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Original contribution

Isolated tumor cells in regional lymph nodes in patients with adenocarcinoma of the esophagogastric junction might represent part of true metastases ☆

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Summary Regional lymph node metastases in patients with carcinoma of the esophagogastric junction (EGJ) are an important prognostic factor. According to the tumor, node, and metastasis classification, isolated tumor cells (ITCs) are single tumor cells or small clusters of tumor cells not exceeding 0.2 mm. Tumor clusters >0.2 mm are classified as metastases. The significance of lymph nodes with ITCs is unclear, although not contributing to the pN category. The aim of this study was to determine the prevalence of regional lymph nodes with ITCs on the primary hematoxylin and eosin–stained slide and to examine how often deeper sections reveal a true metastasis. The study included surgical specimens of 126 patients with adenocarcinoma of the EGJ. Lymph nodes with ITCs were identified. Additional sections were cut and stained with hematoxylin and eosin and with cytokeratin. All slides were evaluated for the presence of tumor cells, and it was determined whether the criteria for a metastasis were met on the additional sections. ITCs were detected in 59 (1.7%) of 3454 lymph nodes and in 41 (32.5%) of 126 patients. In 29 (49.2%) lymph nodes with ITCs on the primary slide, further sections resulted in a changed status from ITCs to a metastasis. In 7 (17.1%) of 41 patients, the pN category was changed. In patients with adenocarcinoma of the EGJ, the presence of ITCs in regional lymph nodes is a common observation. ITCs often represent part of a real metastasis. To obtain a pN category as accurate as possible, we strongly recommend thorough examination of regional lymph nodes with additional sections when ITCs are observed.

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1. Introduction

The incidence of cancer as well as the related mortality is increasing rapidly worldwide. Cancer of the stomach including both cancer of the esophagogastric junction (EGJ) and non-EGJ cancer was reported to be the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death [1]. The main risk factor of non-EGJ cancer is infection with *Helicobacter pylori*. Although the incidence of this subtype is declining, the opposite is seen for cancer of EGJ, with the main risk factors being reflux disease with associated development of intestinal metaplasia in the distal esophagus and obesity [1,2]. Although the diagnostic methods and treatment have improved, a large proportion of the patients suffer from cancer-related death. One of the strongest prognostic predictors for survival is a negative lymph node status, with a decreased 5-year survival rate from 53% to 11% when lymph node metastases are present [3]. Also, recurrence is observed in patients diagnosed with an early-stage cancer with a reported radical resection. This might be attributable to undetected lymph node metastases [4-7].

In 1999, the concept of lymph nodes with isolated tumor cells (ITCs) was introduced by Hermanek et al who proposed that the presence of ITCs should be reported and a distinction between ITCs, micrometastasis (MM) measuring >0.2 mm and <2 mm, and metastases be made to achieve a uniform histopathological staging [8]. Both the Union for International Cancer Control and the American Joint Committee on Cancer subsequently adopted the proposal [9,10]. As no common terminology existed until this time, these small groups of tumor cells have been defined differently and mentioned by several different names including *MM*, *occult lymph node metastases*, and *minimal tumor involvement*. According to the tumor, node, and metastasis (TNM) classification, the present definition of *ITCs* is single tumor cells or small clusters of tumor cells not exceeding 0.2 mm detected by hematoxylin and eosin (H&E) or immunohistochemical (IHC) staining [9,10]. In the TNM supplement by the Union for International Cancer Control, it is also stated that ITCs typically do not show evidence of metastatic activity (eg, proliferation or stromal response) or penetration of vascular or lymphatic sinus walls [9]. The American Joint Committee on Cancer differs only little in the wording, stating that ITCs may represent tumor cells in transit, usually found in the subcapsular nodal sinuses, although they may be seen in the parenchyma [10]. Surgical specimens with no regional lymph node metastases and with absence or evidence of ITCs are recommended to be classified as pN0(i−) and pN0(i+), respectively. Cases not examined for ITCs should be classified as pN0 [9]. The definition has remained unchanged through the TNM sixth, seventh, and eighth edition. In the eighth edition, an additional criterion has been proposed in breast cancer to include fewer than 200 tumor cells in a single cross section in the definition of ITCs. Different numbers have been suggested for other tumor sites, but a definitive number has not been investigated for cancer of EGJ. For cancer of EGJ, however, MMs should not be reported as an independent category but simply classified as metastases. The results of previous studies are divergent, but a tendency toward ITCs as a negative prognostic factor is reported.

Histopathological evaluation of surgical resection specimens is performed to assign an accurate pTNM stage. In most pathology departments, 1 H&E-stained slide from each of the tissue blocks containing primary tumor and all the resected lymph nodes are provided as a routine. No consensus exists on how to handle lymph nodes with ITCs visualized on the primary H&E-stained slide. As a result, the extent of examination depends on tradition, economy, and individual preferences.

The aim of the present study was to determine the prevalence of regional lymph nodes with ITCs on the primary H&E-stained slide in patients with adenocarcinoma of the EGJ and to examine how often deeper sections reveal that ITCs in fact represent part of a true metastasis, as this might influence the pN category and the prognosis.

2. Materials and methods

2.1. Included patients

Patients diagnosed with adenocarcinoma of the EGJ followed by a surgical resection including regional lymphadenectomy performed in the period January 1, 2016, to August 31, 2017, at the Department of Surgical Gastroenterology, Rigshospitalet, Copenhagen, Denmark, were identified. One hundred and twenty-six patients met the inclusion criteria. H&E-stained slides were retrieved from the archives of the Department of Pathology, Rigshospitalet, Denmark. Two pathologists specialized in gastrointestinal pathology independently reviewed all slides containing lymph nodes for the presence of metastases or ITCs. The size criterion of ITCs as stated in TNM eighth edition was followed. Additionally, the ITCs should exhibit malignant features such as nuclear enlargement, hyperchromasia, and pleomorphism. All cases classified by 1 or both pathologists as positive for ITCs were re-reviewed and verified in common. In cases with disagreement, a consensus decision was achieved.

2.2. Additional sectioning, staining, and digitalizing of slides

For all lymph nodes with ITCs, the corresponding formalin-fixed, paraffin-embedded tissue block was retrieved for further analysis. In a few cases, additional sections had already been cut at the primary histopathological evaluation and stained with H&E or cytokeratin. Additional leveling was performed until 6 levels were reached besides the first H&E-stained slide. In cases with an insufficient amount of tissue, as many levels as possible were cut. The thickness of the sections was 4 μm, and the distance between the levels was 200 μm. At each level, 2 sections were cut and stained with H&E and cytokeratin, respectively.

IHC staining with Monoclonal Mouse Anti-Human Cytokeratin, CK-AE, Clones AE1/AE3 (cat. no. M3515, Dako, Glostrup, Denmark) was performed on the Dako Autostainer Link platform. Briefly, dewaxing and antigen retrieval were performed by immersing slides in EnVision FLEX Target Retrieval Solution, High pH (Dako, cat. no. K8004), and heated in the PT module at 97°C for 20 minutes. After pretreatment, staining was done according to the manufacturer's instructions: Slides were incubated with peroxidase for 5 minutes, mouse linker (Dako, cat. no. K8022) for 15 minutes, and then the primary antibody CK-AE (1:200) for 20 minutes. The reactions were detected using EnVision FLEX+ /HRP Detection Reagent and visualized with Envision DAB+ Substrate according to the manufacturer's instructions (Dako, cat. no. K8002). Antibody binding visualization was performed by incubation with a labeled horseradish peroxidase polymer for 20 minutes, and a signal was generated with a 3,3'-diaminobenzidine-tetrahydrochloride (DAB chromogene) for 10 minutes. All sections were counterstained with hematoxylin and mounted with Pertex. Negative controls were performed by omission of the primary antibody. Epithelium in colonic mucosa was used as external positive control, and tissue from a lymph node was used as an internal negative control.

All primary and additional slides were digitalized using Nanozoomer HT 2.0 slide scanner from Hamamatsu Photonics (Hamamatsu, Japan), and subsequently, the digital images were processed using Hamamatsu software.

2.3. Histopathological evaluation

The scanned slides were reviewed by 2 pathologists, and all areas with tumor cells were identified. Digital measurement of clusters of tumor cells was performed, and it was assessed whether the criteria for a metastasis were met. The presence of tumor cells in a lymph node was categorized as a metastasis (1) if a cluster of tumor cells exceeded 0.2 mm on a single cross section or (2) if a cluster of tumor cells was present in next underlying levels with identical localization in the lymph node. In the latter setting, it was assumed that areas with tumor cells in neighboring levels were cohesive, and as the distance between the levels was 0.2 mm, this was categorized as a metastasis.

2.4. Clinical information

Age of the patient at the date of surgery, sex, and neoadjuvant therapy as well as histopathological tumor subtype,

Table 1 Clinical characteristics of patients

	Negative for ITC (n = 85)	Positive for ITC (n = 41)	*P*
Male, n (%)	69 (81.2)	33 (80.5)	1.00
Female, n (%)	16 (18.8)	8 (19.5)	
Age, y (average; range)			1.00
Male	67.3 (44-81)	65.8 (47-84)	
Female	67.4 (54-78)	66.5 (51-83)	
Received neoadjuvant therapy, n (%)	71 (83.5)	37 (90.2)	.4191
Tumor type, n (%)			1.00 (glandular adenocarcinoma *vs* mucinous and poorly cohesive)
Glandular adenocarcinoma	79 (92.9)	39 (95.1)	
Mucinous adenocarcinoma	4 (4.7)	1 (2.4)	
Poorly cohesive adenocarcinoma	2 (2.4)	1 (2.4)	
Differentiation, n (%)			.0027 (well/moderately *vs* poorly)
Well	9 (10.6)	0 (0)	
Moderately	42 (49.4)	16 (39.0)	
Poorly	22 (25.9)	24 (58.5)	
Unknown	12 (14.1)	1 (2.4)	
Total no. of lymph nodes (average per patient)	2208 (26.0)	1246 (30.4)	
Pathological T-stage, n (%)			<.0001 (T0-T2 *vs* T3-T4)
pT0/Tis	10 (11.8)	0 (0)	
pT1	22 (25.9)	2 (4.9)	
pT2	9 (10.6)	3 (7.3)	
pT3	44 (51.8)	33 (80.5)	
pT4	0 (0)	3 (7.3)	
Pathological N-stage before additional sections, n (%)			<.0001
pN0	50 (58.8)	8 (19.5)	
pN1	11 (12.9)	11 (26.8)	
pN2	17 (20.0)	5 (12.2)	
pN3	7 (8.2)	17 (41.5)	

degree of differentiation, and (y)pTN category were registered. All data were derived from the pathology records.

2.5. Statistical analysis

Association between age, sex, neoadjuvant treatment, tumor subtype, degree of differentiation, and (y)pTN category according to presence of ITCs was evaluated by a 2-tailed Fisher exact test or a χ^2 test as appropriate. $P < .05$ was considered statistically significant. All statistical analyses were performed using GraphPad Software (San Diego, California, United States of America), QuickCalcs (https://www.graphpad.com/quickcalcs/, accessed on June 10, 2019).

2.6. Ethics

The study was approved by the Local Committee on Health Research Ethics (record no. RH-2017-267) and the Danish Data Protection Agency (record no. H-17019303) according to the Declaration of Helsinki and Danish law.

3. Results

3.1. Patient characteristics

A total number of 3454 lymph nodes from 126 patients were included in the study. In total, 2970 (86%) lymph nodes were negative for metastases, and 425 (12.3%) lymph nodes were positive. ITCs were detected in 59 lymph nodes (1.7%) from 41 patients (32.5%). ITCs were identified in 1 to 3 lymph nodes from each of these patients.

Clinical characteristics of the patients with and without ITCs are presented in Table 1. No significant differences were observed for sex, age, neoadjuvant treatment, or histological type when comparing the ITCs-negative and -

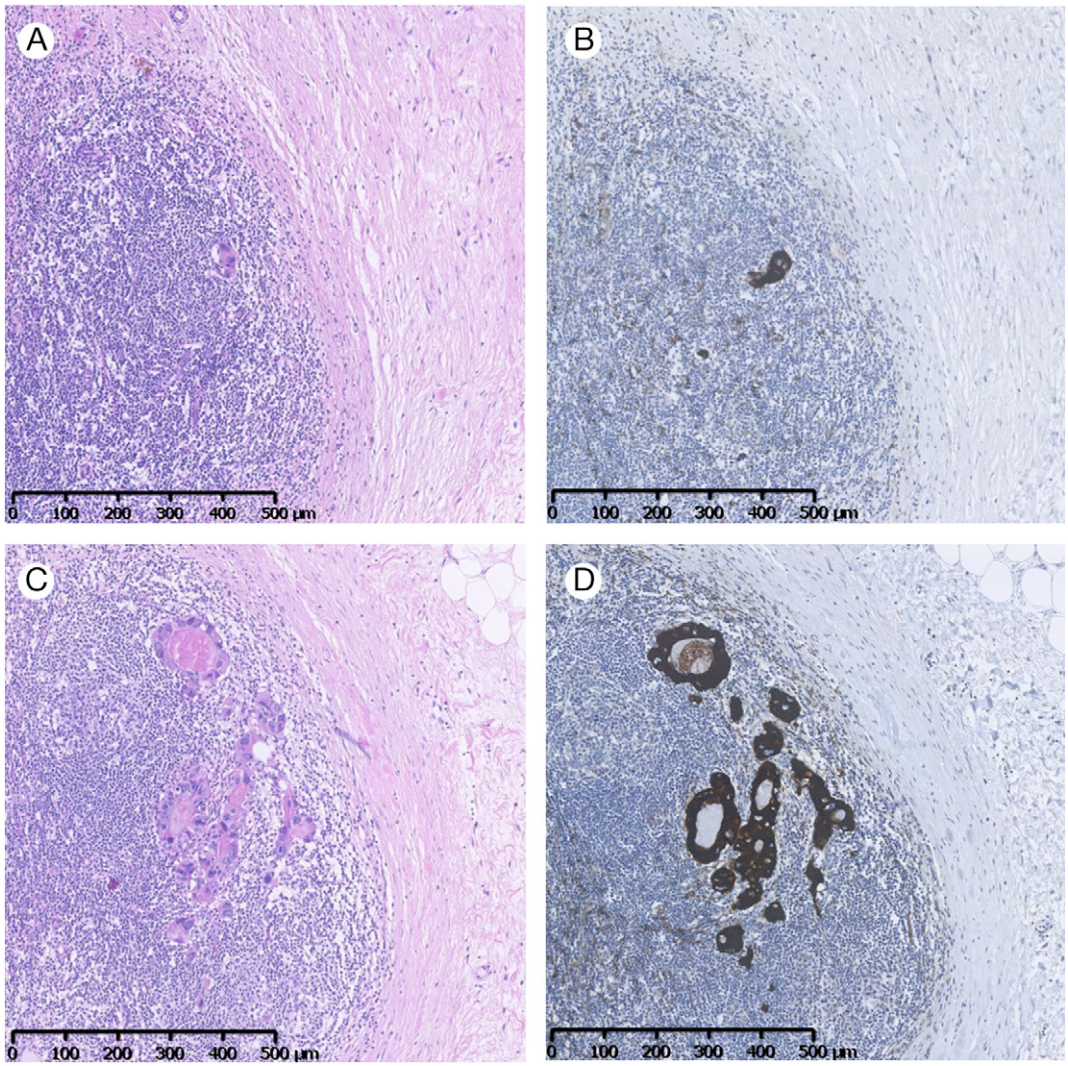

Figure A and B, A lymph node with isolated tumor cells on the first sections; H&E- and cytokeratin-stained slides, respectively. C and D, Deeper sections revealed a metastasis in both H&E- and cytokeratin-stained sections.

Table 2 Data on patients with isolated tumor cells on the first H&E-stained routine section and status after additional sections

No. of lymph nodes with ITCs	59
No. of lymph nodes (%) still classified as ITCs with additional sections	30 (50.8)
No. of lymph nodes (%) with metastasis (>0.2 mm) on a single slide	26 (44.1)
No. of lymph nodes (%) with metastasis (>0.2 mm) by addition of foci with identical location on neighboring levels in lymph nodes	3 (5.1)
No. of patients with ITCs (1-3 lymph nodes per patient)	41
No. of patients (%) where supplementary sectioning revealed metastasis	23 (18.3)
No. of patients (%) with a change in pN category	7 (17.1)

positive groups. The average number of harvested lymph nodes in the ITCs-negative group was 26 compared with 30 in the ITCs-positive group. In patients with poorly differentiated tumors, higher (y)pT category, and lymph node metastases, a significantly higher risk of presence of ITCs was observed.

3.2. Additional sectioning revealed metastases in nearly half of the lymph nodes with ITCs

Of 59 lymph nodes from 41 patients with ITCs on the primary H&E-stained slide, additional sectioning revealed metastases in 29 lymph nodes from 23 patients, with 26 lymph nodes from 21 patients meeting the criteria for metastases on a single slide (Figure). Three more lymph nodes from 3 patients met the criteria for metastases when adding areas on neighboring levels. One patient having 3 lymph nodes with ITCs met both criteria. In total, additional sectioning resulted in a changed pN category in 7 patients (Table 2). A change from pN0 to pN1 was observed in 3 patients, and in 4 patients, the N category changed from pN1 to pN2. In the remaining 34 patients, additional sectioning did not change the pN category; in 18 patients, the criteria for a metastasis were not met, whereas in 16 patients, additional sectioning revealed a metastasis without altering the pN category.

3.3. Comparison of study data with the original pathology report

In the 59 lymph nodes included as ITCs in our study, the original pathology report was checked. In 17 of 59 lymph nodes (28.8%), the primary pathologist had classified the lymph nodes as containing ITCs. Eight lymph nodes (13.6%) were classified as metastasis, whereas the remaining 34 lymph nodes (57.6%) were described as nonmalignant. Additional levels had been cut in 19 of the cases, of which 15 were classified as ITCs and 4 as metastasis. In 6 cases with no additional levels, the primary pathologist had classified the lymph node as ITCs in 2 cases and as metastasis in 4 cases. The number of additional levels varied from 1 to 6. The primary pathologist had categorized the lymph nodes identical to our study classification in 4 of 7 cases (42.9%) with 1 additional level. Four of 8 cases (50%) with 3 additional levels, 2 cases with 4 levels, and 3 cases with 6 additional levels all were categorized identical to our classification.

4. Discussion

Obviously, the more careful lymph nodes are scrutinized, the higher the number of ITCs and metastases to be identified. Our study showed that ITCs were present on the primary H&E-stained slides in one third of the patients and 1.7% of the examined lymph nodes. Deeper sections revealed more extensive tumor involvement in almost half of the lymph nodes. According to the present guidelines, these lymph nodes now fulfilled the criteria of metastases. We found higher (y)pTN categories as well as poor differentiation to be significant risk factors for the presence of ITCs. One or more of these risk factors have also been identified as predictive for ITCs when sectioning primarily negative lymph nodes in several previously published studies [5,7,11-15]. Comparing the primary pathology report with our results revealed that in 57.6% of the lymph nodes classified as ITCs in our study, ITCs were not mentioned in the original pathology report. This might represent cases missed by the primary pathologist, although we cannot be certain about this. According to the TNM classification, the pathologist is not obliged to report the presence of ITCs because ITCs do not contribute to the pN category. When ITCs were identified on routine H&E-stained slides by the primary pathologist, additional levels were not always added, and some cases were wrongly classified as metastasis. Not unexpectedly, the higher the number of primary additional levels, the higher was the agreement with our study classification.

In several lymph nodes, we observed that more than 1 tumor cluster was present. If adding these separately located areas, the criteria for metastases would have been met for an even higher number of lymph nodes. Thus, our study might underestimate the number of metastases, but the present TNM classification does not mention addition of separate foci as an option. Moreover, with 0.2 mm between the levels, we are aware that a large part of the lymph node remains unexamined, representing a risk of missing even further metastases. In addition, we realize that the remaining lymph nodes negative for tumor cells on the primary slides are also at risk of hiding ITCs or a metastasis in deeper sections.

The present study differs from previously published studies concerning ITCs in EGJ which have focused on examination of lymph nodes primarily considered negative. Several researchers have correlated the presence of ITCs in deeper sections of negative nodes to survival. Comparison of studies

is difficult because different definitions of ITCs have continued to be used. Furthermore, the studies are heterogenous due to inclusion of a limited number of patients, sometimes consisting of both adeno- and squamous cell carcinoma, different localization of the primary tumor, and differences in pathological examination and surgical techniques. Although not clear cut, an overrepresentation of studies reporting ITCs to be a negative prognostic factor associated with increased risk of recurrence and decreased survival exists [5,7,12-14,16-19]. Furthermore, in some studies, the presence of ITCs was a negative prognostic factor in a subgroup of patients [20-23], whereas others found no difference in survival or disease-free survival [11,15,24,25]. A recently published systematic review article from our group reduced the heterogeneity by using very strict inclusion criteria only including studies using the definition of ITCs according to the TNM eighth edition [26]. The study found ITCs to be present in 20.3% of the patients, with only 5 studies [7,14,15,18,27] fulfilling the criteria, and although the data were not completely clear, ITCs seemed to be a negative prognostic factor [26].

A further challenge concerning ITCs and MMs is the reported low interobserver reproducibility when using the definitions by the TNM guidelines. Several former studies have been performed including patients with breast cancer [28,29]. A small improvement in agreement was reached in one study by refinement of the criteria with a more detailed description followed by reassessment of the cases [28], whereas the others showed that a training program combined with using only the size-based criteria resulted in very high agreement between 6 pathologists [29]. In our study, we used the size-based criterion, and although interobserver variability was not assessed, disagreement between the 2 pathologists was only observed in few cases. Still, the reported low interobserver agreement may possibly explain part of the controversy concerning the prognostic impact of ITCs.

In breast cancer, a higher number of large-scale both retro- and prospective studies investigating the impact of ITCs and MMs in sentinel nodes have been conducted with conflicting results. Although general agreement concerning the fact that serial sections and IHC staining of negative sentinel nodes reveal a higher number of both MMs and ITCs exists, the influence on long-term survival is unclear [30,31]. Sentinel node surgery allows for a more extensive examination of the lymph nodes at highest risk, and detailed guidelines on gross examination as well as histologic evaluation in breast cancer are available [30,31]. In patients with cancer of EGJ, sentinel node surgery is a debatable and technical challenging procedure, and skip lesions are a common phenomenon [32]. As the surgical procedure and the physiological and anatomical features of the EGJ are very different, data from breast cancer cannot be used as a basis for guidelines concerning EGJ cancer.

The definition of ITCs was made before neoadjuvant treatment was introduced as a standard treatment for many cancers. It can be speculated that the prognosis of patients with ITCs might be different in a treatment-naive patient compared with a patient with a metastasis having been downstaged to ITCs. Today, pre- or perioperative chemo- or chemoradiation is recommended for T3/T4 tumors with any node positivity and may also be considered for T2 tumors with positive nodes for adenocarcinomas in EGJ [33]. Neoadjuvant treatment leads to a spectrum of changes in the primary tumor as well as in the lymph nodes including reduction of the amount of tumor cells, cytological changes of the cells, necrosis, and fibrosis [34]. This results in further challenges in the histopathological assessment, as measuring dispersed tumor cells in a lymph node with fibrosis and inflammation is subject to some degree of uncertainty. In this aspect, the proposed additional TNM criterion of 200 tumor cells in breast cancer can be helpful. Although the number is statistically established, it is still an arbitrarily chosen cutoff value [30,31]. Furthermore, it might seem to be too high a number if used in adenocarcinoma of the EGJ. We observed that a cluster of tumor cells measuring 0.2 mm often consisted of much fewer than 200 tumor cells. In the present study, 86% of the patients had received the same standard treatment, although it was sometimes modified according to adverse effects. The observed histopathological changes in lymph nodes from patients who had received neoadjuvant therapy varied both between individual patients and between lymph nodes from the same patient. In some lymph nodes, no definite changes were observed, whereas in others, varying degrees of the abovementioned changes were seen.

Although most of the previous studies have been published >10 years ago, ITCs still lead to uncertainty in the daily routine, and lymph nodes containing ITCs are not assessed the same way nationally or internationally. Further guidance upon how to examine lymph nodes is necessary for stability in the TNM classification which is warranted to enable comparison of clinical studies concerning prognosis and treatment response. Although the present study was not designed to assess the impact of ITCs on survival, hopefully our results can be used as a basis for estimating the number of patients needed for future studies correlating ITCs to clinical outcome. Tumors classified as pN0 might be the most interesting to study, necessitating additional levels on all negative lymph nodes.

5. Conclusions

In conclusion, the presence of ITCs in regional lymph nodes is a common observation in patients with adenocarcinoma of the EGJ. ITCs often represent part of a real metastasis. To obtain a more precise pN category, we strongly recommend thorough examination of regional lymph nodes with additional levels when ITCs are observed. In daily practice with limited resources, it is not feasible to perform serial sections on all lymph nodes, but it is recommendable as a minimum in cases where a metastasis would lead to a change in the pN category.

Author contributions

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