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Histological study on pN upgrading of oral cancer

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Abstract The International Union Against Cancer (UICC) does not define the number of sections required from each regional lymph node to record pTNM classification. This study was designed to clarify the incidence of occult metastasis and to assess the pN upgrading of patients with oral cancer. Ultimately, this study led to a proposal for appropriate semiserial sectioning guidelines. Five hundred fifty-four nonmetastatic cervical lymph nodes taken from 73 patients with oral cancer were subjected to hematoxylin-eosin (HE) staining and keratin immunohistochemistry. Micrometastases, defined as foci ≤3 mm, were detected in 29 sites of 23 lymph nodes (4.2%) of 16 patients (21.9%). In 9 patients (12.3%) pN upgrading was needed: in 6 from pN0 to pN1, in 1 from pN0 to pN2b, and in 2 from pN1 to pN2b. The remaining 13 lymph nodes with occult metastasis were found in 5 pN2b and 2 pN2c patients, resulting in no pN upgrading. Occult metastasis was also detected in 6 small lymph nodes ≤5 mm in diameter. The average minor axis of the micrometastasis was 1.36±0.85 mm. We propose that the lymph nodes should be cut and examined at 1-mm intervals to detect micrometastatic foci and to evaluate the pN classification accurately.

Key words Oral cancer · pN upgrading · Immunohistochemistry · Micrometastasis · Semiserial sectioning

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Introduction

A significant prognostic factor for oral cancer survival after surgical resection appears to be the nodal status [23]. The 5-year survival rate of patients with multiple metastases is significantly low [14, 15]. Therefore, the number of metastatic lymph nodes involving the micrometastatic foci should be accurately evaluated from the surgical specimens.

Cancer cells invading lymphatic canals and drifting with the lymphatic current are attacked by the immune system, but the settled cells, having acquired immune tolerance in a lymph node, seem to proliferate vigorously. From this viewpoint, the diagnostic significance of the micrometastasis in a regional lymph node is different from the detection of circulating cancer cells in the blood vessel, most of which are likely to be killed and therefore not to proliferate in the lumen. „Micrometastasis“ is defined as a minute involvement in the lymph node that cannot be detected by radiologic and routine histological examination. It has two manifestations: a small metastatic cluster less than 2 [1, 3, 6] or 3 mm [20, 28–30] in diameter that can be seen by microscopy and an earlier stage comprised of a few cancer cells detectable only by means of a genetic diagnosis [5, 19, 21, 25]. Lymph nodes obtained from cancer patients are usually diagnosed as metastatic or nonmetastatic from one specimen sectioned on the greatest cut surface or from a couple of specimens. The diagnostic accuracy of this procedure is low. The genetic diagnosis of micrometastasis based on reverse transcriptase-polymerase chain reaction (RT-PCR) offers highly sensitive information. However, the method lacks morphological evidence and always involves a risk of false positives owing to illegitimate gene expression or contamination. In addition, ectopic salivary gland in the cervical lymph node [26] hampers reliability of the genetic diagnosis. Some authors have indeed reported abnormally high frequencies of occult metastases in oral cancer [18] and colorectal cancer [8], suggesting false positives.

The incidence and prognostic meaning of micrometastasis have been studied in various neoplasms: breast

[3, 20, 31], lung [11, 24], gastric [10, 17], colorectal [2, 4, 7] and esophageal cancer [6, 12]. Authors using semiserial sectioning or additional specimens with or without keratin immunostaining have reported micrometastasis in 3.6–5.8% of lymph nodes and 15.2–31% of patients [7, 17, 24]. Several authors have evaluated the diagnostic significance of this procedure and support the idea that the presence of micrometastases correlates with poor prognosis [7, 11, 17, 24]. In contrast, some authors feel that neither semiserial sectioning [6] nor subsidiary immunostaining [20, 31] is superior to the conventional routine procedure, since these methods did not contribute to the prognosis. To our knowledge, there are few reports on micrometastases of head and neck cancer [1, 28, 30]. In addition, the spacing of the semiserial sections varies among authors. Van den Brekel et al. [28] sectioned 600 nonmetastatic cervical lymph nodes at the deeper level of the paraffin block and demonstrated 7 additional micrometastases in 5 of 64 patients. However, this method is inadequate for detecting minute foci being harbored in the lymph node.

This study was conducted to determine the appropriate interval for semiserial sectioning and to clarify whether the increased number of specimens produces an effect on pN upgrading. We prepared semiserial sections of cervical lymph nodes at 200-µm intervals and in some cases at 50-µm intervals, to analyze size, location, and incidence of micrometastasis and their clinical relevance.

Materials and methods

The initial cohort, consisting of 639 cervical lymph nodes obtained from 73 patients with oral cancer (44 pN0, 8 pN1, 14 pN2b, and 7 pN2c), were fixed in 10% buffered formalin-embedded in paraffin. The site of the primary tumor was the gingiva in 28 cases; the tongue in 21 cases; the floor of the mouth in 11 cases; the buccal mucosa in 6 cases; the maxillary sinus in 6 cases, and the palate in 1. Since 85 lymph nodes showed overt metastasis at the original pathological examination, 554 lymph nodes were eligible for semiserial sectioning. One hundred twenty-five lymph nodes from 10 consecutive patients were sectioned at 50-µm intervals, and the remaining nodes were done at 200-µm intervals. Two continuous sections from every 50- or 200-µm interval were semiserially collected on slide glass. One semiserial series was used for hematoxylin-eosin (HE) staining and the other for immunohistochemistry (IHC). Thus, about 40–100 sections were prepared from each paraffin block. More than 30,000 sections were provided for the study. After deparaffinization, all specimens for IHC were pretreated two times with a microwave for 5 min to restore the cytokeratin antigenicity lost during fixation. A monoclonal antibody AE1/AE3 (Dako-Japan, Kyoto, Japan), a panspecific cocktail of antibodies for human keratins, was applied as a first antibody at a dilution of 1:50. The specimens were then immunostained by the streptavidin–biotin complex method and visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as described previously [22].

The micrometastatic foci ≤3 mm were measured with a micrometer on the subjective lens of a light microscope. The sizes among lymph nodes with micrometastasis (LNMM), lymph nodes with overt metastasis (LNOM), and nonmetastatic lymph nodes were statistically evaluated. Student's *t*-test was used for statistical analysis, and probability values ≤5% were considered significant. Frequency of the micrometastases was analyzed with its clinical relevance. The location of metastatic foci were classified into four levels according to the Japan Society for Head and Neck Cancer guidelines:

Level I: submandibular and submental nodes
Level II: superior internal jugular nodes and prelaryngeal nodes
Level III: mid- and inferior internal jugular nodes
Level IV: spinal accessory nodes

Results

When we prepared semiserial sections at 50-µm intervals, 6 micrometastatic foci were detected in 5 of 125 lymph nodes. All of the foci were observed in 8–22 continuous semiserial sections. Accordingly, we altered the slicing interval to 200 µm. In total, micrometastases were detected in 29 sites of 23 lymph nodes in 16 patients, corresponding to 4.2% of the lymph nodes examined and 21.9% of the patients. Twenty-two micrometastatic foci (75.9%) were located in the marginal sinus, whereas 7 (24.1%) were observed in the medullary sinus of the lymph node. Of 23 LNMM, 10 were detected in 9 patients showing pN upgrading from pN0 to pN1 in 6 patients and to pN2b in 1 patient, and from pN1 to pN2b in 2 patients (Table 1). In contrast, 13 LNMM were located in ipsilateral lymph nodes of 5 pN2b and 2 pN2c patients, resulting in no pN upgrading. Two lymph nodes had three disconnected small foci and 2 had two foci. The mode of invasion of the primary tumor with lymph node micrometastasis was 3 grade II, 10 grade III, and 3 grade IV according to Jakobsson's classification [13]. The degree of differentiation was not associated with occult metastases, since they were seen in 7 of 45 well-differentiated, 8 of 19 moderately differentiated, and 1 of 9 poorly differentiated SCC. They were often located in the upper neck lymph nodes, i.e., 11 level I, 7 level II, 4 level III, and 1 level IV, showing a distribution similar to that of overt metastases (Table 2). A skip lesion, defined as a direct occult metastasis to level III or IV, was seen only in 1 patient with tongue cancer, who had a LNMM in level III. The outcome of 7 patients with pN upgrading from pN0 and the remaining 37 pN0 patients were compared statistically. Four patients with pN upgrading died of recurrence or distant metastasis, showing a higher incidence of recurrence or metastasis (57.1%) than in the pN0 group (13.5%) (P=0.02, Fisher's exact test).

No occult metastases were detected in our review of the original histological sections, and their major axis ranged from 80 µm to 3 mm. All the average major axes, minor axes, and geometric means of the micrometastatic foci were more than 1 mm: 1.36±0.85 mm, 1.17±0.86 mm, 1.28±0.82 mm, respectively. These three parameters of the LNOM were significantly greater than those of lymph nodes without metastases (P<0.01; Table 3). The minor axis and the geometric mean were significantly different between LNOM and LNMM (P=0.05). Early micrometastatic foci ≤0.5 mm were seen in 6 lymph nodes. Micrometastases were detected in 6 small lymph nodes ≤5 mm: 3, 4×3-mm; 1, 4×4-mm, 1, 5×4-mm, and 1, 3×3-mm node. Conversely, no occult metastasis was recognized in 20 of 38 enlarged lymph nodes measuring more than 15 mm in the major axis. These 20 large

Table 1 Summary of the patients with micrometastasis (*MM* micrometastasis, *DR* died of recurrence, *DM* died of distant metastasis)

No.	Primary site	No. of overt metastases	No. of MM	Original pN	Upgraded pN	Degree of differentiation[a]	Major axis of MM (mm)	Outcome
1	Maxillary sinus	0	1	0	1	G1	0.3	DR
2	Gingiva	0	1	0	1	G1	0.4	DR
3	Gingiva	0	1	0	1	G1	1.2	Died of other disease
4	Gingiva	0	1	0	1	G1	0.75	Alive
5	Tongue	0	1	0	1	G2	2.8	DM
6	Gingiva	0	1	0	1	G3	1.35	DR
7	Tongue	0	2	0	2b	G2	0.08, 0.75	Alive
8	Maxillary sinus	1	1	1	2b	G1	2.25; 0.5 (2 foci in a node)	DM
9	Tongue	1	1	1	2b	G2	2.85	DM
10	Tongue	5	1	2b	2b	G1	1.25	DR
11	Gingiva	2	1	2b	2b	G2	0.35, 1.4, 2.25 (3 foci in a node)	Alive
12	Tongue	2	1	2b	2b	G2	2.2	DM
13	Gingiva	2	2	2b	2b	G2	2.07, 1.1 (2 foci in a node); 2.35	DR
14	Tongue	3	3	2b	2b	G2	1; 2; 2	Alive
15	Tongue	2	2	2c	2c	G2	1, 0.6, 0.8 (3 foci in a node); 3	Alive
16	Tongue	3	3	2c	2c	G1	0.5; 1.5; 0.8 29 foci in 23LNMM	DR

[a]According to UICC

Table 2 Level of metastases in the cervical lymph nodes

Level[a]	Overt metastasis	Micrometastasis
I	45 (52.9%)	11 (47.8%)
II	23 (27.1%)	7 (30.4%)
III	14 (16.5%)	4 (17.4%)
IV	3 (3.5%)	1 (4.4%)
	85 (100%)	23 (100%)

[a]According to Japan Society for Head and Neck Cancer

lymph nodes without metastasis often showed the pathological feature of lymphadenitis or fusion of 2 nodes. Ten of them were located in level II, 5 in level I, 4 in level III, and 1 in level IV.

Twenty-six LNMM and 27 LNMM were detected on HE staining sections and on keratin immunostaining sections, respectively. The additional LNMM confirmed by immunohistochemistry showed the end of a small cluster that might have been lost on the HE specimen. All of the LNMM showed a cluster of squamous carcinoma cells linked to each other by desmosomes. Scattered single cancer cells frequently reported in adenocarcinoma of the other organs were observed occasionally at the periphery of the cluster (Fig. 1).

The cervical lymph nodes showed various structural modifications with low-power magnification. These included lymph nodes involving adipose tissue or ectopic salivary gland, lymph nodes with enlarged hilus, and assembly of the small lymph nodes. The ectopic salivary gland, mainly the parotid gland, was involved in 9 of the 554 lymph nodes examined. Furthermore, various degrees of pathological variations, such as sinus histiocytosis, follicular hyperplasia, congestion, and stromal fibrous change, were noted, regardless of the nodal size. Micrometastases were rarely detected in the lymph nodes with sinus histiocytosis and/or follicular hyperplasia.

Table 3 Size of lymph nodes (*LNOM* lymph node with overt metastasis, *LNMM* lymph node with micrometastasis)

	No.	Major axis (mm)	Minor axis (mm)	Geometric mean (mm)
Lymph node without metastasis	531	7.93±3.51	4.65±1.87	5.99±2.33
LNOM	85	13.25±4.59	9.11±3.83	10.87±3.87
LNMM	23	9.15±2.87	5.70±1.94	7.17±2.23

**P*=0.05; ***P*<0.01

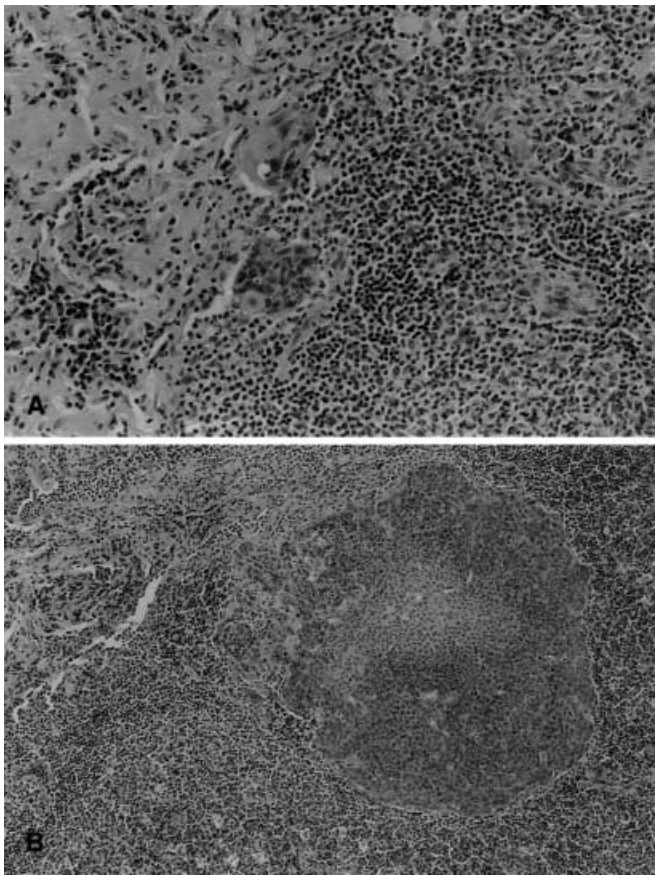

Fig. 1 **A** Two small clusters, 80× 60 μm and 90×75 μm in size, located at the end of the micrometastasis shown in **B**. HE, original magnification ×50 **B** A representative round micrometastasis measuring 750×650 μm in its maximal diameters was detected at the deeper level. Tumor cells were observed in 13 semiserial sections with 200-μm intervals. HE, original magnification ×25

Discussion

The incidence of occult metastases revealed by histological study depends on a sectioning interval of 10 [1], 100 [30], 150 [20], 200 μm [6], or 3–4 levels from a lymph node [3, 24] in the previous studies. As a matter of course, the smaller the interval is, the more sensitive the detection. True serial sectioning, however, is very time-consuming and impractical. If our series were to be cut in 10-μm intervals, more than 600,000 sections would be required on calculation. We began by preparing semiserial sections with 50-μm intervals, which was also cumbersome, and obtained the aforementioned result. A slicing interval of 200 μm is not small, but is appropriate when the size of oral cancer micrometastases and the extreme effort in the laboratory are considered.

Semiserial sectioning in our study detected micrometastases in 4.2% of the lymph nodes examined and in 21.9% of the patients. Of 44 pN0 patients, pN upgrading to pN1 or pN2b was confirmed in 15.9%. This method may be of importance because it concerns a considerable number of patients who would probably benefit from postoperative adjuvant chemotherapy [9, 16]. The detection rate was comparable to or lower than those found in similar studies on regional lymph nodes of breast cancer or colorectal cancer. This is probably due to the differing cell characteristics between squamous cell carcinoma and adenocarcinoma. Invasion and metastasis depend on cell characteristics, including the mode of invasion. As mentioned in Results, 13 of 16 tumors with micrometastasis indeed presented invasive histological features.

Lymph nodes more than 10 mm in diameter are often regarded as suggestive of metastasis, as supported by the evidence that LNOM is significantly larger than nonmetastatic lymph node in our study. On the other hand, most LNMM showed the normal node size and structure without the inflammatory changes that are frequently observed in the upper neck. This may suggest that inflammatory cytokines, such as IL 1, 2, 6, and activated macrophages may inhibit adhesion and proliferation of early cancer cells. Indeed, 20 enlarged lymph nodes more than 15 mm in diameter showed no microinvolvement. Accordingly, a lymph node more than 15 mm in diameter without positive findings on computed tomography (CT) or magnetic resonance imaging (MRI) may ordinarily be considered to be affected by inflammatory hyperplasia.

Keratin immunostaining showed no significant diagnostic superiority to HE staining in our study. We think that it results from meticulous observation of the HE specimens and from the squamous feature of oral cancer. Van den Brekel et al. [28] reported that four additional micrometastases were detected by keratin immunostaining using the same antibody. However, on revision of the original HE slides all micrometastases were confirmed; they had simply been overlooked. The time-consuming keratin staining did not offer any benefit over conventional HE staining, but facilitated detection of a couple of scattered cancer cells in their series. In our study, a discernable nest always appeared on the next section to that with the sparse tumor cells that might be overlooked. From our observations, it is confirmed that a morphological feature of the micrometastasis in oral cancer is to form a small assembly in the marginal or medullary sinus, except for the undifferentiated type. We think that a small cluster more than 25 μm in diameter with squamous feature and keratinization could be diagnosed as micrometastatic by careful observation of HE staining.

It is of great concern whether LNMM significantly influences the outcome of patients. Glickman et al. [6] did not recommend semiserial sectioning for keratin immunostaining, since occult metastasis was not an independent factor in poor prognosis in esophageal adenocarcinoma or squamous cell carcinoma. In contrast, other authors noted that occult metastasis was statistically associated with decreased recurrence-free and overall survival [11, 12]. Similar arguments over the prognostic significance have been reported in the case of other organs. Nasser et al. [20] presented an article in which they evaluated axillary LNMM in 159 node-negative patients with invasive breast cancer. LNMM were detected in 50 patients, and 22 of these consisted mostly of single cells or

very small clusters detected by immunostaining. The patients were divided into two groups by the size of the micrometastasis: smaller than 0.2 mm and greater than 0.2 mm. Nasser et al. concluded that the former group did not present useful prognostic information, while the latter group presented significant information on prognosis.

Woolgar [30] noted detailed topography of cervical lymph node metastases in a series of neck dissections from 154 patients with oral SCC. She classified patients into five categories on the basis of the presence and extent of extracapsular spread (ECS) and the size of the intranodal metastatic deposit for assessment of the prognostic importance. The author concluded that survival was similar (75%) for patients with either macroscopic or microscopic metastases confined to lymph nodes or no evidence of metastasis, though larger nodal involvement with ECS showed poor outcome. From the articles reviewed, it seems likely that very early occult metastasis consisting of single cancer cells or of a minute cluster may not be available as a prognostic factor.

Untreated micrometastases are expected to proliferate gradually and be detected sooner or later as overt metastases. Although the sample size of patients with pN upgrading in our study was small, such patients had a significantly poor outcome. We sometimes encounter recurrence and distant metastasis in node-negative patients with oral cancer after a curative resection. Occult metastasis may be overlooked in such cases, as it is regarded as early evidence [17]. In addition, multiple occult metastases in pN0 patients support biologic metastatic activity of cancer cells. Therefore, it is important to assess them, regardless of the prognostic significance.

Postoperative evaluation of micrometases does not affect the method of neck dissection, although it may benefit postoperative chemoradiotherapy. It is impossible at the moment to detect micrometastases before surgery. There are attempts in progress to use CT and MRI for the detection not only of metastases, but also of micrometastases. In general, CT is superior in the detection of a small lymph node, even though it is difficult to analyze the internal features. Wide et al. [29] discussed whether MRI is available for the assessment of the cervical LNMM. They reported that MRI detected two of five LNMM, even though there was a false-negative rate of 20.9% and false-positive rate of 47.1%, and concluded that MRI lacked sufficient sensitivity and specificity to replace elective neck dissection both for staging and for assessment of prognosis. Magnetic resonance imaging is, indeed, superior to CT with respect to its spatial resolution, depending on the condition of its field of view (FOV) and the contrast. It is, however, difficult to detect the micrometastases accurately by means of image diagnosis, since the spatial resolution is not good enough to demonstrate them and cervical lymph nodes show morphological pleomorphism including adipose tissue or salivary glands. A new development, such as an improved CT utilizing a new enhancer material exclusively taken into the cancer cells, is anticipated for the preoperative detection of the micrometastases.

There are two treatments for the N0 neck. One is prophylactic neck dissection, and another is a concept of „watch and wait." The 5-year survival rate does not differ significantly between these two groups. In the latter group, however, the timing of the neck dissection may be delayed, and some patients reject the second operation on the neck after resection of the primary tumor. The pN upgrading was correlated with the mode of invasion; therefore, the prophylactic neck dissection is recommended, especially for primary tumors with highly invasive characteristics. Our study presents the histological evidence to justify the supraomohyoid neck dissection as a prophylactic procedure, since the LNMM were concentrated in the upper neck and showed no skip lesions to level IV.

Finally, the International Union Against Cancer (UICC) defines pTNM pathological classification in which at least 6 lymph nodes should ordinarily be included for a selective neck dissection and 10 for a radical or modified radical neck dissection to give a diagnosis of pN classification [27]. The number of sections from each lymph node, however, is not defined by the UICC. Semiserial sections with 200-μm intervals in the present study did indeed provide detailed information, but this interval is impractical for a daily examination in the clinical laboratory. Based on the observed average size of micrometastases and their cluster-forming characteristics, we propose that the cervical lymph nodes should be cut with 1-mm intervals for accurate pN grading. The length of a jugulodiagastric lymph node occasionally reaches 20 mm, but its thickness is usually less than 10 mm. Three to ten sections can be mounted on a slide glass. Accordingly, it is feasible to prepare the proposed number of specimens routinely.

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