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Lymphangiogenesis and Lymph Node Metastasis in Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: Tumor lymphangiogenesis plays a key role in lymph node (LN) metastasis in oral squamous cell carcinoma (OSCC). The purpose of this study was to investigate podoplanin and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and their relationship to nodal metastasis and other clinicopathological variables. Patients and Methods: Podoplanin and LYVE-1 expression of the primary tumor and normal tissue were investigated by means of a quantitative real-time PCR assay and immunohistochemistry in samples from 33 cases of OSCC. Results: The mRNA high expression levels of both genes had a statistically significantly higher rate of LN metastasis ($p<0.01$) and histological grade ($p<0.01$ for podoplanin, $p<0.05$ for LYVE-1). High expression of each gene, as shown by immunohistochemistry, had a statistically significant higher rate of LN metastasis ($p<0.01$ for podoplanin, $p<0.05$ for LYVE-1). Conclusion: Podoplanin and LYVE-1 were strongly associated with LN metastasis.*

Oral cancer is a leading cause of cancer death and oral squamous cell carcinoma (OSCC) accounts for more than 90% of cancers in the oral cavity. OSCC is characterized by a high degree of local invasiveness and cervical lymph node (LN) metastasis (1). LN metastasis cannot always be predicted, because some patients with large tumors have no regional LN invasion, whereas some with small primary tumors have cervical LN metastasis. Thus, the important factor which determines the most effective therapeutic strategy in OSCC is the LN status. Recent reports demonstrated that increased tumor lymphatic vessel density/lymphangiogenesis correlates with LN metastasis in head and neck cancer including OSCC (2). Podoplanin has been utilized as a specific marker of lymphatic vessels. Overexpression of podoplanin is a potential marker of LN metastasis (3). Lymphatic endothelial hyaluronan receptor-1 (LYVE-1) is a receptor for hyaluronan, expressed on lymphatic endothelium and involved in leukocyte migration and tumor metastasis (4, 5). However, there is no consensus, yet, whether podoplanin and LYVE-1 can be used for predicting lymphatic status in OSCC. In particular, there are no clinical reports regarding the use of quantitative real-time PCR assay to predict lymphatic status in OSCC. The purpose of this study was to investigate podoplanin and LYVE-1 expression in OSCC and their involvement in nodal metastasis and other clinicopathological variables by means of a quantitative real-time PCR assay and immunohistochemistry.

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Key Words: Oral squamous cell carcinoma, lymphangiogenesis, lymph node metastasis, quantitative real-time PCR, immunohistochemistry.

Patients and Methods

Patients. The present study was a prospective study. This study was conducted in full accordance with the World Medical Association Declaration of Helsinki. All procedures used in this research were approved by the Ethical Committee of Kobe University Hospital (approval number: No. 180171) and informed consent was obtained from each patient. All tumors were resected at the Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine, Japan, between 2013 and 2017. The inclusion criteria were (i) no preoperative chemotherapy, hormone therapy or radiotherapy, and (ii) sufficient tumor tissues and normal tissues available. Patients that did not want to participate were not included in the study. All patients had surgery as their first line of management. Clinical data including age, gender, smoking, alcohol, primary tumor site, pathological T stage, LN status and histological grade were abstracted from the clinical records and are shown in Table I. The median age of the patients was 69 (range=41-88 years) at the time of diagnosis; the group included 20 (60.6%) male and 13 (39.4%) female. A surgical excision with a clear 10 mm margin from the tumor edge was performed for each patient.

Quantitative real-time RT-PCR. The normal and tumor tissues from 33 previously untreated patients with OSCC were snap-frozen at the time of surgery and stored at –80˚C. Briefly,

approximately 100 mg was homogenized using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at room temperature, and total RNA was extracted. The RNA was further cleaned up with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The cDNA was synthesized (600 ng of total RNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), podoplanin and LYVE-1 was analyzed using quantitative real-time polymerase chain reaction (qPCR). GAPDH was used as a reference gene. The quantification of mRNA transcription was performed using a StepOne Real-Time PCR System (Applied Biosystems). Real-time PCR (20 μl) was performed with 0.5 μM forward primer, 0.5 μM reverse primer, and 1 μl of the 10-times diluted cDNA template from the reverse transcription reaction and 10 μl (2×) Power SYBR Green Master Mix (Applied Biosystems). The PCR conditions were as follows: one cycle at 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 1 min. GAPDH primers were obtained from Invitrogen. QuantiTect® Primer Assays (Qiagen: QT02321263, QT00034566) for Podoplanin and LYVE-1 were obtained from Qiagen (Valencia, CA, USA).

Immunohistochemistry. Specimens of complete resections were selected so that both normal and tumor tissues were present on each slide. The paraffin-embedded tissue blocks were sliced into 4 μm thick sections for subsequent histological examinations. These were deparaffinized with xylene, rehydrated in a graded alcohol series, antigen retrieval was performed by proteinase K, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2, and were incubated overnight at 4˚C with the following primary antibodies in Can Get Signal Immuno-stain Solution A (Toyobo, Osaka, Japan); polyclonal sheep anti-human podoplanin (1:200 dilution, AF3670, R&D Systems, Minneapolis, USA), polyclonal goat anti-human LYVE1 (1:200 dilution, AF2089, R&D Systems). Following treatment, sections were incubated with horseradish peroxidase (HRP)-conjugated anti-sheep IgG polyclonal antibody (1:100 dilution in PBS, Abcam, Cambridge, UK) and anti-goat IgG polyclonal antibody (Nichirei, Tokyo, Japan) for 2 h at room temperature. The signal was developed as a brown stain using the peroxidase substrate 3, 3-diaminobenzidine (Nichirei). The sections were counterstained with hematoxylin and were captured under a microscope. The samples were observed with BZ-X700 (Keyence, Osaka, Japan).

Evaluation of stained slides. The slides were analyzed randomly by two authors, blinded to the clinical data. Podoplanin expression was scored as described by Rodrigo *et al.* (6). The proportion of immunoreactive positive cells was scored as 0 (0 %), 1 (less than 10%), 2 (10-29%), 3 (30-49%), 4 (50-79%), or 5 (more than 80%). The staining intensity was rated on a scale of 0-3 (0=negative, 1=weak, 2=moderate, and 3=strong) (Figure 1). German immunoreactive score (IRS) was calculated by multiplying the podoplamin expression and staining intensity scores. An IRS score above the median (7 or higher) was considered as high reactivity and between 0-6 as low reactivity. The scoring method is described in Table II. LYVE-1 expression was counted by lymphatic vessel density (LVD). Three fields with the highest LVD (hot spots) were identified in each sample at ×40 magnification. The number of lymphatic vessels was counted within 3 microscopic fields at a magnification of ×200 and the median was used for statistical analysis (Figure 2). The section was identified as having LYVE-1 high expression when the number of LYVE-1-positive vessels was higher than the median number. The section was identified as having LYVE-1 low expression when the number of LYVE-1-positive vessels was lower than the median number (7).

Table I. *Clinicopthological characteristics of 33 OSCC patients.*

Clinicopathologic variables	Number of patients (%)
Total cases	33
Age (years)	69 (41-88)
Gender	
Male	20 (60.6)
Female	13 (39.4)
Smoking	
Yes	16 (48.5)
No	17 (51.5)
Alcohol	
Yes	17 (51.5)
No	16 (48.5)
Primary tumor site	
Tongue	11 (33.3)
Lower gingiva	10 (30.3)
Upper gingiva	6 (18.2)
Buccal mucosa	4 (12.1)
Floor of mouth	2 (6.1)
Pathological T stage	
T1	4 (12.1)
T2	13 (39.4)
T3	7 (21.2)
T4	9 (27.3)
Lymph node status	
Positive	15(45.5)
Negative	18 (54.5)
Histological grade	
Well	13 (39.4)
Moderate	15 (45.6)
Poor	5 (15.2)

Statistical analysis. The expression of each target gene normalized to an endogenous reference gene relative to the calibrator was calculated by using the ΔΔCT method (Applied Biosystems). The calibrator was the 1× sample, and all other quantities were expressed as an *n*-fold difference. ΔΔCT was calculated (tumor tissues relative to normal tissues) and a two-tailed Mann–Whitney test was used to test statistical significance. The Mann–Whitney test was used for the associations between the mRNA expression levels of each gene and clinicopathological characteristics. The associations between immunohistochemical expression of each gene and primary tumor site were examined using the Mann–Whitney *U*-test. The associations between immunohistochemical expression of each gene and other clinicopathological variables were examined using the chi-square test. The data collection and statistical analyses were performed using IBM SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY, USA). Data was presented as mean±standard error (SE). A value of $p<0.05$ was considered statistically significant.

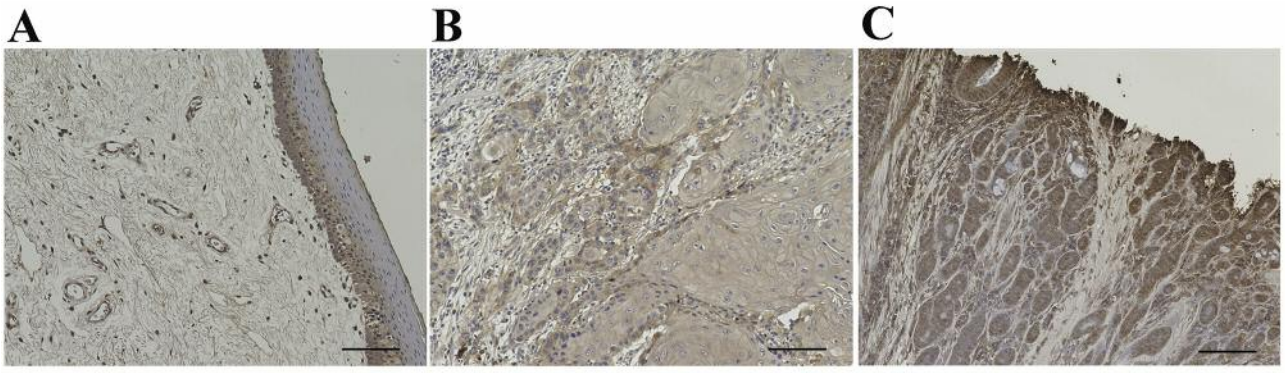

Figure 1. *Expression of podoplanin. The staining intensity was rated on a scale of 0-3 (0=negaive, 1=weak, 2=moderate, 3=strong). (A) Weak: faint staining, (B) Moderate: staining between dark brown and weak staining, (C) Strong: dark brown staining of cells. Scale bar=100 μm.*

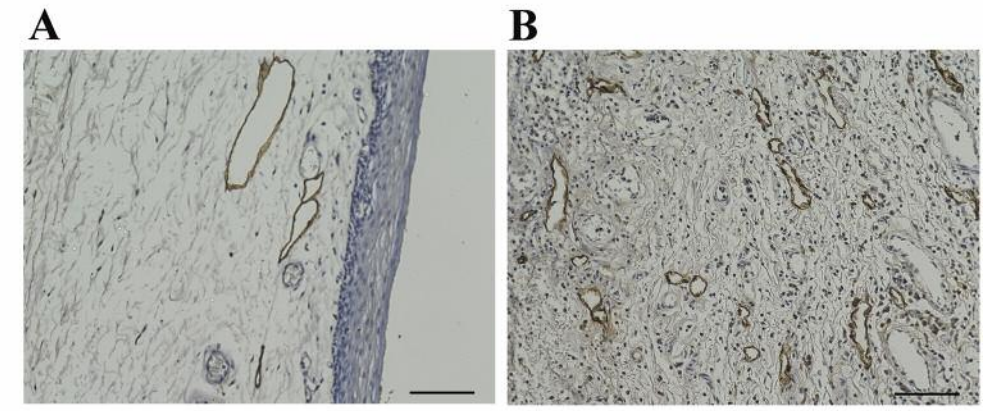

Figure 2. *Expression of LYVE-1. (A) LYVE-1-positive lymphatic vessels were observed in normal tissue. (B) LYVE-1-positive lymphatic vessels were observed in tumor tissue. Scale bar=100 μm.*

Results

Clinicopathological characteristics of OSCC patients. The most common primary tumor sites were the tongue (33.3%) followed by lower gingiva (30.3%), upper gingiva (18.2%), buccal mucosa (12.1%) and floor of mouth (6.1%). Pathological T classifications were T1 in 4 patients (12.1%), T2 in 13 patients (39.4%), T3 in 7 patients (21.2%), and T4 in 9 patients (27.3%). LN metastasis occurred in 15 patients (45.5%) and the others had negative nodes based on pathological examination. Histological grades were well differentiated SCC in 13 patients (39.4%), moderately SCC in 15 patients (45.6%) and poorly SCC in 5 patients (15.2%) (Table I).

Expression of podoplanin and LYVE-1 in clinical OSCC samples. Quantitative real-time PCR showed that mRNA expression levels of podoplanin and LYVE-1 were 13.0±6.5 and 4.8±3.9 in well-differentiated samples, and 53.9±14.5 and 52.3±26.8 in moderately- and poorly-differentiated samples from tumor tissues relative to normal tissues, respectively (Figure 3A and B). The mRNA expression levels of both genes were significantly higher in moderately and poorly-differentiated samples compared to well-differentiated samples ($p<0.01$ for podoplanin, $p<0.05$ for LYVE-1). Quantitative real-time PCR showed that mRNA expression levels of podoplanin and LYVE-1 were 4.2±0.7 and 0.2±0.11 in the LN-negative group, and 78.2±16.1 and 73.7±34.6 in the LN-positive group in tumor tissues relative to normal tissues, respectively (Figure 3C and D). The mRNA expression levels of both genes were significantly higher in the LN-positive group compared to the LN-negative group ($p<0.01$). There was no statistically significant correlation between each gene status and other clinicopathological variables.

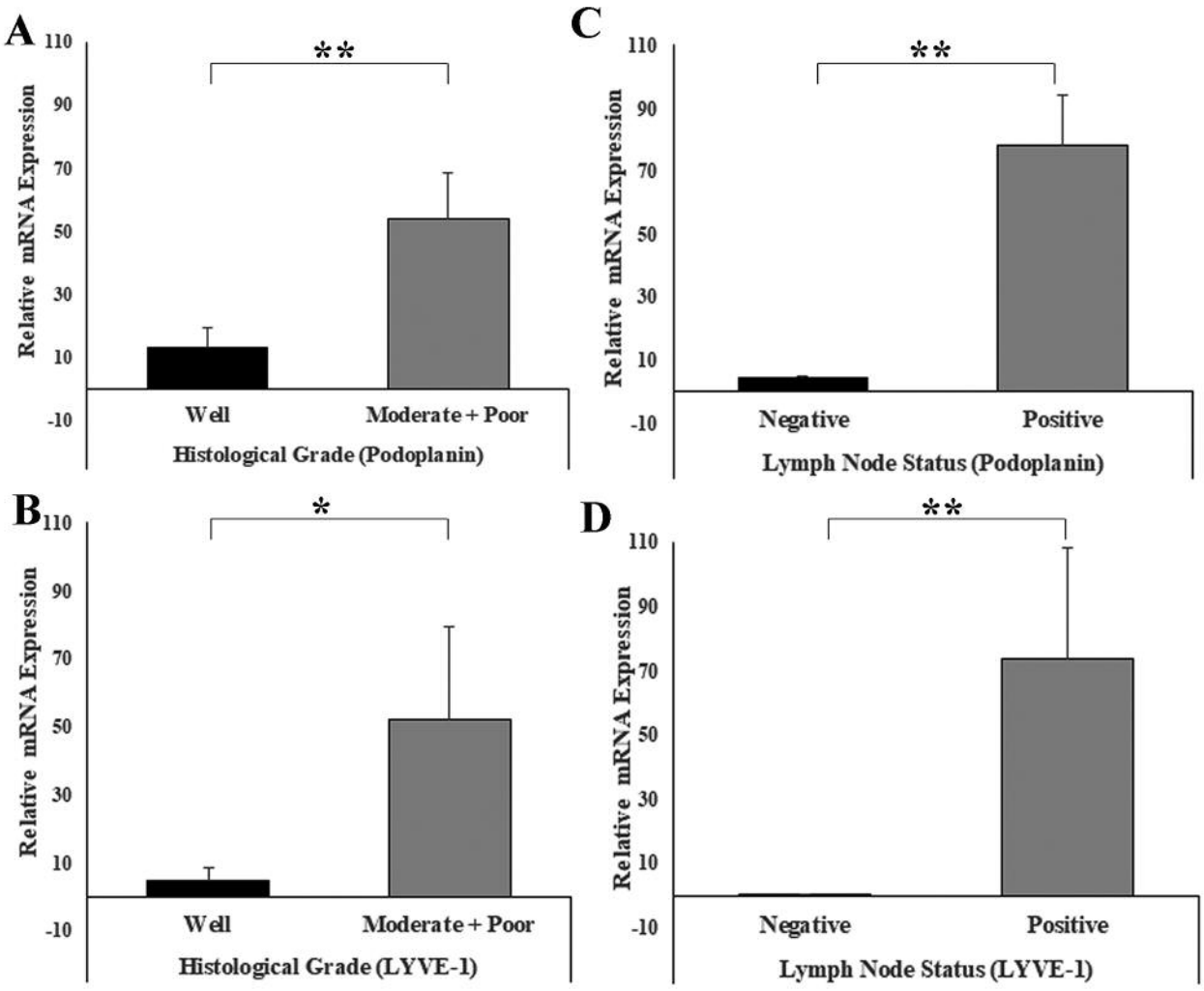

Figure 3. *Expression levels of each gene in well-, moderately- and poorly-differentiated samples, lymph node status obtained from OSCC patients. The mRNA expression levels of podoplanin (A, C) and LYVE-1 (B, D) in tumor tissues relative to normal tissues were evaluated using quantitative real-time polymerase chain reaction (PCR). *p<0.05, **p<0.01.*

Immunohistochemical expression of podoplanin and LYVE-1. As analyzed by immunohistochemistry, high expression of each gene had a statistically significant higher rate of LN metastasis ($p<0.01$ for podoplanin, $p<0.05$ for LYVE-1) (Table III). Immunohistochemically, high expression of podoplanin had a statistically significant higher rate of pathological T stage ($p<0.01$). There was no statistically significant correlation between each gene status and other clinicopathological variables.

Table II. *German immunoreactive score (IRS) scoring method.*

Quantity of positive cells score	Staining intensity score	IRS
0: no positive cells	0: negative	0-6: low reactivity
1: <10%	1: weak	7-15: high reactivity
2: 10-29%	2: moderate	
3: 30-49%	3: strong	
4: 50-79%		
5: more than 80%		

Discussion

Regional LN metastasis is the most prevalent cause of death in OSCC patients. Our results clearly showed that podoplanin and LYVE-1 were expressed in most of the OSCC cases and were strongly associated with LN metastasis.

Podoplanin is a glomerular podocyte membrane mucoprotein (8), specifically expressed in lymphatic endothelial cells, but not in big lymphatic vessels with

Table III. *Clinicopthological characteristics of 33 patients according to the expression of each gene.*

		Podoplanin			LYVE-1		
	Subjects	Low	High	*p*-Value	Low	High	*p*-Value
Age,years							
<65	7	2	5	0.629	2	5	0.312
≥65	26	10	16		13	13	
Gender							
Male	20	8	12	0.591	7	13	0.134
Female	13	4	9		8	5	
Smoking							
Yes	16	7	9	0.392	5	11	0.111
No	17	5	12		10	7	
Alcohol							
Yes	17	8	9	0.188	6	11	0.226
No	16	4	12		9	7	
Primary tumor site							
Tongue	11	5	6	0.599	6	5	0.985
Lower gingiva	10	2	8		5	5	
Upper gingiva	6	1	5		3	3	
Buccal mucosa	4	2	2		1	3	
Floor of mouth	2	2	0		0	2	
Pathological T stage							
T1-T2	17	10	7	0.005**	8	9	0.848
T3-T4	16	2	14		7	9	
Lymph node status							
Positive	15	1	14	0.001**	4	11	0.047*
Negative	18	11	7		11	7	
Histologic grade							
Well	13	7	6	0.09	7	6	0.435
Moderate+Poor	20	5	15		8	12	

*p<0.05, **p<0.01.

smooth muscle and blood endothelial cells (9). Wetterwald *et al*. firstly identified podoplanin in lymphatic endothelial cells in 1996 (10). In normal human tissues, podoplanin is expressed in lymphatic endothelium, skeletal muscle, in the myofibroblasts of the salivary glands, in osteoblasts and in the basal layer of human epidermis. Wada *et al*. demonstrated that the expression level of podoplanin was related to the degree of lymph nodes involvement and lymphovascular invasion in colorectal cancer (11). Braun *et al*. indicated that podoplanin was an important factor for predicting the involvement of lymph nodes in invasive breast cancer (12). Pasad B *et al*. showed that a high level of podoplanin expression is suggestive of a high frequency of LN metastasis and an immature status in the differentiation process of OSCC (13). Previous reports indicated that the expression of podoplanin increased from well to poorly differentiated OSCC (3, 14). Banerji *et al*. first identified LYVE-1 in 1996 (15) where he found that it is a homolog of the vascular endothelium-specific hyaluronan receptor CD44 and was involved in the migration of endothelial cells (16). It was present on normal and tumor-associated lymphatic endothelial cells and was highly expressed in the endothelial cells of lymphatic vessels, but was not detectable in the endothelial cells of blood vessels (Figure 2A). LYVE-1 was associated with tumor progression and metastasis, which could be used to identify tumor-associated lymphogenesis (17). Hara *et al*. showed that LYVE-1 was involved in primary tumor formation and metastasis (18). It was up-regulated in muscle-invasive bladder cancers, exhibiting positive lympho-vascular invasion and LN metastasis compared to non-muscle invasive bladder cancers (19). Ozmen *et al*. showed that the expression level of LYVE–1 in gastric cancer tissues was clearly higher than in para-cancerous tissues and an increased expression level was associated with an increased proportion of lymph node involvement (20). Frech *et al*. reported that a high intratumoral lymphatic vessel density correlated with neck node metastasis in squamous cell carcinoma of the oral cavity (2). Our results showed that the high mRNA expression levels of both genes had a statistically significant higher rate of LN metastasis and histological grade. High expression of each gene, as shown by immunohistochemistry, had a statistically significant higher rate of LN metastasis. Our data suggested

that both genes may predict LN metastasis and histological grade in OSCC patients, using a quantitative real-time PCR assay. If the present results are supported by further studies, analysis of podoplanin and LYVE-1 expression in the biopsied tissues could help clinicians in evaluating the lymph node metastasis risk in OSCC and also these proteins can become biomarkers for advanced grades of OSCC.

This study has certain limitations. First, our sample size was small and further studies may be required to confirm the results of this study. Second, the follow-up period was short. Our findings may contribute to the successful surgical treatment of OSCC patients in the fields of dentistry and oral surgery.

Conflicts of Interest

The Authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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