mi and ITC based on the size of metastasis as recommended by the UICC.

Although immunohistochemically detected mi have been incorporated in the staging system, their clinical implications for gastric carcinoma patients remain controversial. It was the aim of this trial to investigate the prognostic significance of regional LN mi in gastric carcinoma and to examine whether other clinicopathologic parameters might be associated with their occurrence. All patients enrolled were initially classified as node-negative (pN0) by conventional histopathologic examination. The use of immunohistochemistry revealed that LN involvement was not detected in 19 of all 95 cases, 16 of which had mi. This relatively low detection rate of previously undetected tumor cells might be explained by the restriction to node-negative patients and corresponds well with results of other studies that have reported rates between 10.0 and 49.0 % for pN0 patients [6, 10, 12–15, 21–23]. The study could not show a significant decline in overall survival for mi-positive patients although patients with

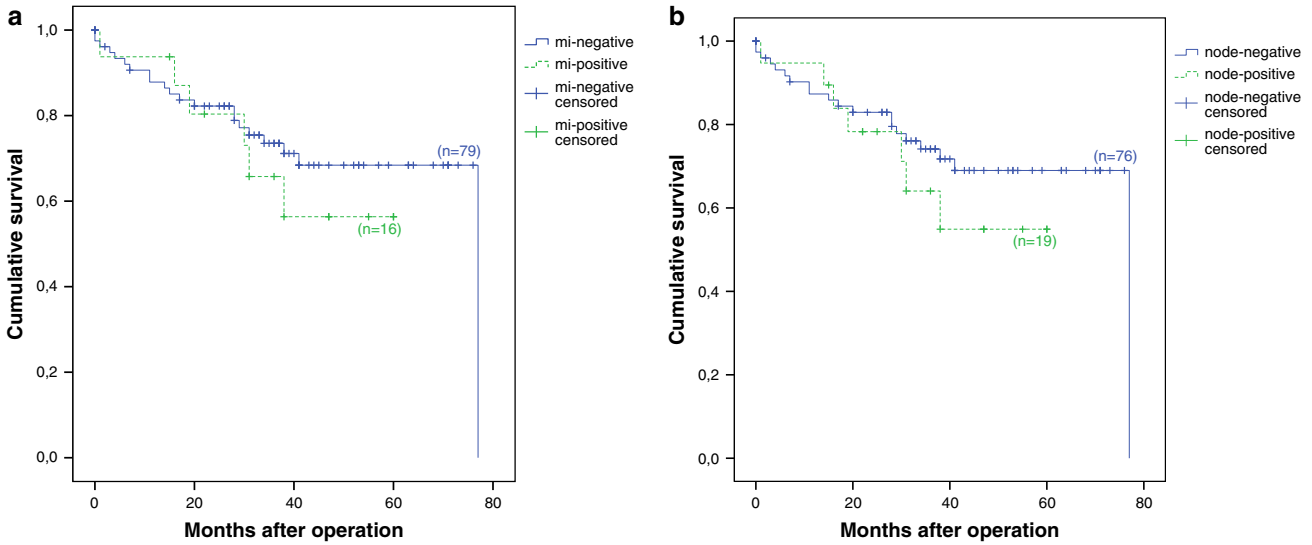

Fig. 3 Kaplan–Meier overall survival curves for all 95 patients with histologically pN0 gastric cancer. There was no significant difference in survival time: **a** neither between patients with immunohistochemically classified mi-positive and mi-negative LN (log-rank test, $p = 0.511$), **b** nor between the groups with node-positive and node-negative findings (log-rank test, $p = 0.386$)

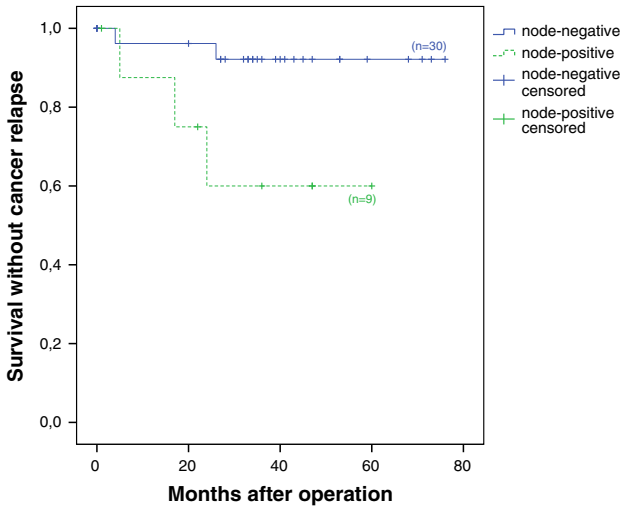

Fig. 4 Kaplan–Meier time to recurrence curves for patients younger than 70 years ($n = 39$) classified as node-positive or node-negative by immunohistochemistry. Node-positive patients showed a significantly higher recurrence rate within this subgroup (log-rank, $p = 0.026$)

micrometastatic nodal involvement had a slightly higher mortality rate (37.5 vs. 26.6 % for mi-negative patients). When mi and ITC were equally regarded as LN involvement and patients younger than 70 years were analyzed separately, a higher rate of cancer relapse was observed for node-positive patients. This finding indicates a prognostic value of tumor cells discovered by immunohistostaining. The effect was significant in the under 70 years group, while time to recurrence curves for node-positive and node-negative patients over 70 years did not differ significantly. In literature, controversy remains about the influence of chronologic age on the nodal status in gastric carcinoma. Most previous reports have negated a significant difference in the rates of LN metastasis between younger and elderly patients [37–40], whereas in cases of early gastric cancer several authors have reported a higher prevalence of LN involvement for elderly patients [41, 42].

Earlier studies have shown conflicting results in the debate about the prognostic value of immunohistochemically detected LN mi in gastric carcinoma: some supporting their clinical impact [6, 8, 10–12, 14–19, 21], and some refuting it [9, 22, 23]. Lee et al. [16] reported a significant decrease in the 5-year survival rate for node-positive (49 %) compared to node-negative patients (76 %) for both early and advanced gastric carcinoma. His results were supported by Cai et al. [14], who investigated the survival of patients with submucosal gastric cancer, and found a significant difference in the 5-year survival rates between patients with nodal involvement and patients with a node-negative status (82 and 100 %, respectively). Contrary to that, Fukagawa et al. [22, 43] detected no influence on survival in two different studies, neither for mi-positive nor for ITC-positive patients with pT2N0M0 gastric carcinoma and D2 dissection. Another approach to LN evaluation was made by Nakamura et al. [44] who classified metastatic LN into a massive type with tumor occupation of the entire LN and a non-massive type with only partial LN infiltration; the 10-year-survival was significantly poorer in the massive type group. In the present study LN mi, which would clearly be regarded as the non-massive type, showed an

association with other histopathologic risk factors and an affection of the recurrence rate for patients younger than 70 years but no direct impact on survival.

The present investigations showed that LN mi detected by immunohistostaining were significantly more frequent in Lauren's diffuse histologic type. The loss of E-cadherin expression in the primary tumor, especially occurring in diffuse type gastric carcinoma [45], might be a possible explanation for this finding as it leads to decreased intercellular adhesion and correlates with tumor cell dissociation [46]. A higher probability for mi and ITC in diffuse histologic type was also reported by other authors [6, 10, 11, 17, 45] leading to the conclusion that LN immunohistostaining is of special importance for this cancer entity. Furthermore, positive correlation between LN mi and total gastrectomy was observed in this study, possibly because total gastrectomy led to a significantly higher number of dissected LN in comparison to patients, who received only partial gastric resection (88.5 % of patients with total gastrectomy had more than 15 LN removed versus 67.6 % of patients with distal gastric resection). It was found that higher numbers of evaluated LN led to an increased detection of LN involvement. Once more, this stresses the importance of systemic lymphadenectomy as it is a precondition for adequate histopathologic examination and offers protection from disease recurrence even in node-negative gastric carcinoma [47]. Another result of the present study was the significantly higher frequency of LN involvement in patients with advancing pT category. The association between LN involvement and the presence of perineural invasion was of borderline significance. Both depth of tumor invasion [1, 33, 48], and perineural invasion [48, 49] have been reported to be risk factors for LN metastases and a worse prognosis in gastric carcinoma. Recently there has been debate whether preoperative chemotherapy in gastric cancer has an influence on LN yield [50, 51] and nodal status [52]. There have been reports about patients with a complete regression of the primary tumor, who still presented with lymph nodes metastases [51]. Even after preoperative chemotherapy LN positivity has been associated with a poorer prognosis in gastric cancer, suggesting that these tumor cells still have proliferative activity [53]. In this context, it is noteworthy that in the present study three of the eight patients who had received preoperative chemotherapy still featured LN mi. One of these patients showed complete response of the primary tumor and yet mi was found in one of his LN. Even though this sample of patients is too small for a statistically significant conclusion, it adumbrates that preoperative chemotherapy does not reduce the need for LN dissection.

In conclusion, immunohistochemistry improves the accurate detection of LN involvement in gastric carcinoma and can be recommended in addition to conventional HE staining, especially in diffuse type carcinoma. Compared to mi-positive patients, no survival benefit could be shown for mi-negative patients, yet the higher rate of cancer relapse for patients with nodal involvement and younger than 70 years indicates a prognostic significance of immunohistochemically detected tumor cells.

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