Clinical significance of L1CAM expression and its biological role in the progression of oral squamous cell carcinoma

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Abstract. L1 cell adhesion molecule (L1CAM) has been implicated in the progression and metastasis of numerous cancers. However, the role of L1CAM in oral squamous cell carcinoma (OSCC) is not well characterized. In the present study, the expression of L1CAM was examined in oral tongue squamous cell carcinoma (OTSCC) tissue samples by immunohistochemistry, the clinicopathological significance of L1CAM expression was evaluated by chi-squared test, and the overall survival (OS) rate was analyzed using Kaplan-Meier method according to the expression of L1CAM. In addition, it was aimed to elucidate the biological role of L1CAM and the underlying molecular mechanisms by which L1CAM functions in OSCC cells in relation to epithelial-mesenchymal transition (EMT) and PI3K/AKT/ERK signaling pathways. Thus, the functions of L1CAM on the OSCC cell proliferation, migration and invasion, and the activation of EMT and PI3K/AKT/ERK signaling pathways were investigated *in vitro*. Positive L1CAM expression was found in 32.5% of OTSCC cases and was significantly correlated with high histologic grade, greater depth of invasion, lymph node metastasis, perineural invasion, advanced stage, and survival status. Patients with positive L1CAM expression had significantly lower OS rate. Particularly in patients with early OTSCC, L1CAM expression was strongly associated with worse prognosis. Overexpression of the recombinant human L1CAM protein significantly increased cell proliferation, migration and invasion. By contrast, L1CAM knockdown using small interfering RNA significantly inhibited cell proliferation, migration, invasion and EMT. Moreover, phosphorylated (p)-PI3K, p-AKT and p-ERK expression levels were significantly reduced by L1CAM knockdown. Taken together, the findings of the present study suggested that L1CAM could be a potential prognostic marker and a promising therapeutic target in OSCC.

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Introduction

Oral cancer is a major health problem. According to the Globocan report (2020), an estimated 377,713 new cases of oral cancer and 177,757 deaths due to oral cancer occur annually across the world (1). Oral squamous cell carcinoma (OSCC) is the most common oral cancer which is characterized by a predilection for cervical lymph node (LN) metastases. OSCC is associated with poor clinical outcomes due to frequent nodal involvement and locoregional recurrence, resulting in survival rates of <50% (2). Along with LN metastasis, perineural invasion (PNI), another form of metastasis, has been widely recognized as a negative prognostic factor in several neurotropic cancers including OSCC (3). Oral tongue squamous cell carcinoma (OTSCC) is particularly neurotropic compared with OSCC in other regions due to its anatomical structure (4). PNI has been shown to correlate with the nodal status at the time of diagnosis, presence of occult neck metastases, and neck recurrence in head and neck SCC, particularly of the oral cavity origin (5). Therefore, developing optimal biomarkers related to both LN metastasis and PNI of OSCC may help predict the clinical outcomes, guide treatment decision-making and develop the therapeutic targets for the treatment of OSCC.

L1 cell adhesion molecule (L1CAM) is a 200-220 kDa transmembrane glycoprotein of the immunoglobulin superfamily that plays a role in neural development by promoting neural cell adhesion and migration (6). L1CAM also exerts its functions as a soluble form released from the cell surface by ADAM (a disintegrin and metalloproteinase) family of proteinases (7). Besides its expression in neural tissues, L1CAM is known to be expressed in normal tissues such as endothelial cells, renal collecting ducts and skin mast cells (8,9). During the past 20 years, research on L1CAM has expanded from the field of neurobiology to tumor biology. A growing body of evidence has revealed that L1CAM overexpression is associated with progression and metastasis of numerous human cancers, including melanoma and ovarian, endometrial and pancreatic cancers (10-13). In addition, L1CAM overexpression has been identified to correlate with PNI in certain

malignancies, including extrahepatic cholangiocarcinoma and pancreatic ductal adenocarcinoma (13,14). Thus, a linkage of L1CAM with LN metastasis and PNI is plausible.

L1CAM is known to activate different signaling pathways involved in tumor progression, such as PI3K/AKT and ERK pathways in several malignancies (9,15-17). Both these pathways have been mainly implicated in the pathogenesis of human cancers by regulating cell growth, differentiation, proliferation, migration and apoptosis (18). Nevertheless, whether L1CAM functions through these pathways in OSCC remains unclear. In the first study on the role of L1CAM in OSCC, Hung *et al* (19) demonstrated that L1CAM knockdown induced retardation of cell cycle at the G1 phase and inhibition of cell proliferation as well as attenuated the migration and invasion of OSCC cells; however, they did not investigate the related signaling pathway. Overexpression or knockdown of L1CAM *in vitro* has been demonstrated to induce changes in the expression levels of epithelial and mesenchymal markers, suggesting the involvement of L1CAM in the epithelial-mesenchymal transition (EMT) process of breast, endometrium, lung and oral cavity carcinomas (19-22). EMT is involved in cancer progression and metastasis and is regulated by multiple intracellular signaling networks, including PI3K/AKT and RAS/RAF/ERK axes (23). Thus, it can be hypothesized that L1CAM is involved in the progression and metastasis of OSCC by activating the PI3K/AKT/ERK pathways. If so, L1CAM appears to be a potential therapeutic target for the treatment of OSCC.

In the present study, the clinical significance of L1CAM was first investigated using OTSCC tissue samples. Next, *in vitro* functional assays were performed to elucidate the biological roles of L1CAM in the progression and metastasis of OSCC and to evaluate the possibility as a potential therapeutic target. Finally, the downstream pathways regulated by L1CAM in OSCC were explored in relation to EMT and PI3K/AKT/ERK pathways.

Materials and methods

Patients and tissue samples. Formalin-fixed paraffin-embedded tissue samples of 80 patients with OTSCC who were surgically treated at the Department of Oral and Maxillofacial Surgery at the Seoul National University Dental Hospital between January 2008 and December 2012 were included in the present study. The age of patients ranged from 27-83 years, with a median age of 56 years. Tumors were staged according to the TNM system recommended by the 8th Edition of the American Joint Committee on Cancer Staging Manual, Head and Neck Section. The clinicopathological characteristics of the patients including age, sex, differentiation status, tumor size (pT), depth of invasion (DOI), LN metastasis (including late metastasis), PNI, TNM stage, and recurrence are shown in Table I. All procedures followed in the present study were in accordance with the guidelines (approval no. CRI 20003) of the Institutional Review Board of the Seoul National University Dental Hospital (Seoul, Korea).

Immunohistochemistry. Tissue specimens were sectioned at 4-μm thickness, deparaffinized in Neo-clear (Merck KGaA) for 25 min, and rehydrated by passage through a graded alcohol series for 25 min. Heat-induced epitope retrieval was performed in Target Retrieval Solution pH 9 (Dako; Agilent Technologies, Inc.) for 10 min using a microwave. Endogenous peroxidase was inactivated by incubation in 3% H_2O_2 solution for 10 min. Immunohistochemical staining for L1CAM was performed using a mouse monoclonal anti-human L1CAM antibody (1:100; clone 14.10; cat. no. 826701; BioLegend Inc.) for 1 h at room temperature (RT). Slides were rinsed in DAKO wash buffer and then incubated for 30 min with peroxidase-labeled polymer conjugated to anti-mouse immunoglobulins (EnVision Detection System; Dako; Agilent Technologies, Inc.). The chromogenic reaction was carried out with DAB chromogen. All sections were counterstained with Mayer's hematoxylin for 1 min at RT.

Evaluation of immunohistochemical staining. All samples stained with anti-L1CAM antibody were independently evaluated by two oral pathologists (K-YO and H-JY) using a light microscope (BX53; Olympus Corporation). Nerve tissues in each slide were used as an internal positive control. L1CAM expression was considered positive if 10% or more of the tumor cells showed moderate to strong membranous staining, as previously described (12).

Cell lines. The Ca9-22, HSC-2, HSC-3, HSC-4 and SAS cell lines were kindly provided by professor Masanobu Shindoh of Hokkaido University (Hokkaido, Japan). The HN22 cell line was generously provided by the School of Dentistry, DanKook University (Cheonan, Korea). The cell lines from Japan were authenticated by Japanese Collection of Research Bioresources (JCRB) Cell Bank using the SRT profiling. Ca9-22 (cat. no. JCRB0625) cell line was derived from gingiva SCC; HN22 and HSC-2 (cat. no. JCRB0622) cell lines from unknown sites of oral cavity; and HSC-3 (cat. no. JCRB0623), HSC-4 (cat. no. JCRB0624) and SAS (cat. no. JCRB0260) cell lines from tongue SCC. All OSCC cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (Welgene, Inc.) containing 10% fetal bovine serum (FBS; Welgene, Inc.) and 100 U/ml penicillin and streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific, Inc.). RNA (1 μg) was reverse-transcribed using an AMPIGENE cDNA Synthesis Kit (Enzo Life Sciences, Inc.) according to the manufacturer's instructions. RT-qPCR for analyzing L1CAM expression level was performed using Applied Biosystems StepOne Plus Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Two independent experiments were performed in triplicate. The primer sequences used for RT-qPCR were as follows: L1CAM forward, 5'-ACGAGG GATGGTGTCCACTTCAAA-3' and reverse, 5'-TTATTGCTG GCAAAGCAGCGGTAG-3'; and β-actin forward, 5'-CACTCT TCCAGCCTTCCTTC-3' and reverse, 5'-AGCACTGTGTTG GCGTACAG-3'. L1CAM mRNA expression was measured using a SYBR Premix Ex Taq™ kit (Takara Bio, Inc.). β-actin expression was used as a reference. The thermocycling conditions suitable for L1CAM mRNA were the following: 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 sec and synthesis at 60˚C for 30 sec. The relative level of L1CAM mRNA was normalized to that of β-actin and calculated by the $2^{-\Delta\Delta Cq}$ method (24).

Table I. Clinicopathological characteristics of 80 patients with oral tongue squamous cell carcinoma.

Clinicopathological characteristics	Number
Median age at diagnosis, years	56
Range	27-83
Sex	
Male	48
Female	32
Differentiation	
Well	60
Moderately	20
Perineural invasion	
No	48
Yes	32
Tumor size	
T1	21
T2	38
T3	16
T4	5
Lymph node metastasis	
No	44
Yes	36
Stage	
I	19
II	24
III	11
IV	26

Western blotting. Whole cells were lysed in RIPA buffer and protein concentration was determined using the DC protein assay kit II (Bio-Rad Laboratories, Inc.). A total of 30 μg protein extract was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After being blocked by 5% non-fat milk at RT for 1 h, the membranes were incubated with the following primary antibodies diluted at 1:1,000: L1CAM (cat. no. SC-53386; Santa Cruz Biotechnology, Inc.), E-cadherin (cat. no. BD610181; BD Biosciences), N-cadherin (cat. no. BD610920; BD Biosciences), vimentin (cat. no. BD550513; BD Biosciences), Snail (cat. no. CST3879; Cell Signaling Technology, Inc.), Slug (cat. no. CST9585; Cell Signaling Technology, Inc.), Twist (cat. no. SC-81417; Santa Cruz Biotechnology, Inc.), AKT (cat. no. CST9272; Cell Signaling Technology, Inc.), p-AKT (cat. no. CST9271; Cell Signaling Technology, Inc.), and β-actin (cat. no. SC-47778; Santa Cruz Biotechnology, Inc.). After incubation with goat anti-rabbit (1:3,000; cat. no. GTX213110-01; GeneTex, Inc.) or anti-mouse secondary antibody (1:3,000; cat. no. GTX213111-01; GeneTex, Inc.) at RT for 2 h, the proteins were identified by SuperSignal West Pico Chemiluminescent Substrate (cat. no. SC-2048; Santa Cruz Biotechnology, Inc.), and immunoreactive bands were visualized using ImageQuant LAS 500 (Cytiva). Densitometric analysis of western blot bands was carried out using ImageJ software (version 1.51 k; National Institutes of Health).

L1CAM overexpression and knockdown. Recombinant human L1CAM (rhL1CAM) was purchased from R&D Systems, Inc. Cell viability was first assessed at concentrations of 0, 50, 100, 200, and 400 ng/ml, and a titer of 100 ng/ml of rhL1CAM was used for subsequent experiments.

ON-TARGETplus SMARTpool small interfering RNA (siRNA) targeting L1CAM (siL1CAM) and Non-targeting Control Pool (siControl) were purchased from GE Healthcare Dharmacon, Inc. This SMARTpool siRNA contains four pooled siRNAs, each targeting a separate region of the L1CAM RNA sequence. ON-TARGETplus Non-targeting siRNA (D-001810-10-20) was also used as a non-targeting control. The SMARTpool siRNA and non-targeting siRNA target sequences are as follows: ON-TARGETplus SMARTpool siRNA J-011069-05, L1CAM Target Sequence: CACUAC ACCUUUAGGGUUA; ON-TARGETplus SMARTpool siRNA J-011069-06, L1CAM Target Sequence: GCAAGA GACAUAUCCACAA; ON-TARGETplus SMARTpool siRNA J-011069-07, L1CAM Target Sequence: GAUACAAUG UGACGUACUG; ON-TARGETplus SMARTpool siRNA J-011069-08, L1CAM Target Sequence: ACACAAUGGUGA CCCAAUG; and ON-TARGETplus Non-targeting pool Target Sequences: UGGUUUACAUGUCGACUAA, UGGUUUACA UGUUGUGUGA, UGGUUUACAUGUUUUCUGA and UGGUUUACAUGUUUUCCUA.

Transfection was performed using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, anti-L1CAM siRNA and Lipofectamine 2000 were mixed according to the protocol and allowed to form the siRNA-Lipofectamine 2000 lipoplexes at RT for 20 min. HSC-4 or HN22 cells was seeded on 60-mm plates and transfected transiently with 100 nM transfection complex. After 24 h of transfection, subsequent wound healing or Transwell migration/invasion experiment was performed.

Cell proliferation assay. A total of $3x10^5$ cells were seeded in six-well plates and incubated overnight. After treatment with 100 ng/ml of rhL1CAM or 100 nM of siL1CAM for 24 h, cells were incubated with 5% CO_2 at 37˚C for 24, 48 and 72 h. Viable cells were stained with 0.4% trypan blue solution (Gibco; Thermo Fisher Scientific, Inc.) at RT for 5 min and counted using a hemocytometer at each time-point.

Wound healing assay. Wound healing assay was carried out as previously described (25). Ca9-22, HSC-2, HSC-4, and HN22 ($3x10^5$ cells) were seeded in six-well plates. After being cultured for 24 h, cells were treated with rhL1CAM (100 ng/ml) or siL1CAM (100 nM) using Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.). After 24 h, the center of the cell monolayers was scratched vertically with a sterilized 100-μl pipette tip. After rinsing with PBS three times for removing cell debris, the cells were incubated in 5% FBS-containing medium for 12 h to enable wound healing. Images were captured at 0 and 12 h using an inverted microscope (CKX53; Olympus Corporation) and the wound dimensions were measured using ImageJ software (version 1.51 k; National Institutes of Health).

Transwell migration and Matrigel invasion assays. For Transwell migration assay, Ca9-22 ($0.8x10^5$), HSC-2 ($0.8x10^5$), HSC-4 ($1.8x10^5$), and HN22 ($1.8x10^5$) cells in serum-free

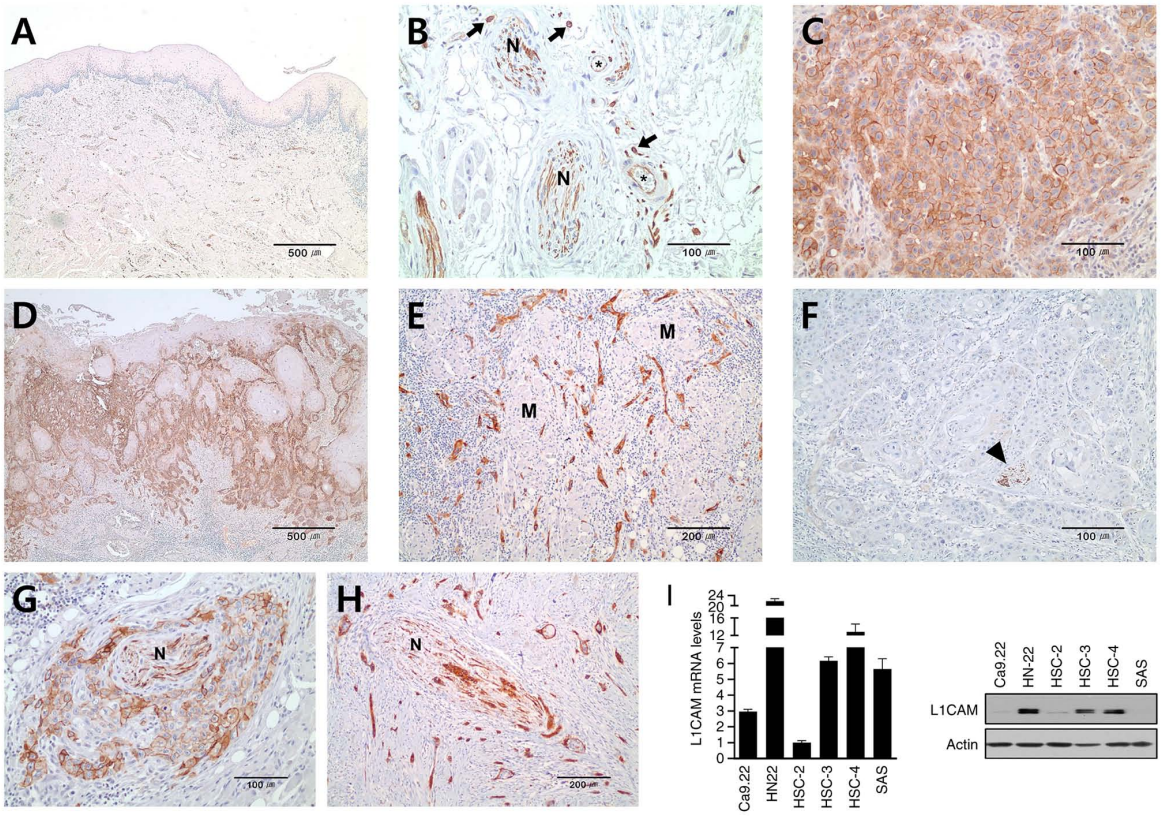

Figure 1. L1CAM expression in oral tongue squamous cell carcinoma tissues and OSCC cell lines. (A) Negative expression in the epithelium of normal tongue mucosa. (B) Positive expression in the nerve tissues (N), vascular endothelial cells (asterisks), and mast cells (arrows) in the connective tissue area. (C) Strong membranous expression in the tumor cells. (D) Strong expression at the invading area of OSCC as compared with the negative expression in upper well-differentiated cells. (E) Strong expression in the spindle-shaped tumor cells infiltrating into the muscle (M) bundles. (F) Negative expression in a relatively well-differentiated, keratinizing tumor (Arrowhead indicates nerve tissue as an internal positive control). (G) Strong expression in the tumor cells showing perineural invasion. (H) L1CAM expression in the tumor cells inside the nerve (N). (I) Expression levels of L1CAM mRNA and protein by reverse transcription-quantitative CR and western blot analysis. Relative high expression was detected in HN22 and HSC-4 cell lines. L1CAM, L1 cell adhesion molecule; OSCC, oral squamous cell carcinoma.

medium were seeded in the upper chambers of 24 well plate with Collagen type I-coated PET membrane of 8.0-μm pore size (BD Biosciences). For invasion assay, culture inserts of 24-well plate were coated with Matrigel (BD Biosciences) in a 37˚C incubator for 2 h. Ca9-22 ($1.0x10^5$), HSC-2 ($1.0x10^5$), HSC-4 ($1.2x10^5$), and HN22 ($1.2x10^5$) cells in serum-free medium were seeded in the upper chambers. Lower chambers were filled with the media containing 10% FBS as a chemoattractant. After incubation for 24 h, the non-migratory or non-invasive cells in upper chamber were removed with a cotton swab. Cells on the lower surface of the filter were fixed with 100% methanol for 2 min and stained with H&E solution. Images of migratory or invasive cells were captured under an inverted light microscope (CKX53; Olympus Corporation) and the number of migratory or invasive cells were counted in randomly selected areas in three different microscopic fields (magnification, x100).

Statistical analysis. Statistical analyses were performed using SPSS software (version 26.0; IBM Corp.). Correlation of L1CAM expression with clinicopathological parameters was assessed with Pearson's Chi-squared test or Fisher's exact test. Survival curves were generated using the Kaplan-Meier method and between-group differences assessed using the log-rank test. For *in vitro* experiments, the mean ± standard deviation values from three independent experiments are reported. Statistical significance was analyzed using a two-tailed Student's t-test for paired samples. One-way ANOVA followed by Tukey's post hoc test analyses were used for multiple-group comparisons. For all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

L1CAM expression in OSCC tissues and cell lines. L1CAM expression was not detected in the epithelium of the normal oral mucosa (Fig. 1A), but was strongly expressed in the peripheral nerve tissue, vessels and mast cells (Fig. 1B). In OTSCC tissue samples, tumor cells showed a membranous pattern of expression of L1CAM (Fig. 1C). Of the 80 clinical samples, 26 (32.5%) samples were positive for L1CAM. Tumor cells exhibiting moderate to strong expression were frequently found at the invasive front of tumors or in the less-differentiated and usually spindle-shaped tumor cells (Fig. 1D and E), while negative expression was observed in relatively well-differentiated tumors (Fig. 1F). In the area of PNI, tumor cells expressing L1CAM were found in the perineural tissues or even inside the nerves (Fig. 1G and H).

mRNA and protein levels of L1CAM were examined in six OSCC cell lines (Fig. 1I). HSC-3, HSC-4 and HN-22 presented relatively high levels of L1CAM compared with other cell lines

Table II. Correlation of PNI with clinicopathologic parameters in oral tongue squamous cell carcinoma.

Clinicopathological characteristics	Total cases	PNI		P-value
		Negative (%)	Positive (%)	
	n=80	n=48	n=32	
Age				0.235[a]
<56	39	26 (66.7)	13 (33.3)	
≥56	41	22 (53.7)	19 (46.3)	
Sex				0.192[a]
Male	48	26 (54.2)	22 (45.8)	
Female	32	22 (68.8)	10 (31.3)	
Differentiation				0.292[a]
Well	60	38 (63.3)	22 (36.7)	
Moderately	20	10 (50.0)	10 (50.0)	
Tumor size				0.001[a]
T1 + T2	59	42 (71.2)	17 (28.8)	
T3 + T4	21	6 (28.6)	15 (71.4)	
Depth of invasion				<0.001[a]
≤0.5 cm	30	28 (93.3)	2 (6.7)	
0.5~1.0 cm	25	15 (60.0)	10 (40.0)	
>1.0 cm	25	5 (20.0)	20 (80.0)	
Lymph node metastasis				<0.001[a]
No	44	35 (79.5)	9 (20.5)	
Yes	36	13 (36.1)	23 (63.9)	
Stage				0.001[a]
I + II	43	33 (76.7)	10 (23.3)	
III + IV	37	15 (40.5)	22 (59.5)	
Local recurrence				0.734[b]
No	71	42 (59.2)	29 (40.8)	
Yes	9	6 (66.7)	3 (33.3)	
Survival status				0.002[a]
Alive	62	43 (69.4)	19 (30.6)	
Dead	18	5 (27.8)	13 (72.2)	

PNI, perineural invasion; [a]Pearson's chi-squared test; [b]Fisher's exact test.

(Ca9.22, HSC-2 and SAS). Thus, Ca9.22 and HSC-2 were used for confirming the effect of L1CAM upregulation induced by treatment with rhL1CAM. By contrast, HSC-4 and HN-22 were used for determining the effect of L1CAM downregulation using siL1CAM.

L1CAM overexpression in OSCC significantly correlates with poor clinical outcomes. To assess the correlation of L1CAM expression with clinicopathological parameters, including PNI, PNI was determined more objectively according to the commonly accepted definition by Liebig *et al* (3): i) Tumor in close proximity to nerve and involving at least 33% of its perimeter or ii) tumor cells within any of the three layers of the nerve sheath. PNI was detected in 40.0% of 80 OTSCC samples. PNI, which is a known adverse prognostic factor in OSCC, revealed a significant correlation with pT (P=0.001), DOI (P<0.001), LN metastasis (P<0.001), advanced clinical stage (P=0.001) and survival status (P=0.002) (Table II). L1CAM expression showed significant correlation with high histologic grade (P=0.013), greater DOI (P=0.009), LN metastasis (P<0.001), presence of PNI (P<0.001), advanced stage (P=0.004), and survival status (P=0.018) (Table III). These findings suggested high clinical relevance of L1CAM in relation to cancer invasion, metastasis, and progression.

Kaplan-Meier survival analysis was performed to evaluate the prognostic significance of LN metastasis, PNI, and L1CAM expression for overall survival (OS) of patients with OSCC. OTSCC patients with PNI or LN metastasis demonstrated significantly worse OS rate (P=0.001 and P=0.007, respectively; Fig. 2A and B). As for a novel marker, L1CAM, patients with positive expression of L1CAM showed significantly lower OS than those with negative expression (P=0.014; Fig. 2C).

Table III. Correlation of L1CAM expression with clinicopathologic parameters in oral tongue squamous cell carcinoma.

		L1CAM		
Clinicopathological characteristics	Total cases	Negative (%)	Positive (%)	P-value
Age				0.747[a]
<56	39	27 (69.2)	12 (30.8)	
≥56	41	27 (65.9)	14 (34.1)	
Sex				0.079[a]
Male	48	36 (75.0)	12 (25.0)	
Female	32	18 (56.3)	14 (43.8)	
Differentiation				0.013[a]
Well	60	45 (75.0)	15 (25.0)	
Moderately	20	9 (45.0)	11 (55.0)	
Tumor size				0.238[a]
T1 + T2	59	42 (71.2)	17 (28.8)	
T3 + T4	21	12 (57.1)	9 (42.9)	
Depth of invasion				0.009[a]
≤0.5 cm	30	26 (86.7)	4 (13.3)	
0.5~1.0 cm	25	16 (64.0)	9 (36.0)	
>1.0 cm	25	12 (48.0)	13 (52.0)	
Lymph node metastasis				<0.001[a]
No	44	37 (84.1)	7 (15.9)	
Yes	36	17 (47.2)	19 (52.8)	
Perineural invasion				<0.001[a]
No	48	40 (83.3)	8 (16.7)	
Yes	32	14 (43.8)	18 (56.3)	
Stage				0.004[a]
I + II	43	35 (81.4)	8 (18.6)	
III + IV	37	19 (51.4)	18 (48.6)	
Local recurrence				0.710[b]
No	71	47 (66.2)	24 (33.8)	
Yes	9	7 (77.8)	2 (22.2)	
Survival status				0.018[a]
Alive	62	46 (74.2)	16 (25.8)	
Dead	18	8 (44.4)	10 (55.6)	

L1CAM, L1 cell adhesion molecule; [a]Pearson's chi-squared test; [b]Fisher's exact test.

L1CAM expression was found to have a more significant effect on the decrease of OS rate in patients with T1/2 tumor, N0 tumor, or stage I/II tumor (P=0.012, P=0.001 and P=0.003, respectively; Fig. 2D-F). These findings suggested that expression of L1CAM in early OSCC may be an indicator of poor prognosis.

Overexpression of L1CAM increases cell proliferation, migration and invasion of OSCC cells in vitro. To evaluate the function of L1CAM in cell proliferation, migration and invasion in OSCC, Ca9-22 and HSC-2 cell lines were treated with rhL1CAM. The viability of Ca9-22 cells increased in a concentration-dependent manner up to 400 ng/ml of concentration of rhL1CAM, while viability of HSC-2 cells increased gradually up to 100 ng/ml of concentration and there was no change >100 ng/ml (**P<0.01 and ***P<0.001; Fig. 3A). Overexpression of L1CAM significantly increased proliferation capacity of both cell lines at each time-point (***P<0.001; Fig. 3B). Next, wound healing assay revealed that upregulation of L1CAM significantly increased the migration of both cell lines (***P<0.001; Fig. 3C). Wound closure in the rhL1CAM-treated groups increased more than two-fold compared with that of control groups in both cell lines. Lastly, Transwell migration and Matrigel invasion assays also revealed significantly increased migration and invasion ability in rhL1CAM-treated groups of both cell lines compared with the corresponding control groups (***P<0.001; Fig. 3D). Collectively, these findings indicated that overexpression of L1CAM can promote cell

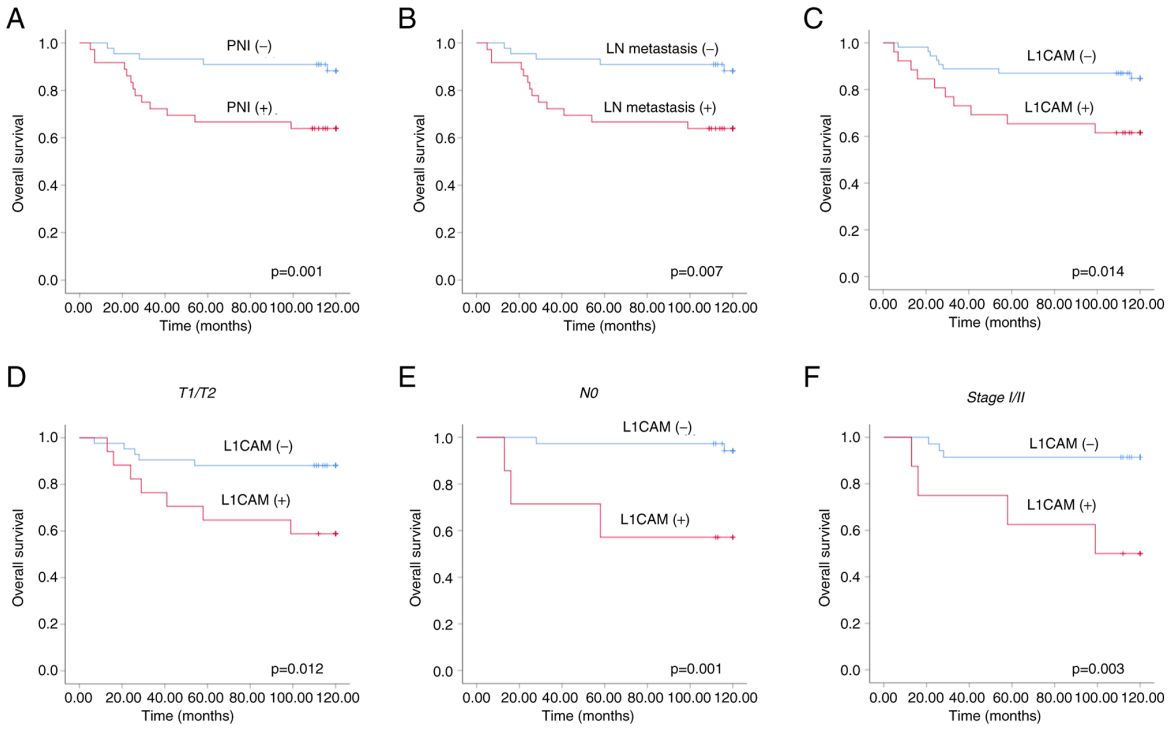

Figure 2. Kaplan-Meier survival curves. (A-C) OS in relation to (A) PNI, (B) LN metastasis and (C) expression of L1CAM. Overexpression of L1CAM is significantly associated with the presence of PNI and cervical LN metastasis. (D-F) In patients with early oral squamous cell carcinoma, L1CAM overexpression is predictive of worse OS in relation to (D) T1/2 tumor, (E) N0 tumor or (F) stage I/II tumor. OS, Overall survival; PNI, perineural invasion; LN, lymph node; L1CAM, L1 cell adhesion molecule.

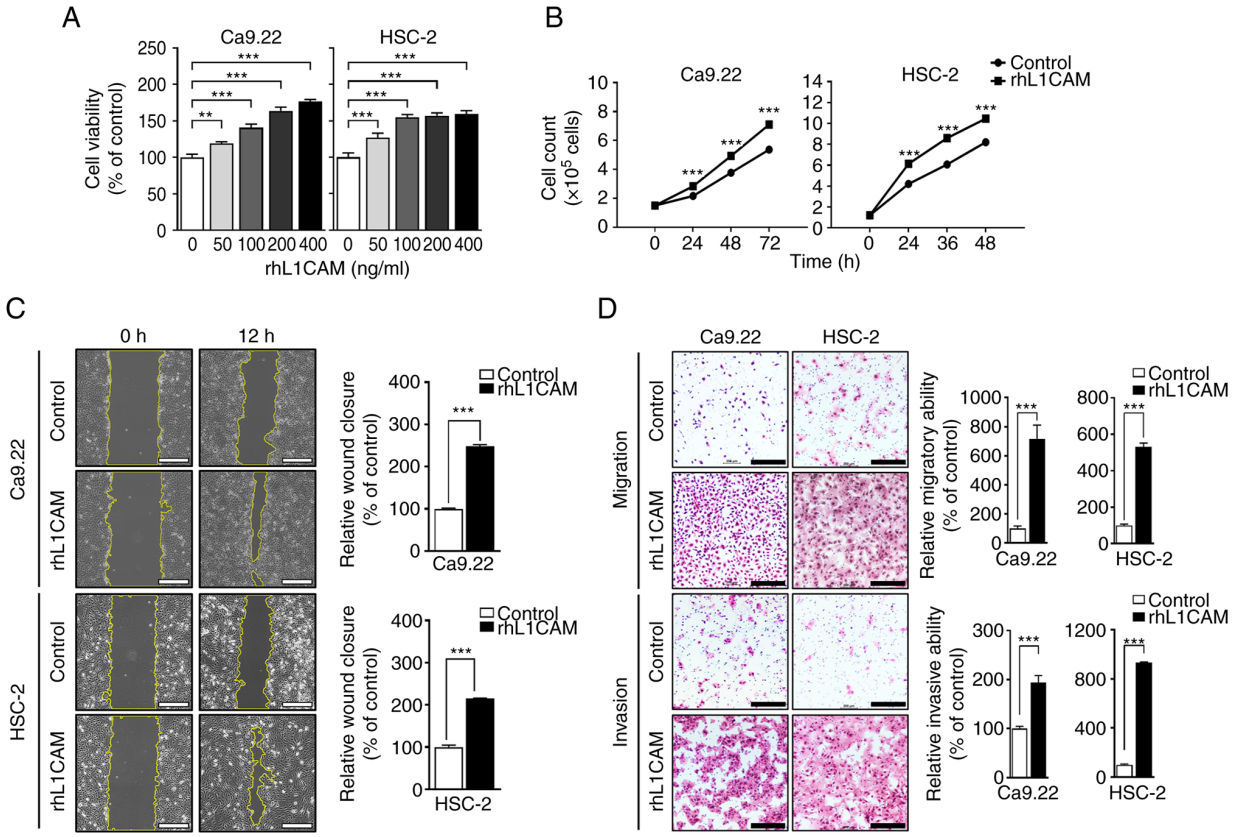

Figure 3. Effect of L1CAM overexpression on the cell proliferation, migration and invasion of oral squamous cell carcinoma cells. (A) With the treatment of rhL1CAM protein, cell viabilities of Ca9-22 and HSC-2 cells increased in a concentration-dependent manner. (B) There was statistically significant increase in the proliferation of both Ca9-22 and HSC-2 cell lines at each time point. (C) Wound healing assay demonstrated significant increase of the cell migration in both cell lines (Scale bar, 400 μm). (D) Transwell migration and Matrigel invasion assays revealed significant increase in the migration and invasion abilities of both cell lines compared with the control groups (Scale bar, 400 μm). **P<0.01 and ***P<0.001. L1CAM, L1 cell adhesion molecule; rh-, recombinant human.

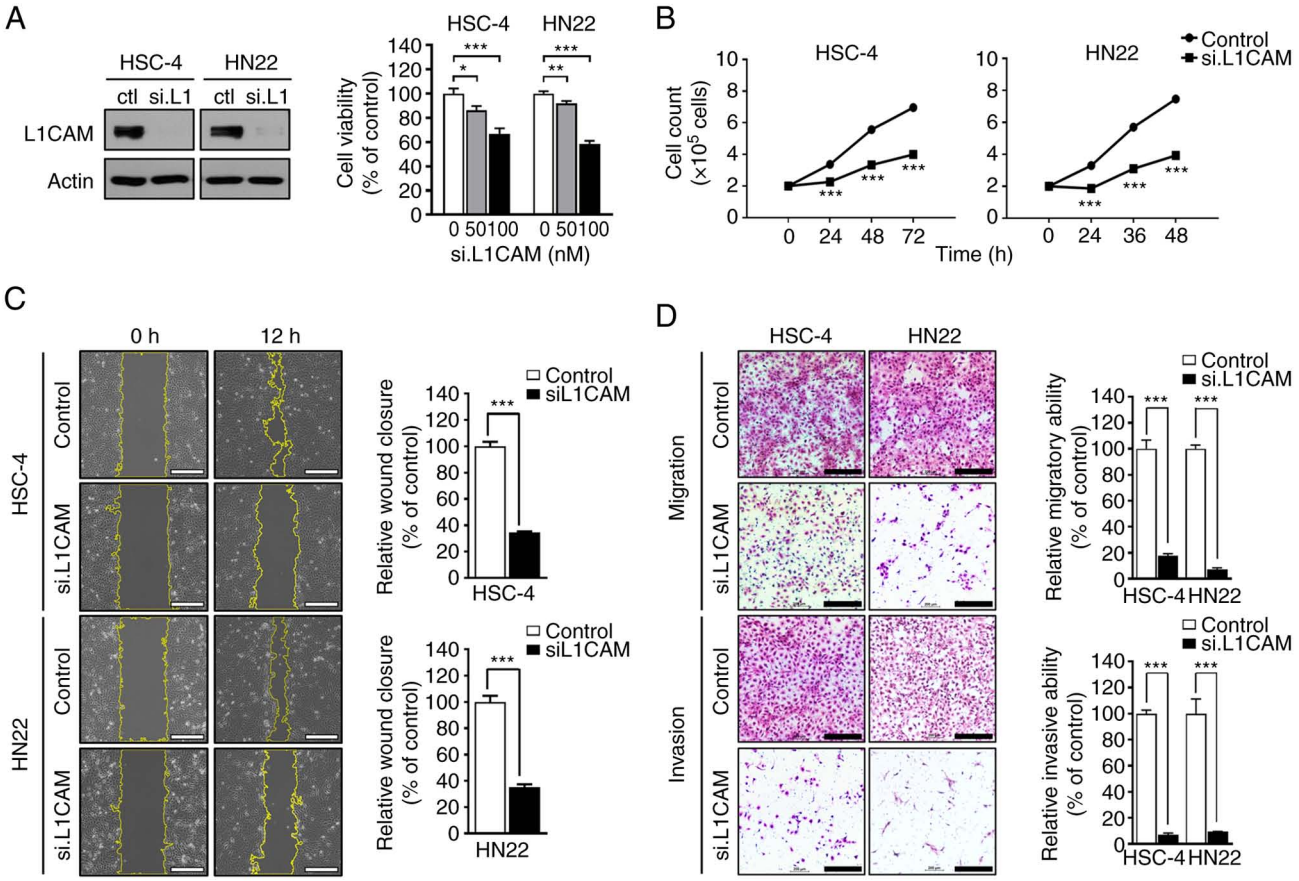

Figure 4. Effect of L1CAM knockdown on the cell proliferation, migration and invasion of oral squamous cell carcinoma cells. (A) After the treatment of anti-L1CAM siRNA, HSC-4 and HN22 cell lines revealed significant reduction of cell viability in a concentration-dependent manner. (B) Knockdown of L1CAM expression significantly reduced the proliferation capacity of both HSC-4 and HN22 cell lines at each time-point. (C) Wound healing assay showed a significant decrease of the migration in both cell lines (Scale bar, 400 μm). (D) Transwell migration and Matrigel invasion assays demonstrated significant suppressions of the migration and invasion abilities in both cell lines (Scale bar, 400 μm). *P<0.05, **P<0.01 and ***P<0.001. L1CAM, L1 cell adhesion molecule; si-, small interfering.

proliferation, migration, and invasion, suggesting its important role in the progression of OSCC.

Downregulation of L1CAM inhibits cell proliferation, migration and invasion and induces apoptosis of OSCC cells in vitro. To assess the effect of L1CAM knockdown, both HSC-4 and HN22 cells were treated with 100 nM siL1CAM for 24 h. HSC-4 and HN22 cell lines revealed decreased cell viability in a concentration-dependent manner (*P<0.05, **P<0.01 and ***P<0.001; Fig. 4A). Suppression of L1CAM expression significantly decreased the proliferation capacity of both cell lines at each time-point (***P<0.001; Fig. 4B). Next, in wound healing assay, L1CAM knockdown significantly affected the migration ability of both cell lines. The ability of wound closure in siL1CAM-treated groups of both cell lines decreased more than three-fold compared with that in the corresponding control groups (***P<0.001; Fig. 4C). Lastly, Transwell migration and Matrigel invasion assays demonstrated significantly decreased cell migration and invasion ability in siL1CAM-treated groups of both cell lines compared with the control groups (***P<0.001; Fig. 4D). These results indicated that knockdown of L1CAM can inhibit proliferation, migration and invasion, suggesting its possibility to serve as a therapeutic target.

L1CAM is involved in EMT and PI3K/AKT and ERK signaling pathways during tumor progression. Based on the effect of L1CAM in enhancing the migration and invasion of OSCC cells, the association of L1CAM with EMT was assessed. After L1CAM knockdown, changes in the expression of EMT-associated markers were assessed by western blotting. As revealed in Fig. 5A, expression of epithelial cell marker E-cadherin was increased, whereas mesenchymal markers including N-cadherin, vimentin, snail, slug and twist were significantly decreased after L1CAM knockdown. In other words, suppression of L1CAM was found to reverse EMT. These results suggested the involvement of L1CAM in the EMT process of OSCC cells.

The PI3K/AKT and ERK pathways are the main signaling pathways regulating cell proliferation, differentiation, survival, motility and apoptosis. Dysregulation of these pathways is known to contribute to carcinogenesis and cancer progression (18). In order to determine whether the functions of L1CAM in OSCC are mediated through the PI3K/AKT and ERK signaling pathways, the effect of L1CAM knockdown on the activation of these pathways was investigated. As revealed in Fig. 5B, no change was observed in the expression of PI3K/AKT and ERK1/2 in the HSC-4 and HN22 cell lines; however, there was significant decrease

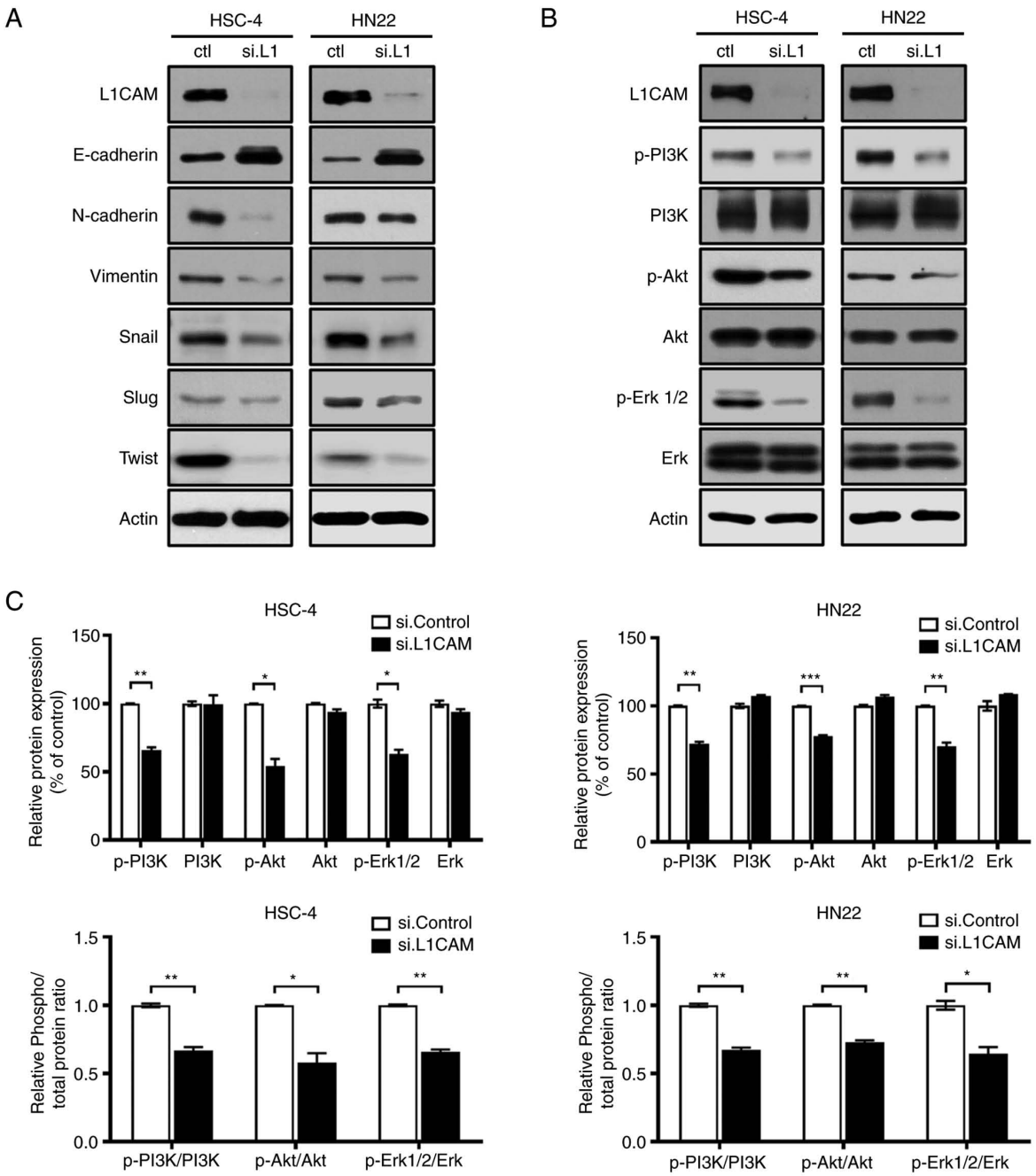

Figure 5. L1CAM is involved in the epithelial-mesenchymal transition process and functions through the PI3K/AKT and ERK signaling pathways. (A) When L1CAM expression is knocked down, expression of E-cadherin is significantly increased, whereas expression levels of N-cadherin, vimentin, snail, slug, and twist are decreased correspondingly. (B) Knockdown of L1CAM expression induces a remarkable decrease of p-PI3K, p-AKT, and p-ERK1/2 levels. (C) Relative protein expression levels and the ratio of phosphorylated/total protein were shown as bar-chart graphs (*P<0.05, **P<0.01 and ***P<0.001). L1CAM, L1 cell adhesion molecule; p-, phosphorylated; si-, small interfering.

of p-PI3K, p-AKT and p-ERK1/2 levels. According to the densitometric analysis, relative protein expression levels and the ratio of p-/total protein were evaluated with bar-chart graphs (*P<0.05, **P<0.01 and ***P<0.001; Fig. 5C). These data indicated that L1CAM likely plays a role in the progression of OSCC via both the PI3K/AKT and ERK pathways.

Discussion

Over the past two decades, L1CAM overexpression has been identified in numerous different types of cancer including ovarian, endometrial, pancreatic, colorectal and gastric cancers. Moreover, L1CAM expression has been identified to be a valuable prognostic marker in these cancers (6,11-13,16,26). In these tumors, L1CAM expression is significantly related with aggressive tumor characteristics including high histologic grade, LN metastasis, advanced stage and worse survival. Moreover, L1CAM expression has been shown to significantly correlate with the presence of PNI in extrahepatic cholangiocarcinoma and pancreatic ductal adenocarcinoma (13,14). In a previous study on OSCC by Hung *et al* (19), L1CAM overexpression was closely associated with high histological grade of

the tumor; however, the association of L1CAM overexpression with other clinicopathological parameters was not assessed. In addition, a recent study by Adnan *et al* (27) demonstrated that L1CAM expression was not correlated with any clinicopathological parameters and survival of patients in OSCC. In the present study, however, a significant correlation between L1CAM expression and high histologic grade, greater DOI, cervical LN metastasis, presence of PNI and advanced TNM stage was observed in OTSCC. These results were inconsistent with those of Adnan *et al* (27). This may be due to difference in the scoring method of immunohistochemical staining. Adnan *et al* (27) scored by multiplying the intensity of staining with the percentage of positive cells, whereas a cutoff (10% of tumor cells) was used in the present study as it was used in most studies of endometrial carcinoma in which the prognostic value of L1CAM has been well established (12,28,29). Although Adnan *et al* (27) reported that L1CAM had no effect on either OS or disease-free survival in OSCC, our survival data from 10 years of follow-up showed significantly reduced OS in patients with positive L1CAM (P=0.014). In the present study, the most notable result was that L1CAM expression was more significantly associated with worse OS rate in patients with early OTSCC, such as T1/2, N0, or stage I/II tumors (P=0.012, P=0.001 and P=0.003, respectively). To solve the discrepancy between the different studies, further studies on the reliable evaluation method for L1CAM immunostaining in OSCC are needed in the future. According to the present results, L1CAM appears to be a potential biomarker that is closely related to the progression of OSCC and predict poor prognosis in patients with OSCC.

Although OSCC cell lines originating from various parts of the oral cavity were used for *in vitro* experiments, the cohort was intentionally constituted of patients only with OTSCC due to the anatomical preference of tongue for PNI and the easiness to observe the resected whole specimen by serial sections. Gingival SCC tissues were excluded to avoid errors in PNI detection resulting from demineralization process which could interfere the observation of H&E-stained soft tissue and induce inaccurate results of immunohistochemical staining. In the present study, a significant association was observed between L1CAM and PNI in OTSCC (P<0.001). This is very notable due to several reasons. First, PNI is a key pathologic feature of head and neck SCC; however, the reported rates of PNI have ranged from 5.2-90% due to methodological inconsistencies (e.g., detection in a limited number of H&E-stained slides and subjective interpretation of PNI) (5). Therefore, auxiliary diagnostic markers are required for a more objective detection of PNI. Although the association between L1CAM and PNI has been reported only in certain cancers (13,14), L1CAM is considered as one of the molecules involved in the development of PNI through the paracrine interaction between Schwann cells and cancer cells (30). This suggests a potential role of L1CAM as a marker of PNI. Second, PNI has been considered as an independent predictor of cervical metastasis, neck recurrence and worse survival in head and neck SCC, including OTSCC (4,5,31). Particularly, cN0 T1/2 OTSCC with PNI was suggested as an indication for adjuvant radiation or elective neck dissection due to the high risk of occult metastasis leading to neck recurrence (32,33). Therefore, use of potential molecular marker of PNI, possibly L1CAM, may help inform more rigorous evaluation of PNI by microscopy and facilitate optimal neck management.

Regarding the expression pattern in OTSCC tissue samples, L1CAM expression was more frequently found at the invasive front or in less-differentiated, spindled tumor cells. These findings are consistent with those of previous studies on endometrial, pancreatic and colorectal cancers (21,34,35). Moreover, L1CAM expression was significantly associated with DOI (P=0.009) and presence of LN metastasis (P<0.001) in the present study of OTSCC, suggesting that L1CAM may play a role in the invasion and metastasis of OSCC cells during tumor progression. The present *in vitro* functional assays revealed the direct effects of L1CAM on the migration and invasion of OSCC cells. Overexpression of L1CAM induced significant increase in proliferation, migration and invasion of both Ca9.22 and HSC-2 cells, whereas suppression of L1CAM significantly reduced these attributes in both HSC-4 and HN22 cells. In a previous study by Hung *et al* (19), shRNA knockdown of L1CAM in SCC4 cells (tongue SCC cell line) overexpressing L1CAM strongly attenuated cell proliferation, migration and invasion. Besides, it was found that these phenomena were parallel to changes in the expression of EMT-related molecules in SCC4 cells. These findings are consistent with the current results that L1CAM knockdown could reverse the EMT phenotype, possibly resulting in significant inhibition of migration and invasion of tongue SCC cell line (HSC-4 cell line). The ability of L1CAM to promote migration and invasion has also been confirmed in breast, gastric, pancreatic and esophageal cancer cell lines (15,20-22,36,37). Several studies have also demonstrated the relationship between L1CAM and EMT process of carcinoma (20-22). Shtutman *et al* (20) suggested for the first time the functions of L1CAM during EMT process in MCF7 breast carcinoma cells. In case of endometrial carcinomas, L1CAM was upregulated at the invasive front of tumor, whereas expression of E-cadherin, one of key epithelial markers, was downregulated in the same area (21). In lung SCC tissues, L1CAM expression was increased at the tumor-stroma interface rather than at the tumor center, and at the same time, E-cadherin expression was decreased, and slug expression was increased in the same area (22). Collectively, L1CAM can play a role in the progression of OSCC by participating in the EMT process similar to that observed in other malignancies.

Given the involvement of L1CAM in the PI3K/AKT or ERK signaling pathways in several cancers (9,13,15-17,26), it was investigated whether inhibition of L1CAM expression affects the activity of those downstream molecules in OSCC cell lines. Downregulation of L1CAM reduced the phosphorylation of PI3K, AKT and ERK, indicating that L1CAM is linked to both signaling pathways in OSCC and regulates cell proliferation, migration and invasion possibly through these pathways. In the study by Silletti *et al* (38), L1CAM was revealed to induce sustained activation of the ERK pathway and the concomitant expression of ERK-related gene products such as integrin $\alpha_v\beta_3$, resulting in increasing cell mobility and invasion. Additional studies have demonstrated a close association between L1CAM and ERK in pancreatic, gastric and colorectal cancers (13,15,16). Moreover, L1CAM-dependent activation of the PI3K/AKT signaling pathway has been reported in bile duct and gastric cancers (17,26). L1CAM overexpression or knockdown was found to increase or decrease phosphorylation of AKT in

cholangiocarcinoma (17). In gastric cancer, inhibition of PI3K or AKT suppressed L1CAM-induced cancer cell migration and invasion (26). Given that inhibition of ERK/PI3K/AKT pathway-associated molecules is an attractive anticancer therapeutic strategy, further in-depth studies are needed on the exact mechanism by which L1CAM promotes OSCC progression through these pathways.

Considering that L1CAM contributes to various cellular events during tumor progression, *in vitro* and *in vivo* studies have been conducted to develop the therapeutic approach targeting L1CAM (17,39,40). In the present study, inhibition of L1CAM by siRNA significantly reduced proliferation and migration/invasion of OSCC cells *in vitro*, which suggests that L1CAM may be a potential molecular target to hinder OSCC progression. Hung *et al* (19) also demonstrated that downregulation of L1CAM by shRNA could suppress the tumor growth and metastasis of OSCC cells in an animal model. Arlt *et al* (39) investigated the effects of anti-L1CAM monoclonal antibodies on ovarian carcinoma. It was found that L1CAM-directed antibody significantly inhibited the proliferation of ovarian carcinoma cells *in vitro*; in addition, it reduced pT and inhibited peritoneal growth and dissemination of cancer cells in ovarian carcinoma-bearing mice. Several subsequent studies using tumor xenograft models have demonstrated the inhibitory effect of L1CAM-blocking antibodies on tumor growth and metastasis in intrahepatic cholangiocarcinoma, pancreatic and ovarian carcinoma (17,40). Schäfer *et al* (40) reported the therapeutic potential of combined treatment with L1CAM antibodies and cytostatic drugs in pancreatic and ovarian carcinomas. These cancers are representative of tumors that are associated with poor prognosis and show poor response to conventional chemotherapy; therefore, a novel antibody treatment targeting L1CAM may overcome the limitation of traditional treatment. In addition to antibody-based approach, *in vivo* targeting by intratumoral administration of liposome-encapsulated L1CAM siRNAs effectively inhibited prostate cancer growth in mouse bone (41). These findings strongly suggested the plausibility of L1CAM being a major driver of tumor growth and metastasis, and it is likely to serve as a promising therapeutic target in OSCC. However, further *in vivo* studies are required to confirm the effect of L1CAM inhibition on OSCC. Subsequent study using mouse model is in progress in the authors' lab.

In conclusion, expression of L1CAM in OSCC tissues showed significant correlation with aggressive tumor characteristics including DOI, LN metastasis, presence of PNI and poor survival rate. Based on the high association of L1CAM with LN metastasis and PNI, L1CAM could be considered a potential prognostic biomarker in patients with OSCC, particularly in those with early tongue tumor. Furthermore, L1CAM was found to play a role in the progression of OSCC by promoting cell proliferation, migration and invasion, likely via both the PI3K/AKT and ERK signaling pathways. These findings suggested that L1CAM could serve as a promising therapeutic target in OSCC.

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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

JHK contributed to the conduction of *in vitro* experiments, data acquisition and interpretation, and preparation of the manuscript. KWL contributed to the conduction of immunohistochemical study, data acquisition and analysis, and preparation of the manuscript. DGA contributed the collection of clinical data and statistical analysis. KYO contributed to the editing of figures and statistical analysis. HJY contributed to the conception, design of experiment, revision of the manuscript, and supervision of the overall aspects of the project. KYO and HJY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures followed in the present study were in accordance with the guidelines (approval no. CRI 20003) of the Institutional Review Board of the Seoul National University Dental Hospital (Seoul, Korea). A waiver of informed consent was approved by the IRB due to the retrospective nature of the study and the analysis of anonymized data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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