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Expression of Sphingosine Kinase-1 Is Associated with Invasiveness and Poor Prognosis of Oral Squamous Cell Carcinoma

KOICHIRO KATO[1], MIYAKO SHIMASAKI[2], TAKAO KATO[1], NATSUKI SEGAMI[1] and YOSHIMICHI UEDA[2]

[1]*Department of Oral and Maxillofacial Surgery,* [2]*Department of Pathophysiological and Experimental Pathology, Kanazawa Medical University, Ishikawa, Japan*

Abstract. *Background/Aim: The expression of sphingosine kinase-1 (SphK1) has been reported in several cancers. However, the exact roles of SphK1 in cancer progression still remain unknown. The aim of the present study was to investigate SphK1 expression in oral squamous cell carcinoma (OSCC) and clarify the involvement of SphK1 in the proliferation and invasiveness of OSCC and its prognostic implications. Materials and Methods: Expression of SphK1, E-cadherin, vimentin, and Ki-67 were examined in 69 OSCC tissues immunohistochemically, as well as by western blot, and correlations between their expression and relationships with tumor invasiveness and prognosis were analyzed. Results: SphK1 was expressed in the tumor cells of 38 of 69 OSCCs, particularly at the invasion front. Patients with OSCCs with high SphK1 expression showed higher invasive grades and unfavorable survival rates. SphK1 expression correlated with acquisition of vimentin expression and loss of E-cadherin expression; there was no significant difference in Ki-67 labeling indices between OSCCs with high and low SphK1 expression. Conclusion: These results demonstrate the involvement of SphK1 in the invasiveness of OSCC and in unfavorable prognosis, indicating its role in the epithelial-mesenchymal transition of OSCC cells.*

Some oral squamous cell carcinomas (OSCCs) aggressively invade the surrounding tissues, while some are as stable as benign tumors (1). The most important cause of treatment failure is local recurrence attributable to inadequate surgical resection. Cancer cells persisting after resection may spread with unexpectedly high invasiveness. Thus, invasiveness of the tumor needs to be carefully evaluated to select a suitable treatment for OSCC.

Relationships between histological characteristics and SCC progression have been reported in various organs, including OSCC (2, 3). Four grades of mode of invasion were proposed by Jakobsson for the evaluation of cell invasiveness and proliferation of SCC in the larynx (4): grade 1 has a well-defined borderline; grade 2 has a less-marked borderline; grade 3 has groups of cells and no distinct borderline; and grade 4 has diffuse growth. These grades have been proven to be associated with the malignant potentials of SCC not only in the larynx, but also in the oral cavity (2, 3, 5). Furthermore, Yamamoto *et al.* suggested that, according to the sensitivities of OSCCs to bleomycin treatment, grade 4 malignancy proposed by Jakobsson should be subdivided into two grades, 4C and 4D; grade 4C has deep invasiveness as a cord-shaped microscopic tumor nest, while grade 4D has diffuse invasiveness in the deeper portion as a single or a few tumor cells (5). These five grades of cancer cell invasion correlate closely with rates of lymph node metastasis and of local recurrence, as well as with the prognosis of patients with OSCC (5, 6).

The epithelial-mesenchymal transition (EMT) is considered essential for tumor cells to acquire motility and invasiveness (7). There have been several reports of regulators of invasiveness/EMT of OSCC (8, 9). The precise mechanism of the invasiveness of OSCC, however, which is clinically applicable to the evaluation of tumor aggressiveness, remains unclear.

Sphingosine kinase-1 (SphK1) is an enzyme that regulates ceramide, sphingosine, and sphingosine-1-phosphate (S1P) metabolism (10, 11). Ceramide and S1P are well known to have opposite effects on cancer cells: ceramide induces apoptosis and cell growth arrest; S1P prevents cell death and stimulates cell proliferation. Moreover, recent studies have demonstrated novel functions of S1P in tumor biology related to cellular motility,

Correspondence to: Koichiro Kato, Department of Oral and Maxillofacial Surgery, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, Japan. Tel: +81 762862211, Fax: +81 762862010, e-mail: k-kato@kanazawa-med.ac.jp

Key Words: Sphingosine kinase-1, oral squamous cell carcinoma, invasiveness, epithelial-mesenchymal transition.

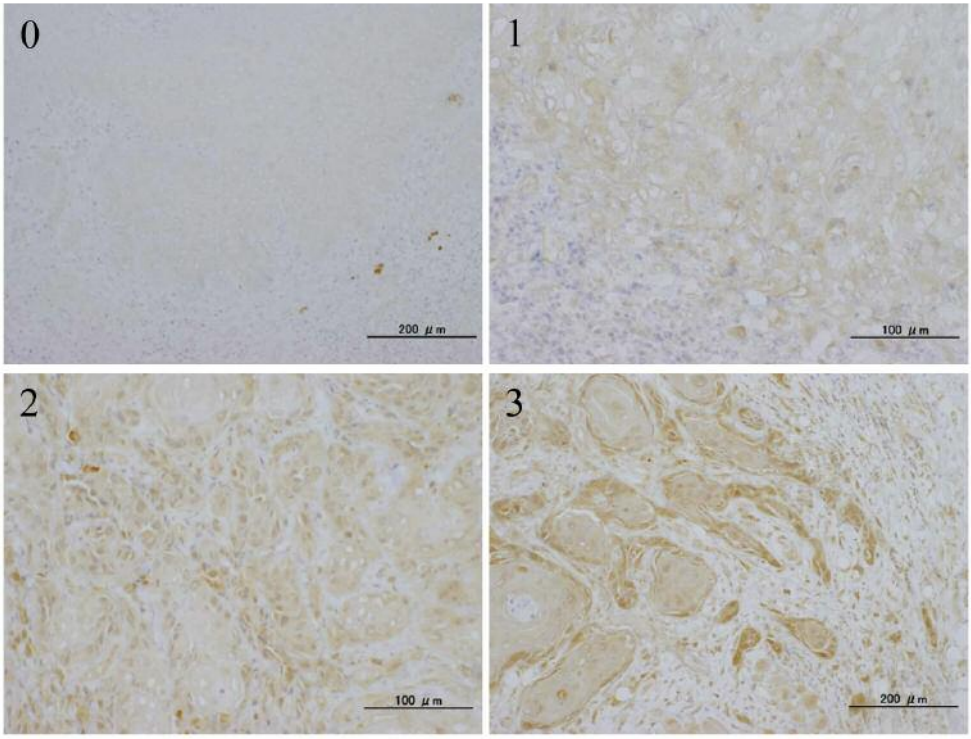

Figure 1. *Staining intensity scores of immunostaining for sphingosine kinase-1. The number in each panel shows representative staining intensity score.*

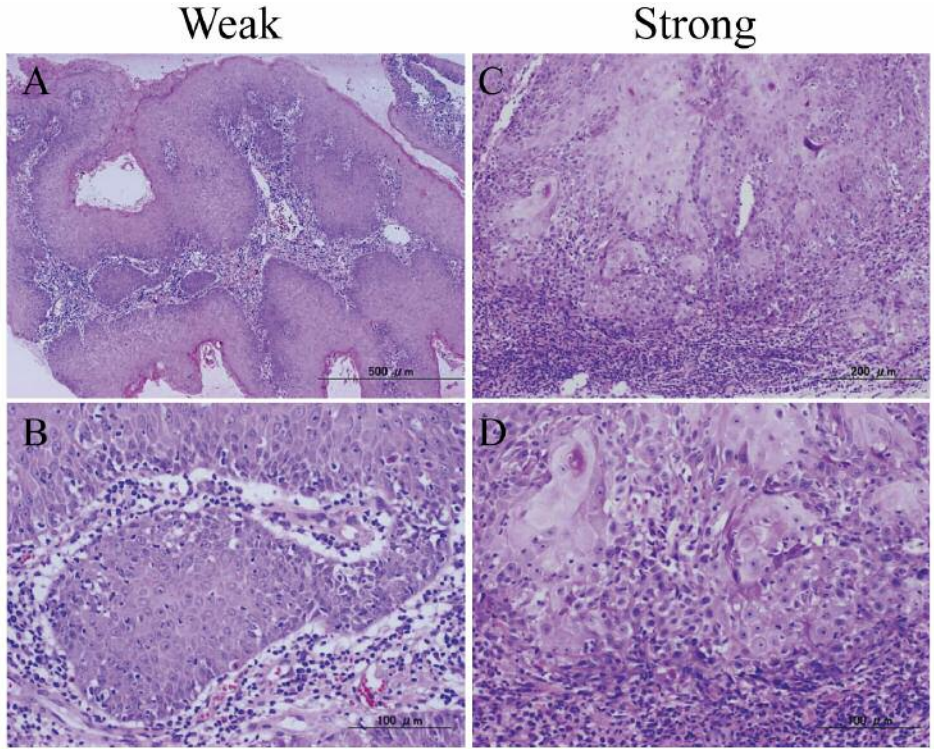

Figure 2. *Stromal fibroblastic reactions at the front of invasion of oral squamous cell carcinomas. Figures showing representative stromal fibroblastic reactions at the front of invasion of oral squamous cell carcinomas. A, B Show weak reaction; C, D show strong reaction. B, D are higher magnification of A, C, respectively. Hematoxylin and eosin stain.*

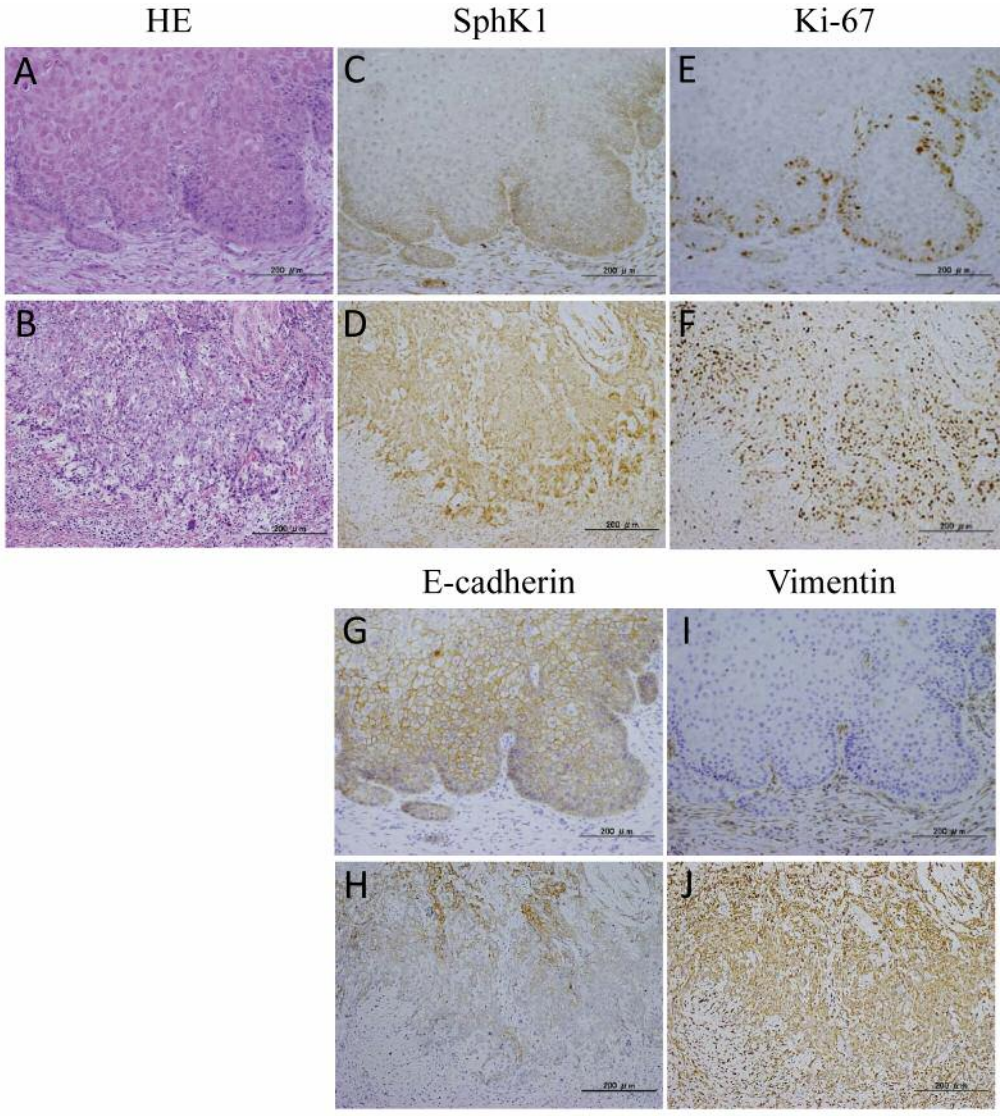

Figure 3. *Expression of sphingosine kinase-1, Ki-67, E-cadherin, and vimentin in oral squamous cell carcinoma of invasion grades 2 and 4C. A, C, E, G, and I are oral squamous cell carcinoma (OSCC) of invasion grade 2; B, D, F, H, I are OSCC of invasion grade 4C. A, B are hematoxylin and eosin stains; C, D immunostains for sphingosine kinase-1 (SphK1); E, F for Ki-67; G, H for E-cadherin; I, J for vimentin. OSCC of invasive grade 4C shows increased expression of SphK1, Ki-67 labeling, reduction of E-cadherin, and acquisition of vimentin expression at the front of invasion (D, F, H, I).*

invasiveness, and angiogenesis (12). The balance between the activities of ceramide and of S1P is critically regulated by SphK1, and it is called the sphingosine rheostat theory (13-15). Expression of SphK1 and its roles in cell proliferation/differentiation, apoptosis, angiogenesis, and prognosis of patients have been reported in esophageal, head and neck, breast, thyroid, and ovarian cancers, as well as astrocytomas (16-21). In OSCC, the expression, localization, and precise roles of SphK1 in tumor progression have not yet been clarified. In this report, the expression of SphK1 in human OSCCs was studied, and a correlation of SphK1 expression not with proliferation, but with invasive grades and prognosis was found, providing data suggesting that SphK1 facilitates the EMT.

Materials and Methods

Patients and tissue specimens. This study included 69 OSCC cases diagnosed and treated at the Department of Oral and Maxillofacial Surgery of Kanazawa Medical University Hospital from 1996 to 2014. The study was approved by the Ethics committee of Kanazawa Medical University. All tissue samples were biopsied prior to neo-adjuvant chemoradiotherapy. Histopathological classification and staging followed the criteria of the World Health Organization.

Immunohistochemistry. Immunostaining for SphK1, Ki-67, E-cadherin, and vimentin was performed using 10% buffered, formalin-fixed, paraffin-embedded thin sections according to the previous report (16). For antigen retrieval, sections were autoclaved at 121˚C for 4 min for SphK1, at 121˚C for 15 min for Ki-67, or microwaved for 15 min for E-cadherin and vimentin. After the quenching of endogenous peroxidase activity with 3% (v/v) hydrogen peroxide in methanol for 10 min and blocking non-specific binding of secondary antibodies with 10% normal goat or rabbit serum for 30 min, the sections were incubated overnight at 4˚C with affinity-purified anti-SphK1 rabbit polyclonal antibody (2.0 μg/ml, Sigma-Aldrich Inc., St. Louis, MO, USA), anti-Ki-67 mouse monoclonal antibody (0.46 μg/ml, Dako Denmark A/S, Glostrup, Denmark), anti-E-cadherin mouse monoclonal antibody (0.25 μg/ml, Dako North America Inc., Carpinteria, CA, USA), or anti-vimentin mouse monoclonal antibody (0.32 μg/ml, Novacastra, Newcastle-upon-Tyne, UK). The antibodies were visualized using the streptavidin-biotin-peroxidase technique (Histofine SAB-PO kit, Nichirei, Tokyo, Japan), followed by chromogen detection with diaminobenzidine (DAB, Nichirei, Tokyo, Japan). The sections were counterstained with Mayer's hematoxylin. Controls for immune-specificity were included in all experiments by omission of the primary antibody and its replacement with phosphate-buffered saline or with matching concentrations of normal rabbit or mouse IgG (data not shown).

Evaluation of staining. Immunostaining was evaluated at the invasive front of each tumor tissue. SphK1 staining was evaluated semi-quantitatively using a staining index and scoring system according to Li *et al*. (16). The sections were examined at 200× magnification using light microscopy, and, based on the percentage of positive tumor cells (PP), frequencies of expression were categorized into four grades: 0, no positive tumor cells; 1, <10% positive cells; 2, 10-50% positive cells; and 3, >50% positive cells. The staining intensity (SI) in cancer cells was categorized into four grades: 0, nil staining; 1, weak light yellow staining similar to SI in adjacent normal squamous epithelium; 2, staining in yellowish-brown; and 3, brown (Figure 1). When different extents of SI were evident within a sample, 10 areas were randomly selected, and the most intense was chosen as representative of immunoreactivity. The staining index score (SIS), a comprehensive criterion for immunoreactivity, was calculated as the multiplication product of PP and SI. The SISs were 0, 1, 2, 3, 4, 6, or 9; scores 0-4 were defined as low expression, and those ≥6 were defined as high expression. Immunoreactivities of E-cadherin and vimentin were evaluated with the same staining index and scoring system. The Ki-67 labelling index was calculated by counting 200 tumor cells. The extent of tumor cell invasion was classified according to the five grades proposed by Yamamoto *et al*. (5). The extent of fibroblastic reaction in the cancerous stroma at the invasive front was evaluated as nil, weak, or strong (Figure 2). All tissue sections were analyzed independently by two pathologists who were blinded to the clinicopathological variables, and the average values were calculated.

Western blotting. Immunoblotting for SphK1 of 12 OSCC tissues and 4 non-neoplastic oral mucosa tissues was performed according to Liu *et al*. (22). Proteins were extracted using buffer (50 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM magnesium acetate, 0.2 mM ethylenediamine tetraacetic acid, 1 mM phenyl-methyl-sulfonyl fluoride, and 1% sodium dodecyl sulfate). Extracted protein (10 μg) was applied to and electrophoresed on a 10% polyacrylamide gel, and then transferred to nitrocellulose membrane (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4˚C with anti-SphK1 rabbit polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) at a concentration of 2.0 μg/ml. After subsequent incubation with peroxidase-labeled goat anti-rabbit IgG antibody (Dako North America Inc., USA) for 1 hour at room temperature with vigorous washing, the nitrocellulose membrane was incubated with a chemiluminescence luminal reagent (Pierce, Rockford, IL, USA) and photographed digitally using the ImageQuant LAS4000 (GE Health Care Japan, Tokyo, Japan). All samples were standardized by immunoblotting using anti-β-actin mouse monoclonal antibody (Sigma-Aldrich Inc., St. Louis, MO, USA).

Statistical analysis. Correlations between the immunohistochemical reactivities of SphK1 and those of other proteins were evaluated using Spearman's rank correlation coefficients. Associations between immunohistochemical expression of SphK1 and clinicopathological parameters were calculated using the Mann-Whitney *U*-test. Survival curves were generated using the Kaplan-

Table I. *Clinicopathological characteristics and sphingosine kinase-1 expression*.

Characteristics	Mean±SD or n (%)	SphK1		*p*-Value
		Low (n=31)	High (n=38)	
Age, years	68.8±12.74	68.4±13.3	69.1±12.4	0.918
Gender				0.226
Male	39 (56.5%)	20	19	
Female	30 (43.5%)	11	19	
Primary				0.278
Tongue	23 (33.3%)	13	10	
Lower jaw gingival	18 (26.1%)	7	11	
Upper Jaw gingival	10 (14.5%)	6	4	
Buccal mucosa	9 (13.0%)	2	7	
Oral floor	6 (8.7%)	1	5	
Soft palate	2 (2.9%)	1	1	
Lip	1 (1.4%)	1	0	
Stage				0.749
I	12 (17.4%)	6	6	
II	27 (39.1%)	13	14	
III	12 (17.4%)	5	7	
IVA	16 (23.2%)	7	9	
IVB	2 (2.9%)	0	2	
Lymph node metastasis				0.016*
–	53 (76.8%)	28	25	
+	16 (23.2%)	3	13	
Mode of invasion (grade)				<0.001**
1	2 (2.9%)	2	0	
2	12 (17.4%)	11	1	
3	37 (53.6%)	17	20	
4C	14 (20.3%)	1	13	
4D	4 (5.8%)	0	4	
Fibroblastic reaction				0.043*
Weak	33 (47.8%)	19	14	
Strong	36 (52.2%)	12	24	

*p-Value <0.05, **p-Value <0.01. SD: Standard deviation; n: number; SphK1: sphingosine kinase-1.

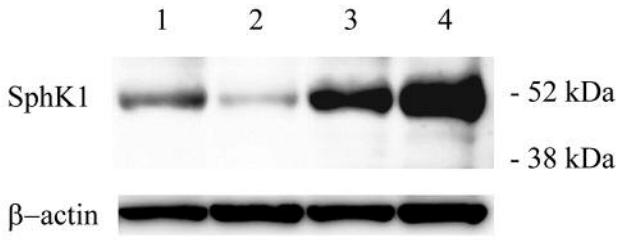

Figure 4. *Western blot for sphingosine kinase-1 in non-neoplastic oral mucosa and oral squamous cell carcinoma with various invasion grades. Lane 1, non-neoplastic oral mucosa; lane 2, oral squamous cell carcinoma (OSCC) of invasion grade 1; lane 3, OSCC of invasion grade 3; lane 4, OSCC of invasion grade 4C. Sphingosine kinase-1 (SphK1) is expressed at a molecular weight of about 52 kDa.*

Meier method and compared with the log-rank test. Survival data were evaluated by univariate and multivariate Cox's regression analyses. A p-value <0.05 was considered significant. All statistical analyses were performed using SPSS ver. 21.0 software for Windows (IBM, Armonk, NY, USA).

Results

Clinical information of patients examined. All clinicopathological data are summarized in Table I. The average age of the 69 patients examined was 68.8±12.7 years. There were 39 (56.5%) males and 30 (43.5%) females. The most common anatomical site was the tongue 33% (23/69), followed by the lower gingiva, the upper gingiva, the buccal mucosa, and the oral floor. By stage, 27 cases were stage II, followed by stage IVA, I, and III. Cervical lymph node metastases were seen on imaging evaluation in 16 patients (23.2%). With respect to invasiveness according to the grading system proposed by Yamamoto *et al*. (5), 37 tumors (53.6%) were grade 3, followed by grades 4C and 2; four tumors were grade 4D. A strong fibroblastic reaction at the invasive front was seen in 36 tumors (52.2%).

Expression of SphK1 in OSCC. Immunohistochemically, SphK1 was strongly expressed in the cytoplasm, as well as the cell membrane, of invading OSCC cells, particularly at the invasive front (Figure 3). SphK1 was also expressed weakly in the non-neoplastic epithelial and stromal tissues adjacent to the OSCC. Expression of SphK1 with molecular weight of about 52 kDa was demonstrated both in the non-neoplastic and the OSCC tissues. The average signal intensity of OSCC tissue was 6.23-fold stronger than that of normal tissue (Figure 4).

Expression of SphK1 and its correlations with clinicopathological features. Regarding the expression of SphK1, 31 OSCCs had low SIS values, and 38 OSCCs had high SIS values. High-SIS OSCCs metastasized to the cervical lymph nodes more frequently than low-SIS OSCCs (13/38, 34% *vs*. 3/31, 10%, p=0.016), although the SphK1 SIS was not correlated with the primary site or clinical stage. Mode of invasion had a strong relation with the SphK1 SIS (p<0.001); 20/37 (54%) of grade 3 OSCCs and almost all grade 4 OSCCs had a high SIS, while most grade 1 and grade 2 OSCCs had a low SIS. Moreover, a high SphK1 SIS was associated with a strong fibroblastic reaction adjacent to the invading tumor cells (p=0.043) (Table I).

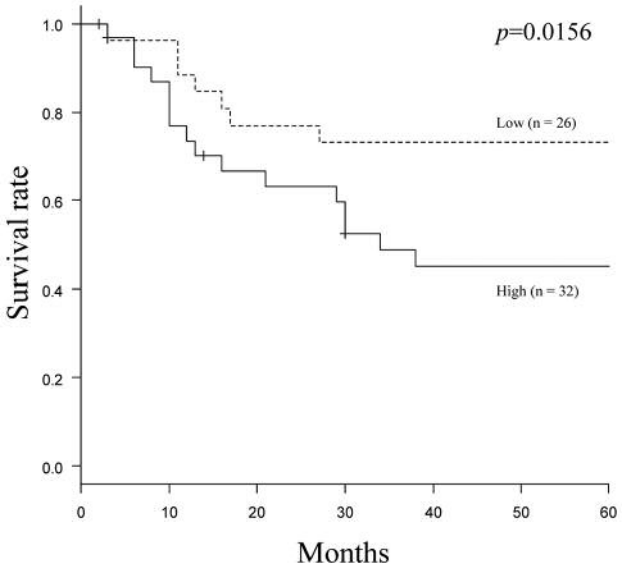

Figure 5. *Survival curves of patients with oral squamous cell carcinoma with low and high expressions of sphingosine kinase-1. Kaplan–Meier survival curves of patients with oral squamous cell carcinoma (OSCC) with low expression of sphingosine kinase-1 (SphK1) (dotted line) and of patients with OSCC with high expression of SphK1 (bold line). The 5-year survival rate of patients with high SphK1 expression is 45.1% (n=32), whereas that of patients with low SphK1 expression is 73.1% (n=26) (p=0.0156 by log-rank test).*

Correlation of SphK1 expression with patient survival. OSCC patients with a high SphK1 SIS had poorer survival rates than OSCC patients with a low SphK1 SIS (p=0.0156) (Figure 5); the 5-year survival rates of patients with OSCCs expressing high and low SphK1 SIS values were 45.1% and 73.1%, respectively. Univariate analysis showed that clinical stage, grades of invasion, and Sphk1 SIS and vimentin SIS were significant prognostic factors for OSCC patients; multivariate Cox's regression analysis showed that only clinical stage was an independent prognostic factor for OSCC patients (p=0.017); SphK1 SIS was a marginally independent prognostic factor (p=0.102) (Table II).

Table II. *Univariate and multivariate analyses of prognostic factors in patients with oral squamous cell carcinoma.*

Parameters	Univariate analysis *p*-Value	Multivariate Cox's regression analyses				
		HR	95%CI			*p*-Value
			Lower		Upper	
Age		1.018	0.974	-	1.063	0.433
Gender	0.427	1.472	0.582	-	3.723	0.414
Stage	0.0077**	1.780	1.110	-	2.855	0.017*
Mode of invasion (grade)	0.0245*	1.140	0.489	-	2.662	0.761
Fibroblastic reaction	0.613	0.488	0.166	-	1.434	0.192
SphK1	0.0156*	2.829	0.814	-	9.830	0.102
Ki-67 labelling index		0.982	0.937	-	1.028	0.434
E-cadherin	0.625	1.064	0.251	-	4.509	0.933
Vimentin	0.0261*	2.381	0.784	-	7.230	0.126

*p-Value <0.05, **p-Value <0.01. HR: Hazard ratio; CI: confidence interval; SphK1: sphingosine kinase-1.

Correlations of SphK1 expression with factors associated with proliferation and the epithelial-mesenchymal transition. OSCC with a high SphK1 SIS showed a higher Ki-67 labeling index than OSCCs with a low SphK1 SIS, but there was no significant difference between them (26.2±9.74 *vs.* 22.0±8.39, p=0.0599) (Table III). On the other hand, the SphK1 SIS was significantly correlated with decreased expression of E-cadherin and increased expression of vimentin in the tumor cells at the invasive front (Figure 3E-H): 36/38 (95%) of OSCCs with a high SphK1 SIS showed a low E-cadherin SIS, and 14/38 (39%) of OSCCs with a high SphK1 SIS expressed a high vimentin SIS, while OSCCs with a low SphK1 SIS showed a low E-cadherin SIS and a high vimentin SIS, 22/31(71%) and 3/31 (19%), respectively (p=0.0073 and 0.0092, respectively) (Table III).

Table III. *Correlations between sphingosine kinase-1 expression and molecules associated with proliferation and the epithelial-mesenchymal transition.*

	SphK1, mean±SD or n		*p*-Value
	Low (n=31)	High (n=38)	
Ki-67 labelling index (%)	22.0±8.39	26.2±9.74	0.0599
E-cadherin			0.0073*
Low	22	36	
High	9	2	
Vimentin			0.0092*
Low	28	24	
High	3	14	

*p-Value <0.01. SphK1: Sphingosine kinase-1; SD: standard deviation; n: number.

Discussion

The present immunohistochemical study demonstrated the expression of SphK1 in OSCC tumor cells, particularly at the front of invasion. The increased expression, which was confirmed by western blotting, correlated with the grade of invasion, degree of fibroblastic reaction adjacent to the invasive tumor cells, the frequency of metastases to cervical lymph nodes, and the prognosis of patients with OSCC. Furthermore, increased expression of SphK1 was associated not with proliferative activity, but with features of EMT, such as loss of E-cadherin and acquisition of vimentin expression of the invading OSCC cells.

SphK1 is an enzyme that synthesizes biologically-active S1P through phosphorylation of sphingosine derived from phospholipid of the cell membrane (23). SphK1 is, therefore, postulated to be localized near the cell membrane. The present immunostaining of SphK1, however, showed a positive reaction not only near the cell membrane, but also diffusely in the cytoplasm of tumor cells. Intracellular localization of SphK1 has previously been shown in in vitro studies, which may alter the access to substrate pools, but does not affect the degradative fate of S1P (24, 25). In the present study, the validity of intracellular localization of SphK1 was confirmed by its association with aggressive features and unfavorable prognosis of patients with OSCC with increased cytoplasmic expression of SphK1, as well as by the results of Western blot analysis.

S1P, being balanced with ceramid/sphingosine, was originally thought to be involved in cell proliferation and inhibition of apoptosis (10, 11, 13-15). Recent studies, however, disclosed novel functions of S1P, including cellular

migration, invasion, and angiogenesis (12). Studies of the expression of SphK1, which is a critical enzyme in synthesizing S1P from sphingosine, in several tumors reported correlations of SphK1 expression not only to proliferation and inhibition of apoptosis, but also to angiogenesis, invasiveness, and patient prognosis (16-20). OSCC is a unique neoplasm, whose histological invasion grades are closely related to aggressiveness, lymph node metastases, and patient prognosis (3, 5, 6). The close association of increased SphK1 expression with higher invasion grades, such as grades 3, 4, and 4, of OSCC demonstrated in the present study further supports the important role of SphK1 in cancer cell invasion. The increased expression of SphK1 was related to both metastases to cervical lymph nodes and an unfavorable prognosis of OSCC patients, although the expression of SphK1 was not an independent prognostic factor for OSCC patients. These results indicate that SphK1 is one of the important molecules involved in the invasive growth of OSCC.

EMT is a critical event in the invasion and metastasis of tumor cells, where disrupted cell-cell adhesion and acquisition of mesenchymal cytoskeletons permit tumor cells to migrate and invade a secondary site. Loss of cell adhesion and protein E-cadherin and gain of vimentin are a hallmark of the EMT (26). The present study showed the correlations of SphK1 expression to both reduced E-cadherin expression and acquisition of vimentin expression at the invasive front of OSCC, indicating a pivotal role of SphK1 in the EMT of OSCC. Involvement of SphK1 in the EMT of invasive carcinoma cells was suggested by previous studies, (27, 28) although the exact mechanism has not been clarified. Tamashiro *et al*. (2014) reported that SphK1 overexpression increased epidermal growth factor (EGF)-induced EGFR/ERK and AKT activities, increased matrix metalloproteinase (MMP)-2/9, and reduced E-cadherin through modulation of IL-6/STAT3 signaling in cultured SCC cells (29). Degradation of E-cadherin by MMP-9 can be involved in the reduction of E-cadherin (30). S1P, which is released by SphK1 activity and binds to S1P receptors, has been shown to be involved in the EMT by activating Rac-ERK, PI3K-AKT-rac, phospholipase C, and rho (31).

The microenvironment of tumor cells is also an important factor for invasion and metastasis (32, 33). The present study indicated the role of overexpressed SphK1 in activating stromal fibroblasts, which has not been reported so far, although previous studies showed increased tumor angiogenesis by SphK1 (34, 35). Elucidation of the exact mechanism of activation of tumor-associated fibroblasts of SphK1 is another important subject for a future study.

SphK1 is well known to increase cell proliferation. The present study, however, failed to demonstrate a correlation of SphK1 and the Ki-67 labeling index of OSCC cells at the invasive front. It may be one of the reasons that tumor cells surrounding the tumor nest, as well as basal cells of the non-neoplastic squamous epithelium, generally show high Ki-67 labeling.

A limitation of the present study is the lack of demonstration of S1P, a phosphorylated product of SphK1, and its receptors (S1PRs) in the OSCC tissues. We have been immunohistochemically examining the expressions of S1PR1, 2, 3, 4, and 5 in OSCC tumor tissues, and the data will be reported in our next publication. Furthermore, quantification of S1P in OSCC tissues by liquid chromatography is being planned.

In conclusion, the present study demonstrated that SphK1 is involved in the invasive growth of OSCC by facilitating EMT, and it is related to an unfavorable prognosis in OSCC patients, suggesting that the expression of SphK1 seems to be an objective factor that could be used to evaluate the invasiveness of OSCC. Moreover, SphK1 may be a promising target for controlling the aggressiveness of intractable OSCC.

Conflicts of Interest

None declared.

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