Clinical significance of cytokeratin in the cervical lymph nodes of patients with mandibular gingival squamous cell carcinoma

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Abstract. The present study aims to analyze the expression of broad spectrum cytokeratin in the cervical lymph nodes of patients with mandibular gingival squamous cell carcinoma and explore the metastasis of mandibular gingival squamous cell carcinoma in cervical lymph nodes. This study included 42 patients with mandibular gingival squamous cell carcinoma, which was staged according to the clinical staging criteria by International Union Against Cancer 2002 (UICC) and the Level staging method of cervical lymph node by American Academy of Otolaryngology-Head and Neck Surgery 1991. Monoclonal mouse anti-human cytokeratin (AE1/AE3) antibody was used in immunohistochemical examination and hematoxylin and eosin (H&E) staining. All positive sections by H&E staining were also positive by immunohistochemistry (IHC). The positive rate of routine H&E staining and serial-section H&E staining was 8.03 and 9.57%, respectively, the positive rate of IHC was 12.82%. The positive rate of IHC was significantly different with that of routine H&E staining (χ^2=7.17, P<0.01), yet not significantly different with that of serial-section H&E staining (χ^2=3.10, P>0.05). Lymph node metastasis was mainly in Level I, II and III, both serial-section H&E staining and IHC showed lymph node metastasis in Level IV for advanced patients. IHC showed 19 lymph node micrometastasis in 12 patients, while neither serial-section nor routine H&E staining showed micrometastasis. Lymph node dissection of hyoid bone (mainly in Level I, II and III) could be used for early patients, and the dissection could be expanded to Level IV for advanced patients.

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Introduction

Malignant tumors pose great threats to human health and life. Global governments, universities, pharmaceuticals companies and charities have spent huge money in the study of malignant tumors. With the development of surgical technology, many adjuvant therapies have been applied clinically. Yet, unfortunately, the cure rate and survival of patients with malignant tumors have not been improved significantly. It has been recognized that the metastasis and relapse due to micrometastasis is a major reason of mortality caused by malignant tumors. Micrometastasis is important for the diagnosis, clinical staging and treatment of tumors, and the key factor for the prognosis of tumors. Appropriate assessment of the micrometastasis in lymph nodes is very important in tumor surgery (1,2). Recently, cytokeratin has gained increasing interest in the detection of micrometastasis in lymph nodes. In the present study, we performed routine pathological examination, serial-section hematoxylin and eosin (H&E) staining and IHC against cytokeratin (AE1/AE3) to detect the metastasis in 585 cervical lymph nodes from 42 patients with mandibular gingival carcinoma, the detection rates of these methods were compared. The clinical significance of metastasis of mandibular gingival carcinoma in cervical lymph nodes was explored.

Patients and methods

Clinical information. Forty-two patients with mandibular gingival carcinoma were included from the Oral and Maxillofacial Surgery in The Affiliated Hospital of Qingdao University Medical College (Qingdao, China) and Hangzhou First People's Hospital (Hangzhou, China) between July 2009 to December 2012, including 27 male and 15 female with a mean age of 54.1 years (27-77 years). This study was approved by the Ethics Committee of Hangzhou First People's Hospital. Signed written informed consents were obtained from all participants before the study. All patients underwent primary combined dissection of gingival, mandible and neck, no patient underwent radiotherapy or chemotherapy before surgery. Mandibular gingival squamous cell carcinoma were confirmed for all patients by histopathological examination after surgery.

Table I. The distribution and metastasis of mandibular gingival squamous cell carcinoma in cervical lymph nodes by routine pathological examination.

	Distribution in cervical lymph node									
	Level I		Level II		Level III		Level IV		Level V	
Stage/No.	P	N	P	N	P	N	P	N	P	N
T1/9	1	20	1	19	1	17	0	14	0	12
T2/16	5	46	2	48	1	50	0	38	0	18
T3/6	4	34	6	18	2	20	0	24	0	5
T4/11	12	43	7	28	5	32	0	35	0	17
In total	22	143	16	113	9	119	0	111	0	52
Metastatic rate (%)	13.33		12.4		7.03		0		0	

N, negative; P, positive.

Forty-two patients were staged according to the International Union against Cancer (UICC) Criteria 2002, i.e., T1 stage (9), T2 (16), T3 (6) and T4 (11). Cervical lymph nodes were collected following Haagensen's method, then were numbered and sent for pathological examination. In total, 585 cervical lymph nodes were obtained. Routine pathological examination and H&E staining showed 47 positive lymph nodes and 438 negative lymph nodes. 165 lymph nodes were in Level I with 22 micrometastasis (13.33%), 129 lymph nodes were in Level II with 16 micrometastasis (12.40%), 128 lymph nodes were in Level III with 9 micrometastasis (7.03%), 163 lymph nodes were in Level IV and V without any micrometastasis (Table I).

Methods. A total of 585 formalin-fixed, paraffin-embedded (FFPE) tissue specimens were sectioned at 1 mm intervals using a microtome to produce 2 serial sections (4 μm thick, one section for H&E staining, the other section for IHC). These sections were observed under under light microscope (BX-42; Olympus, Tokyo, Japan) to determine the percentage of metastasis of mandibular gingival squamous cell carcinoma in cervical lymph nodes.

Instruments and reagents. Automatic tissue dehydration machine (TP1020; Shandon Pathcentre, Cheshire, UK); Tissue embedding machine (BMJ-111; Shandon Pathcentre); H&E staining kit (Beijing Reagan Biological Technology Co., Ltd., Beijing, China); monoclonal mouse anti-human cytokeratin antibody (AE1/AE3) (1:100; cat. no. BM0034) was purchased from Merck KGaA (Darmstadt, Germany).

Procedures

H&E staining. Firstly, the sections were deparaffinized in xylene and were gradually hydrated through graded alcohol, i.e., 100, 95, 80 and 70% ethanol, respectively. Secondly, the sections were stained in hematoxylin solution and differentiated in 1% hydrochloric alcohol, then were rinsed with tap water and with distilled water until the nuclei becoming blue, then was dehydrated in 95% ethanol. Thirdly, the sections were counterstained in 1% eosin solution, washed with 70% ethanol twice and absolute ethanol, and then were cleared in 2 changes of xylene. Lastly, the sections were mounted with neutral balsam and observed under microscopy (BX-42; Olympus).

IHC. Firstly, the sections were deparaffinized in xylene and gradually hydrated through graded alcohol to water. Secondly, the sections were immersed in H_2O_2 and heated in a microwave oven, washed with phosphate-buffered saline (PBS; pH 7.4) and immerse in citrate buffer solution (pH 6.0). Thirdly, the sections were blocked in nonimmune serum, stained with primary antibody (AE1/AE3) and then secondary rabbit anti-mouse (HRP) IgG antibody (1:1000; cat no. ab6728; Abcam, Cambridge, MA, USA). The sections were incubated with SP (streptavidin-perosidase) and then freshly prepared DAB solution for color development. Lastly, the sections were counterstained with hematoxylin, cleared in water, mounted with neutral balsam and observed under microscopy (BX42; Olympus).

Interpretation of the results. The results of pathological and immunohistochemical examinations was evaluated by 2 senior pathologists, who were blind to the location of the lymph nodes and the patients' diagnosis before the examination. In IHC, lymph node metastasis was determined by: The staining of AE1/AE3 was positive in carcinoma cells; cytokeratin was expressed in the cytoplasm rather than in the nucleus and the expression of cytokeratin was heterogeneous; the intrinsic components in lymph nodes were negative; positive lymph nodes included those lymph nodes with micrometastasis and isolated carcinoma cells. Lymph node micrometastasis were defined by that the size of the metastasis lesion was <2 mm, the carcinoma cells were confined in the lymph nodes, whose structures were not significantly destroyed. In H&E staining, lymph node metastasis was determined by: Deformation and destruction of lymph sinuses, abnormal lymphatic structure, anomalous cells with the nucleus larger than lymphocytes.

Statistical analysis. All statistical analysis was performed by Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS, Inc., Chicago, IL, USA). Comparison between groups was done using one-way ANOVA test followed by post hoc test (Least Significant Difference). $P<0.05$ was considered as significant difference.

Table II. The distribution and metastasis in cervical lymph nodes by routine H&E staining, serial-section H&E staining and IHC.

		Routine H&E staining		Serial-section H&E staining		IHC	
Levels	No.	Positive	(%)	Positive	(%)	Positive	(%)
I	165	22	13.33	22+2	14.54	22+9	18.78
II	129	16	12.4	16+3	14.72	16+7	17.82
III	128	9	7.03	9+3	9.37	9+8	13.28
IV	111	0	0	+1	0.90	+4	3.60
V	520	0	0	0	0	0	
Total	585	47	8.03	47+9	9.57	47+28	12.82

IHC, immunohistochemistry; H&E, hematoxylin and eosin.

Table III. The metastasis in cervical lymph nodes by routine H&E staining, serial-section H&E staining and IHC.

		Routine H&E staining		Serial-section H&E staining		IHC	
Staging	No.	Metastasis	(%)	Metastasis	(%)	Metastasis	(%)
T1	9	2	22.22	2	22.22	2+1	33.33
T2	16	5	31.25	5+1	37.5	5+2	43.75
T3	6	3	50	3	50	3+1	66.66
T4	11	5	45.45	5+1	54.54	5+3	72.72
Total	42	15	35.71	15+2	40.47	15+7	52.38

IHC, immunohistochemistry.

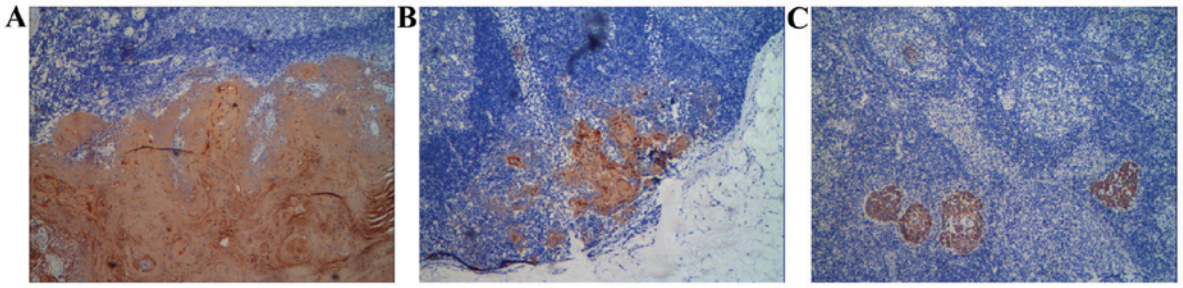

Figure 1. Representative of lymph node micrometastasis by IHC. (A) Patchy lymph node metastasis (AE1/AE3 positive) (magnification, x40). (B) Nest-like lymph node micrometastasis and subcapsular micrometastasis (2 mm) (magnification, x40). (C) Disseminated nest-like lymph node micrometastasis (AE1/AE3 positive). IHC, immunohistochemistry.

Results

All sections that were positive in H&E staining were also positive in IHC of AE1/AE3 (Table II). The serial sections with positive H&E staining (n=56, 9.57%) were more than those with positive H&E staining (n=47, 8.03%). One lymph node in Level IV (T4), 1 lymph node (T2) and 1 lymph node (T3) were negative in routine H&E staining. However, serial-section H&E staining showed 1 and 2 lymph node metastasis in Level I and II, respectively, without any micrometastasis (Fig. 1).

IHC detected 75 positive lymph nodes (n=75/585, 12.82%) which were more than those detected by routine H&E staining (n=47/585, 8.03%) or those detected by serial-section H&E staining (n=56/585, 9.57%) (Table II). Four lymph nodes were in Level IV (2 at T4 and 2 at T3). Seven sections were negative by routine H&E staining, however, IHC showed 12 positive lymph nodes in these sections, including 7 from 4 sections (T1), 3 from 2 (T2) and 2 from 1 section (T3). In total, 19 lymph node micrometastasis from 13 patients were shown. The percentages of lymph node micrometastasis between serial-section H&E staining (56/585, 9.57%) and routine H&E staining (47/585, 8.03%, χ^2=0.86, P>0.05) or IHC (75/585, 12.82%, χ^2=3.10, P>0.05) were not significantly different, however, the percentages of lymph node micrometastasis

between routine H&E staining and IHC were significantly different (χ^2=7.17, P<0.01).

The percentages of metastasis in cervical lymph nodes detected by routine H&E staining, serial-section H&E staining and IHC were 35.71, 40.47 and 52.38%, respectively, the difference among 3 methods was not significant (χ^2=2.53, P>0.05) (Table III). Two positive cervical lymph nodes detected by serial-section H&E staining and 7 positive cervical lymph nodes detected by IHC were negative by routine H&E staining, these were considered as missed diagnosis.

Discussion

Metastasis in lymph nodes was an important factor for the prognosis of head and neck squamous cell carcinoma. Both the local control rate and survival of the patients with metastasis in lymph nodes were 50.0% less than those without metastasis in lymph nodes (3,4). The incidence of metastasis in lymph nodes was closely associated with the location, seize, type, degree of pathological differentiation and infiltration depth of the primary lesion.

Infiltration and metastasis are important biological characteristics of malignant tumors, and main reasons of death. Control or elimination of malignant tumors and minimization of dysfunction are the goal of medical scientists and practitioners. Thus, precise assessment of primary lesion, early diagnosis of metastasis in lymph nodes and elucidation of the micrometastasis pattern were beneficial for the treatment and prognosis of tumor patients (5,6).

Gingival carcinoma is a common malignant tumor in head and neck. It is highly invasive and can infiltrate into the jaw bones and adjacent nerves, muscles, vessels and skin directly, and can transfer to cervical lymph nodes through lymphatic system, leading to high mortality (3,6,7).

The diagnosis of lymph node metastasis is confirmed by pathological examinations, which include frozen section procedure, routine H&E staining, special staining, IHC, reverse transcription polymerase chain reaction (RT-PCR) and serial-section H&E staining. Routine H&E staining is most commonly used clinically.

In the present study, 585 cervical lymph nodes from 42 patients with mandibular gingival squamous cell carcinoma were collected. Routine pathological examination showed 47 lymph node metastasis (8.03%), all in Level I, II and III. Serial-section H&E staining showed 56 lymph node metastasis (9.57%), which were not significantly different from those by routine pathological examination (χ^2=0.86, P>0.05), 1 lymph node (T4) in Level IV, 1 lymph node (T2) and 1 lymph node (T3) were negative in routine H&E staining, however, serial-section H&E staining showed 1 and 2 lymph node metastasis in Level I and Level II, respectively. These results demonstrated that serial-section H&E staining was more accurate than routine H&E staining, especially serial-section H&E staining detected lymph node metastasis in Level IV, indicating that it was inappropriate for lymph node dissection to be limited to the Level I, II and III of hyoid bone in patients with mandibular gingival carcinoma, for T4 patients, the lymph node dissection of hyoid bone should include Level IV.

With the development of immunology and molecular biology, the studies in tumor markers have been increasing in order to provide accurate diagnosis, treatment and prognosis (6,8-10). Cytokeratin is an important tumor marker of epithelium origin, it had been investigated in the studies of many epithelium derived malignant tumors (11,12). Cytokeratin is extensively distributed in epithelium and an intermediate filament in the dynamic network structure of cytoskeleton. It can maintain cellular structure and functions, and play key roles in cell differentiation, proliferation and nuclear change. Cytokeratin is a highly conservative protein. When neoplasia or canceration occurs in epithelial tissues, cytokeratin remains in epithelial tumor and migrate with tumor cells, and the expressed type of cytokeratin is still the same with that in normal tissues (13,14), therefore, cytokeratin is of unique value in the identification of epithelial tissues and provides evidence for the detection of lymph node metastasis by anti-cytokeratin antibody. Epithelial component is not present in normal lymph nodes, the expression of cytokeratin can only be possible in the case of lymph node metastasis of squamous cell carcinoma, thus cytokeratin can be used to determine metastasis (15-17).

In the present study, monoclonal mouse anti-human cytokeratin antibody (AE1/AE3) was used in IHC to label keratinized epithelium, stratified squamous epithelium, stratified epithelium, hyperplastic keratinized epithelium and simple epithelium. The cytoplasm and cell membrane of epithelium-derived carcinoma cells were brown, which was easy to be identified, this overcame the limitation of diagnosis only by cell morphology in routine H&E staining. This study showed that metastasis lymph nodes were mainly distributed in Level I, II and III, only a few metastasis lymph nodes in T3 and T4 patients were in Level IV. IHC detected 75 lymph node metastasis, the detection rate (12.82%) was significantly higher than those by routine H&E staining and serial-section H&E staining. The detection rate of routine H&E staining was not significantly different with serial-section H&E staining, yet was significantly different with IHC (χ^2=7.17, P<0.01). Notably, IHC identified micrometastasis or isolated tumor cells in 19 lymph nodes that were negative in routine H&E staining, and IHC showed metastasis in 5 out of 19 lymph nodes in which routine H&E staining did not.

Tumor micrometastasis usually occurs in the progress of tumor, the disseminated tumor cells in blood, lymph tract, bone marrow and organs do not form metastatic nodule or present any clinical manifestation, thus cannot be detected by routine pathological or imaging examinations, however, these tumor cells can grow rapidly to form recurrence and metastasis lesions (18). Micrometastasis typically refers to metastasis with a maximum diameter <2 mm. International Union Against Cancer (UICC) defines the metastasis <0.2 mm as isolated tumor cells or clusters, and the lesion >0.2 and <2.0 mm as micrometastasis. Many experts consider micrometastasis as an important factor for prognosis and selecting surgery (19,20). This study demonstrated that IHC could detect micrometastasis that was not detected by H&E staining, this indicated that IHC was more sensitive than H&E staining and could reduce missed diagnosis of lymph node metastasis, thus could be complementary to routine H&E staining.

Cervical lymph node dissection is an indispensable treatment of gingival carcinoma and can decrease the mortality of gingival carcinoma significantly (21). The selection of cervical lymph node dissection is based on the characteristics of lymph node metastasis. Previous studies showed that the metastasis of gingival carcinoma in cervical lymph nodes was mainly

distributed in Level I, II and III, and lymph node dissection of hyoid bone could achieve eliminate the metastasis (22,23). This was consistent with the present study, yet IHC showed metastasis in Level IV in advanced patients.

In conclusion, this study showed that the metastasis of mandibular gingival carcinoma in cervical lymph nodes was mainly distributed in Level I, II and III, and also in IV in advanced patients. Lymph node dissection of hyoid bone could be used for early patients undergoing combined dissection of gingival, mandible and neck, and the dissection could be expanded to Level IV for advanced patients. IHC was more sensitive for the detection of metastasis or micrometastasis in cervical lymph nodes than H&E staining, thus was of important clinical value.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

QD and MX designed the study and performed the experiments, XZ and MJ collected the patient data, RY and MG analyzed the patent data, QD and MX prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Hangzhou First People's Hospital (Hangzhou, China). Signed written informed consents were obtained from all participants before the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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