

High MMP-21 expression in metastatic lymph nodes predicts unfavorable overall survival for oral squamous cell carcinoma patients with lymphatic metastasis

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Abstract. The aim of the present study was to examine the clinical significance of lymph node metastatic (LNM) foci in predicting the overall survival of oral squamous cell carcinoma (OSCC) patients with LNM. MMP-21 was screened based on the LNM animal model of OSCC. Then four proteins, matrix metalloproteinase (MMP)-2, MMP-21, vascular endothelial growth factor (VEGF)-C and VEGF receptor (VEGFR)-3 were examined by immunohistochemistry in 63 OSCC specimens, including the primary tumors (PTs) and the corresponding LNM foci. The expression levels between the PTs and LNM foci were compared by Wilcoxon paired test. Relationships between expression of the four proteins and patient overall survival were assessed by Kaplan-Meier based on the median of the labeling index. The Cox proportional hazards model was used to assess the relative hazard factors. MMP-21 and VEGF-C expression levels were higher in the LNM foci than levels in the PTs. Results showed that MMP-2 and VEGF-C expression levels in the PTs and MMP-2, MMP-21 and VEGF-C expression in the LNM foci correlated with the overall survival of the OSCC patients with lymphatic metastasis. MMP-21 expression level in the LNM foci was the most reliable predictor among all the tested factors. These results suggest that high MMP-21 expression in LNM foci can be used to predict survival in OSCC patients with LNM. Characteristics of LNM foci may be more reliable than PT characteristics in predicting the overall survival of OSCC patients with lymphatic metastasis.

Introduction

Despite preventative and therapeutic advances, the 5-year survival outcome of oral cancer patients still remains at 50% with no significant improvement over the past several decades (1). This is mainly due to the high metastatic rates to cervical lymph nodes (2,3), particularly in oral squamous cell carcinoma (OSCC), which accounts for more than 90% of all oral cancer cases (4). In addition, nearly 45% of OSCC patients present with lymph node metastasis (LNM) (5) and their overall survival decreases to 28% (6).

Lymphatic metastasis progresses insidiously. Migration and invasion are essential stages underlying metastasis (7). Matrix metalloproteinases (MMPs) promote tumor cell migration and invasion to normal tissues by destructing the extracellular matrix and by influencing the tumor microenvironment (8). Among the MMP family members, MMP-2 is closely related to the development and progression of tumors (9). Human MMP-21, which was expressed higher in LNM foci than that in the primary tumor (PTs) in the lymphatic metastasis animal model of OSCC established in the present study, is a new member of the MMP family (10). MMP-21 expression has been associated with embryogenesis and tumor progression (11). MMP-21 has been reported to be related to cell differentiation such that its high expression has been suggested as a marker of highly differentiated pancreatic cancer cells (12). In gastric (13) and colorectal cancer (14) patients, high MMP-21 expression has been associated with poor overall survival. In esophageal squamous cell cancer, MMP-21 expression has been associated with tumor invasion, inflammation, apoptosis and highly differentiated cells (15). VEGF-C and VEGFR-3 have also been closely correlated with tumor progression and LNM (16).

Currently, the PT is considered important in investigating lymphatic metastasis and predicting the outcome of cancer patients whereas metastatic foci are ignored. The present study is the first to investigate LNM foci in assessing the prognosis of OSCC patients with LNM.

Materials and methods

Cells and cell culture. CAL-27 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco)

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supplemented with 10% fetal bovine serum (FBS; HyClone) in a humidified 5% CO₂ incubator at 37°C. Cells in mid-logarithmic growth (~75% confluence) were used for the following experiments.

Establishment of the LNM animal model. The present study was approved by the Medical Ethics Committee of the Peking University School and Hospital of Stomatology. Six-week-old male BALB/c nude mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were placed under general anesthesia with 1% pentobarbital sodium (Sigma). CAL-27 cells (5×10⁶) were injected into the tongue of the nude mice. After 40 days, the PTs and LNM foci were dissected to generate tumor cells named CAL-27-PT and CAL-27-LNM, respectively via primary culture. Both of the two types of cells were identified by short tandem repeat (STR) profiling. DNA was extracted from CAL-27-PT and CAL-27-LNM cells using STR profiling. PCR was performed in the ABI 3100 genetic analyzer machine with the use of 10 ng of DNA as a template and the Goldeneye TM16C STR detection kit (Fig. 1).

Real-time PCR. mRNA was extracted using TRIzol reagent (Invitrogen). The GoScriptTM reverse transcription system (Promega) was used to make complementary DNA. Relative quantitative PCR was carried out using SYBR-Green Master (Roche Diagnostics). Reactions were examined using the ABI 7500 real-time PCR machine (Applied Biosystems) coupled with SYBR-Green chemistry. All PCR reactions were in 20 µl of total volume containing 10 µl of SYBR-Green PCR Master Mix, 50 ng cDNA, 250 nM of each primer (MMP-2 forward primer, GCCCAGACAGGTGATCTTG and reverse primer, GCTTGCGAGGGAAGAAGTTGT; MMP-21 forward primer, TCGACATCAAGCTGGGCTTT and reverse primer, ACCTTGAGAAGGCTGATGCC; VEGF-C forward primer, ACGTTCCTGCCAGCAACAC and reverse primer, TCA TCCAGCTCCTTGTTTGGTCC; VEGFR-3 forward primer, CAGGATGAGCGGCTCATCTAC and reverse primer, ACA GGTGAGGCGGTACCAG; GAPDH forward primer, ATG GGAAGGTGAAGGTCG and reverse primer: GGGGTC ATTGATGGCAACAATA). All amplifications were conducted in triplicate for each sample and repeated three times independently. The thermal cycling consisted of 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec, and at 60°C for 60 sec. The specificity of amplification was monitored using the dissociation curve of the amplified product. Relative expression of the target genes was determined using the 2^{-ΔΔC_t} method.

Western blot analysis. CAL-27-PT and CAL-27-LNM cells were lysed in RIPA buffer (Appligen Technologies, Beijing, China) with protease inhibitors and phosphatase inhibitors (Appligen Technologies). Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific). Proteins (40 µg of each sample) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Appligen Technology) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk for 1 h and probed with antibodies against MMP-2 (1:1,000; Epitomics), MMP-21 (1:5,000; Epitomics, Q8N119), VEGF-C (1:1,000 dilution;

Abcam), VEGFR-3 (1:50; Abcam) and GAPDH (1:1,000; Santa Cruz Biotechnology; sc-25778) separately at 4°C overnight. After incubation with HRP-linked secondary antibodies, immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) reagent (Appligen Technology).

Patients. The present study was approved by the Medical Ethics Committee of the Peking University School and Hospital of Stomatology. From 2008 to 2010, 63 OSCC patients with pathologically confirmed cervical lymph node metastasis at the time of diagnosis who were surgically treated at the Department of Oral and Maxillofacial Surgery, Peking University School of Stomatology, were enrolled after providing informed consent. Patients with recurrent cancer or distant metastasis were excluded. The metastatic area was divided into 6 levels according to the American Academy of Otolaryngology Head and Neck Surgery Foundation (17). The pathological stage was diagnosed by pathologists at the Department of Pathology, Peking University School of Stomatology. The 63 patients comprised 40 men and 23 women (median age, 59 years). The tumor sites were the tongue (27 patients), gingiva (17 patients), palate (10 patients), buccal mucosa (5 patients) and floor of the mouth (4 patients). All patients had regular follow-up visits every 2 months for the first year, every 3 months for the second year, and every 6 months thereafter.

Immunohistochemical staining. Tissue sections were embedded in paraffin. Sections (4-µm) were mounted on slides for immunohistochemical staining. After dewaxing, slides were dipped in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After washing thrice in phosphate-buffered saline, antigen retrieval (citrate, 0.01 M, pH 6.0) was performed using a 3-min-long, high-pressure protocol. After washing, the slides were incubated with antibodies against MMP-2 (used as supplied, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China; ZM-0330), MMP-21 (1:200; Epitomics; Q8N119), VEGF-C (used as supplied, Beijing Zhongshan Golden Bridge Biotechnology; ZA-0266), and VEGFR-3 (1:100; Abcam; ab27278) in a humidified chamber at 4°C overnight. After washing, the slides were treated with a horseradish peroxidase-conjugated secondary antibody for 1 h at ambient temperature. Following washing, immunoreaction was detected using 3,3'-diaminobenzidine (DAB; Beijing Zhongshan Golden Bridge Biotechnology; ZLI-9017) incubation for 30 sec. Negative controls were performed using phosphate-buffered solution instead of the primary antibodies. Slides were counterstained with hematoxylin and visualized using an Olympus DP controller (Olympus, Tokyo, Japan). Immunostaining results were semi-quantitatively evaluated by three independent researchers who were blinded to information concerning the specimens. Target protein expression was determined in 10 random fields of the microscope in tumor tissue of every section. The labeling index was defined as the intensity of staining (strong, moderate, weak and negative, scored as 4, 3, 2 and 1, respectively) and multiplied by the percentage of positive cells. If the results were not consistent, a discussion was held in order to reach a consensus. High or low expression levels were determined based on the median of the labeling index.

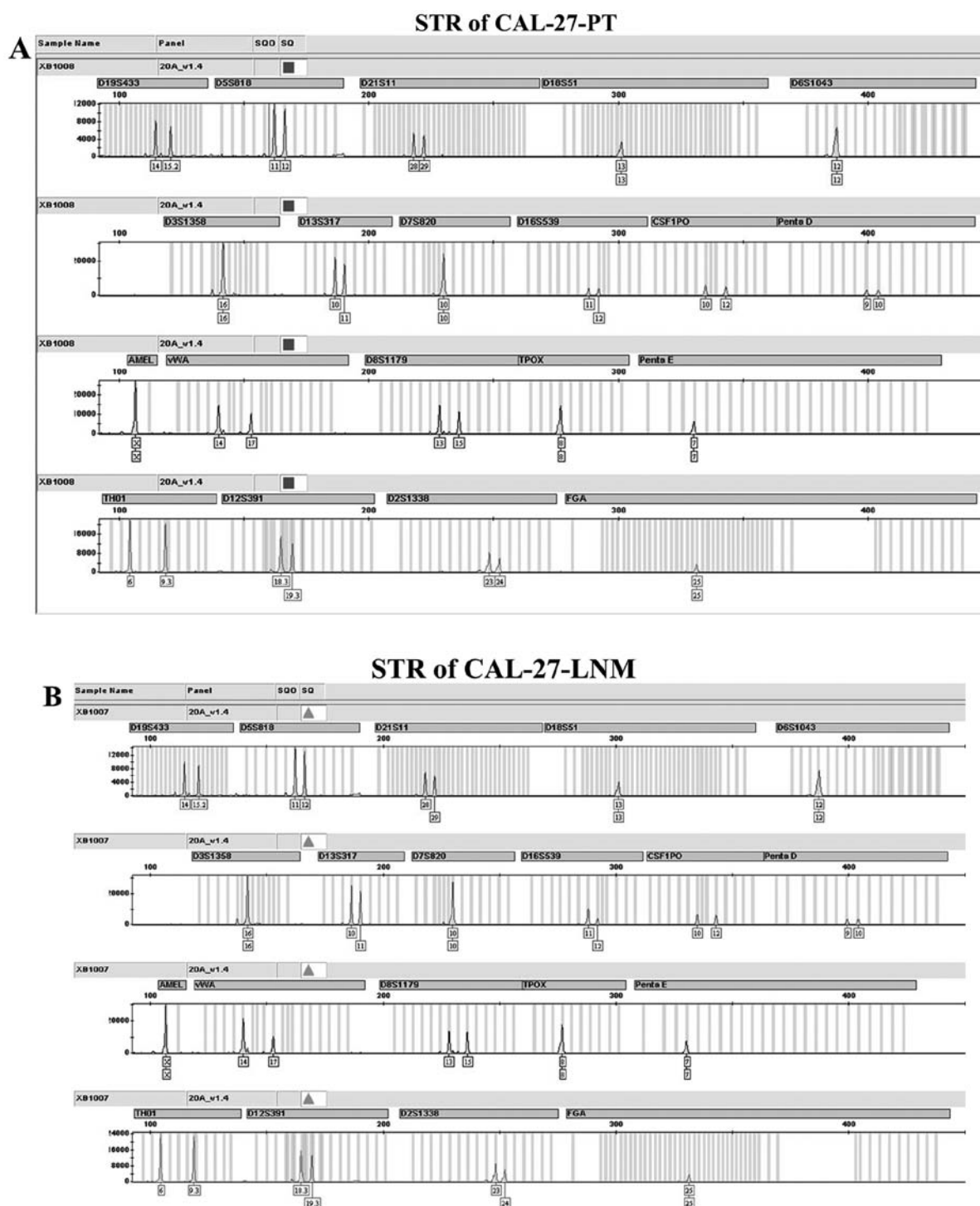


Figure 1. Short tandem repeat (STR) analysis of CAL-27-PT and CAL-27-LNM cells. The results showed that the STR profiling of (A) CAL-27-PT and (B) CAL-27-LNM cells were identical. The 20 foci of CAL-27-PT and CAL-27-LNM are in keeping with oral tongue squamous cell carcinoma cell line CAL-27 (ATCC no. CRL-2095).

Statistical analysis. SPSS 13.0 software was used for analyzing the results. Data are expressed as means \pm SD and were compared using the Wilcoxon paired test and the Pearson's and Spearman's correlation coefficients as indicated (in Results). Patient survival times were defined from the day of surgery to October 2013 or death. The product-limit method and Kaplan-Meier curves were used to calculate survival times. The log-rank test was used to compare the survival curves among the patient groups. Cox proportional hazards model was used

to assess the risk factors. $P < 0.05$ was considered to indicate a statistically significant result.

Results

MMP-21 was screened from the LNM animal model. After 40 days, the PTs and LNM foci were clearly identifiable (Fig. 2A and B). Real-time PCR and western blot analysis revealed that mRNA and protein levels of MMP-21 were higher in the LNM

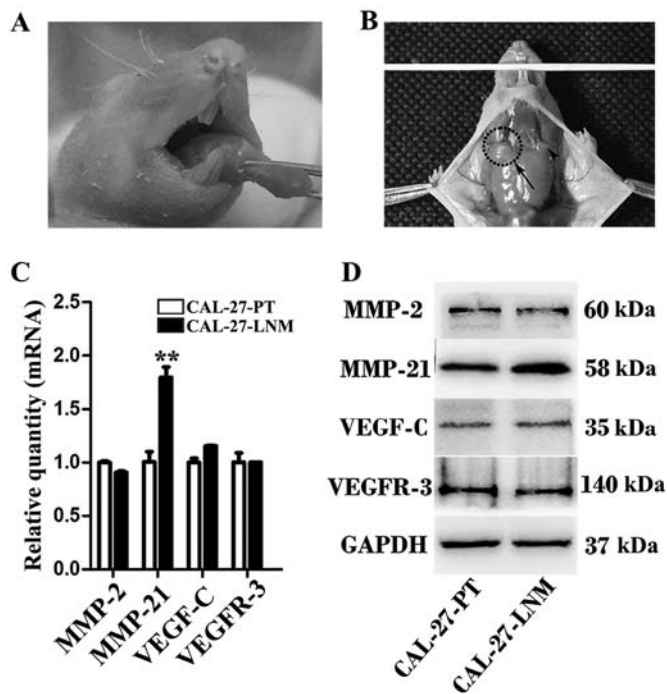


Figure 2. MMP-21 was screened from the LNM animal model. (A) Tumor tissue was clearly observed 40 days after tongue injection of tumor cells. (B) A white lump embedded in the swollen lymph node was considered to be an excellent marker for lymphatic metastasis. (C) Real-time PCR showed that MMP-21 was upregulated in the LNM foci when compared with that in the PT tissue (bars, SD). (D) The results of the western blot analysis are in accordance with real-time PCR. ** $P < 0.01$.

Table I. Association between characteristics and overall survival of the 63 OSCC patients with lymphatic metastasis.

Characteristics	No. of patients (%)	P-value
Gender		
Female	23 (37)	0.91
Male	40 (63)	
Age (years)		
≤59	32 (51)	0.123
>59	31 (49)	
Pathological stage		
High differentiation	20 (32)	0.454
Moderate differentiation	35 (56)	
Poor differentiation	8 (12)	
Neck dissection		
Unilateral	48 (76)	0.816
Bilateral	15 (24)	
No. of metastatic lymph nodes		
1	27 (43)	0.185
>1 (2-9)	36 (57)	
Metastatic side		
Unilateral	55 (87)	0.28
Bilateral	8 (13)	
Metastatic area		
1	38 (60)	0.044
>1 (2-5)	25 (40)	

Table II. Relationships between pathological stage and the expression of the tested proteins in the 63 OSCC patients with lymphatic metastases.

Characteristics	Pathological stage			P-value
	1	2	3	
No. of patients	20	35	8	
Expression in PTs ^a				
MMP-2	76±73	81±67	29±40	0.40
MMP-21	113±82	165±89	96±82	0.45
VEGF-C	42±54	52±62	38±48	0.82
VEGFR-3	71±77	110±91	111±113	0.24
Expression in LNM				
MMP-2	76±85	92±86	104±99	0.30
MMP-21	147±76	172±92	160±114	0.46
VEGF-C	59±61	72±73	123±72	0.04
VEGFR-3	59±64	90±85	132±81	0.03

^aMean ± standard deviation. OSCC, oral squamous cell carcinoma; PT, primary tumor; LNM, lymph node metastasis.

foci than levels in the PTs. The results of real-time PCR and western blot analysis were consistent (Fig. 2C and D).

Characteristics of patients and relationship with overall survival. Among all the characteristics, the metastatic area showed a significant relationship with overall survival of the patients ($P=0.044$). Others including gender, age, pathological stage, neck dissection, number of metastatic lymph node and metastatic side demonstrated no association with overall survival of the 63 patients based on the Kaplan-Meier and log-rank test (Table I). The number of metastatic lymph nodes in every patient ranged from 1 to 9 (1 in 27 patients, 2 in 14 patients, 3 in 8 patients, 4 in 4 patients, 5 in 2 patients, 6 in 5 patients, 7 in 0 patients, 8 in 2 patients and 9 in 1 patient, respectively). The metastatic areas were determined to be 1 in 38 patients, 2 in 13 patients, 3 in 8 patients, 4 in 3 patients and 5 in 1 patient, respectively. Among all the metastatic areas, level I (the submental and submandibular triangles) accounted for 44%, level II (the upper jugular nodal group) accounted for 36%, level III (the middle jugular nodal group) accounted for 12%, level IV (the lower jugular nodal group) accounted for 5%, level V (the posterior triangle of the neck) accounted for 3%, level VI (the pre-laryngeal, pre-tracheal and paratracheal nodes) accounted for 0%.

Expression of the tested proteins in the clinical specimens. MMP-2, MMP-21, VEGF-C and VEGFR-3 expression patterns were assessed in clinical specimens, including the PTs and the corresponding LNM foci. All of the tested proteins were expressed in the tumor cell cytoplasm. The rates of MMP-2, MMP-21, VEGF-C and VEGFR-3 positive staining were 73.02%, 95.24%, 63.49%, and 84.13%, respectively in the PTs. The corresponding positive expression rates were 76.19%, 95.24%, 63.49% and 76.19% in the LNM specimens. Protein expression

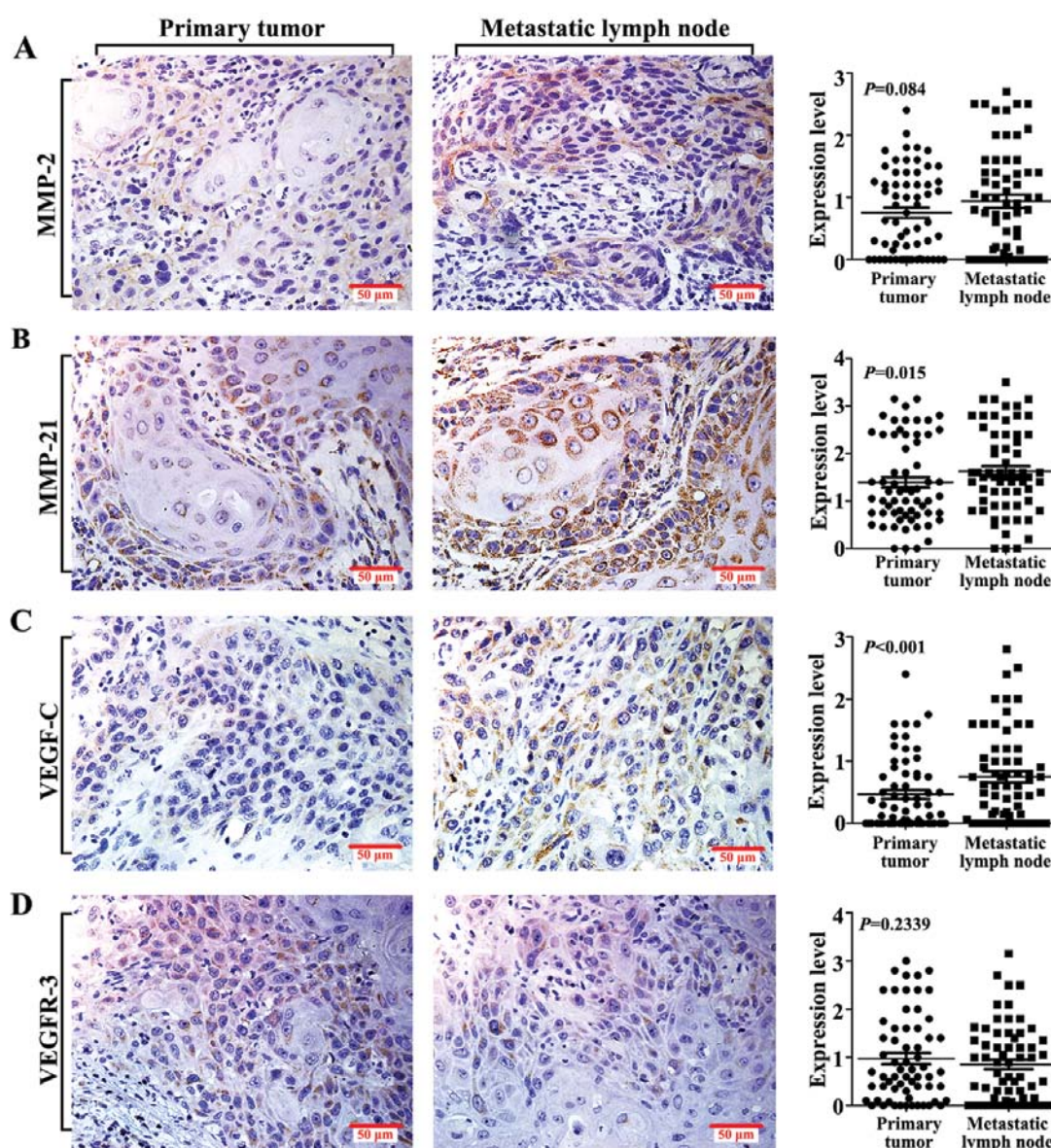


Figure 3. MMP-2, MMP-21, VEGF-C and VEGFR-3 immunostaining in 63 OSCC specimens, including paired PTs and the corresponding LNM foci. (B) MMP-21 and (C) VEGF-C expression levels were higher in the LNM foci than levels in the PT tissues ($P<0.05$). MMP-2 (A, $P=0.08$) and VEGFR-3 (D, $P=0.23$) expression levels were not significantly different between the PTs and the LNM foci. Data were analyzed using the Wilcoxon paired test.

levels were assessed using the labeling index as described above. Our results showed that MMP-21 and VEGF-C expression levels were higher in the LNM foci than levels in the PTs (Fig. 3B and C), while MMP-2 and VEGFR-3 showed no significant expression between the paired specimens (Fig. 3A and D). These data were analyzed using the Wilcoxon paired test.

Relationship between protein expression and clinicopathological characteristics. Relationships between expression of the tested protein and the clinicopathological characteristics were assessed using Spearman's correlation coefficients. VEGF-C and VEGFR-3 expression levels in the LNM foci were associated with the OSCC pathological stage. However, the pathological cancer stage showed no correlation with expression of the four proteins in the PTs or MMP-2 and MMP-21 expression in the LNM foci (Table II). Expression levels of the four tested proteins in the PTs were not correlated with patient

age, gender, neck dissection, number of metastatic lymph nodes, metastatic side or metastatic area (data not shown).

Relationships between protein expression and overall survival. MMP-2, MMP-21, VEGF-C and VEGFR-3 expression levels in the PTs and LNM specimens were assessed in relation to patient overall survival. Patients with high MMP-21 ($P=0.001$) expression in the LNM foci had an unfavorable overall survival when compared with those with low MMP-21 expression. Patients with PTs and LNM foci that had high MMP-2 ($P=0.06$, $P=0.012$) and VEGF-C ($P=0.008$; $P=0.031$) expression levels presented with significantly poor overall survival. However, the MMP-21 expression level in the PTs and the VEGFR-3 expression level in the PTs and LNM foci displayed no significant relationship with overall survival ($P>0.05$; Fig. 4). The results were analyzed using the Kaplan-Meier method and the log-rank test.

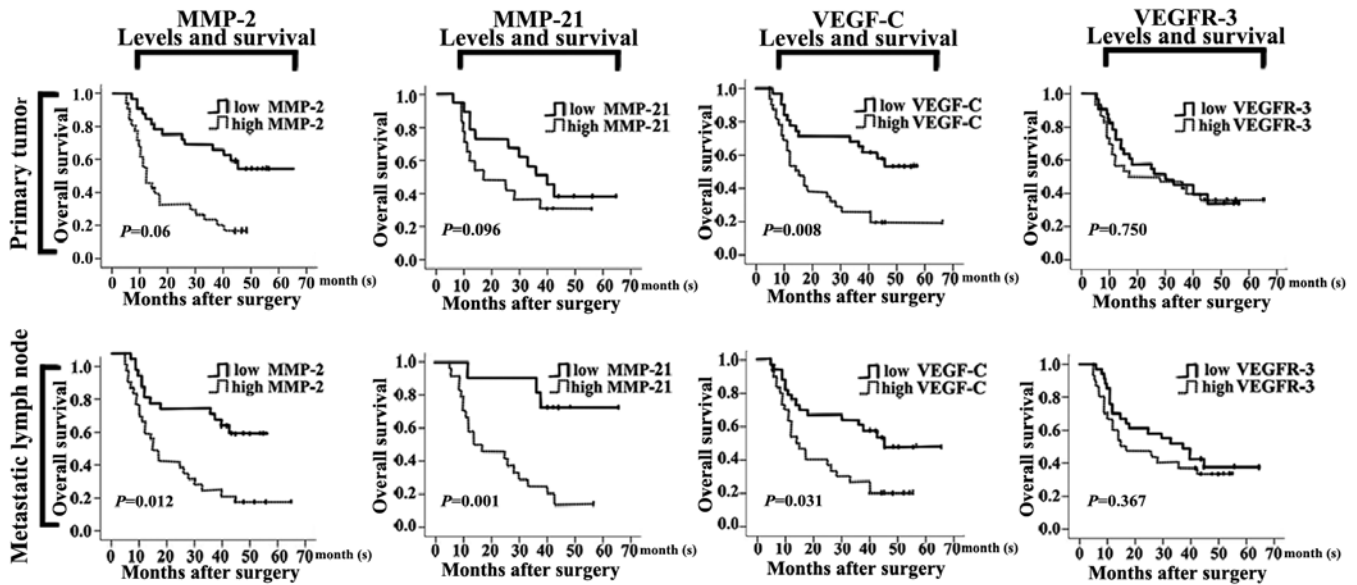


Figure 4. Relationships between MMP-2, MMP-21, VEGF-C and VEGFR-3 expression in OSCC specimens and the overall survival of patients with lymphatic metastasis. High MMP-2 and VEGF-C expression levels in the PTs were significantly correlated with patient overall survival ($P < 0.05$). High MMP-2, MMP-21 and VEGF-C expression levels in the LNM foci predicted poor overall survival ($P < 0.05$). In addition, the MMP-21 expression level in the PTs and the VEGFR-3 expression level in the LNM foci were not related to the overall survival of OSCC patients with lymphatic metastasis. Data were analyzed using the Kaplan-Meier method.

Table III. Cox proportional hazards analysis was performed to select the relative risk factors for the 63 OSCC patients with lymphatic metastasis.

Target proteins	RR	95% CI		P-value
		Lower	Upper	
Primary tumor				
MMP-2	1.465	0.682	3.147	0.328
MMP-21	1.307	0.669	2.554	0.433
VEGF-C	1.767	0.815	3.830	0.149
VEGFR-3	1.121	0.555	2.263	0.750
Lymphatic metastasis				
MMP-2	1.466	0.675	3.183	0.333
MMP-21	2.560	1.093	5.998	0.030
VEGF-C	1.150	0.511	2.591	0.735
VEGFR-3	1.491	0.722	3.079	0.280

RR, relative risk; 95% CI, 95% confidence interval.

MMP-21 expression in LNM foci is a significant predictor of overall survival. MMP-2, MMP-21, VEGF-C and VEGFR-3 expression levels in the PT and LNM specimens were analyzed using the Cox proportional hazards model. Three parameters, including 'overall score', 'change from the previous step', and 'change from the previous block', were used to assess the significance of the model. Results by the omnibus testing of model coefficients were statistically significant ($P = 0.002$; data not shown). Among the markers, MMP-21 expression in the LNM foci was most closely associated with the overall survival ($P = 0.03$; Table III). The associated relative risk was

2.56, which meant that the mortality rate of OSCC patients would likely increase by 2.56 times in patients with a high MMP-21 expression level. In contrast to MMP-21, the expression levels of the other proteins in the LNM foci were not closely correlated with overall survival (Table III).

Discussion

Lymphatic metastasis follows a complex course of progression (18). At present, researchers have focused on the relationships between the microenvironment of the PT and mechanisms underlying LNM. Therefore, expression of a large number of metastasis-related genes in the PT has been found to correlate with cancer patient overall survival. However, metastatic foci may be more crucial for predicting the prognosis of patients with LNM, particularly OSCC patients with an ~45% chance of suffering metastasis to the neck lymph nodes (5).

OSCC tends to metastasize to the cervical lymph nodes (19). PT and LNM cancer cells have been compared by two opposing hypotheses. First, it is hypothesized that PT and LNM cells differ characteristically (20). The second hypothesis suggests that these differences are negligible (21). Since the identification of cancer stem cells, it is generally suggested that metastatic cells are identical to cancer stem cells (22). LNM tumor cells are believed to express a higher proportion of cancer stem cell markers than PT cells since stem cell markers are found to be overexpressed in circulating tumor cells originating from metastatic breast cancer (23).

In the 63 paired specimens, levels of MMP-21 and VEGF-C expression, but not MMP-2 or VEGFR-3, were higher in the LNM foci than levels in the PT cells. This observation suggests that some tumor cells with higher expression of MMP-21 and VEGF-C are strongly capable of invasion and metastasis and these tumor cells metastasize to lymph nodes to grow and

accumulate a higher proportion of tumor cells expressing the two tested proteins.

To the best of our knowledge, the relationship between protein expression levels in the LNM foci and patient overall survival has not been extensively studied. MMP-2 and VEGF-C expression in the PT has been proven to correlate with the overall survival of OSCC patients (24,25). Here, we found that high MMP-2 and VEGF-C expression in the PT specimens or in the corresponding LNM foci was significantly correlated with overall survival of the 63 OSCC patients. Previous studies have shown that VEGFR-3 can potentially promote lymphatic metastasis (26), but cannot predict OSCC patient overall survival (27). Here, we also found that VEGFR-3 did not correlate with patient overall survival. A high MMP-21 expression level in the PTs was previously found to be associated with metastasis and poor overall survival in colorectal cancer (14,21) as well as gastric cancer patients (13). In the present study, we found that high MMP-21 expression in the LNM foci but not in the PTs was associated with poor overall survival of OSCC patients with metastatic lesions. This finding may suggest that investigation of metastatic lesions could provide convincing information for predicting the overall survival of OSCC patients with LNM. The underlying mechanisms warrant further investigation.

Among all the tested proteins, the positive MMP-21 expression rate was the highest. Thus, MMP-21 may be used to identify OSCC cells. Meanwhile, MMP-21 expression in the LNM foci was most closely associated with patient overall survival as found using the Cox proportional hazards model. Although MMP-2 and VEGF-C have been recognized as significant predictors of the overall survival of OSCC patients (24,25), MMP-21 expression in the LNM lesions was a more sensitive predictor of overall survival in the OSCC patients with lymphatic metastasis. This suggests that investigation of LNM foci is important for OSCC patients with lymphatic metastasis.

In conclusion, our results may further strengthen the hypothesis that cellular differences between PT and LNM foci are characteristically specific. Inhibiting tumorigenesis and metastasis is the key to curing cancer patients; however, this is a long and complicated process, which requires tremendous scientific research. While we are facing the ever-increasing number of cancer patients with LNM, investigation of LNM foci may help explain the mechanisms underlying lymphatic metastasis.

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