치의과학박사 학위논문

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ABSTRACT

The role of L1CAM in oral squamous cell carcinoma and its clinical relevance

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Objectives: L1 cell adhesion molecule (L1CAM) is a 200-220 kDa transmembrane glycoprotein which has been involved in not only the development of nerve system but also the progression and metastases of many different types of cancers. However, the roles of L1CAM in the progression of oral squamous cell carcinoma (OSCC) have not been clearly established yet. In the present study, the oncogenic roles of L1CAM in OSCC and the possibility as a therapeutic target were investigated by the *in vitro* functional assays. In addition, the molecular mechanisms underlying the L1CAM-induced tumor progression in OSCC were examined in relation to epithelial-mesenchymal transition (EMT) and PI3K/AKT/MAPK signaling pathways. Finally, the expression of L1CAM was examined in OSCC tissue samples to analyze the correlations between L1CAM expression and clinicopathological parameters including perineural invasion (PNI).

Methods: Effects of L1CAM on cell viability, migration, and invasion were investigated using four OSCC cell lines (Ca9.22, HSC-2, HSC-4, and HN22) by *in vitro* assays. Changes in the expression of various markers related to EMT and the PI3K/AKT and MAPK signaling pathways were assessed by western blot analysis. Eighty tissue samples from tongue OSCC patients were immunohistochemically stained for evaluating L1CAM expression. The associations of L1CAM expression with clinicopathological parameters were analyzed with Fisher's exact or Pearson's chi-squared tests.

Results: When L1CAM was overexpressed by the recombinant L1CAM protein in Ca9.22 and HSC-2 cell lines showing relatively low L1CAM expression, there were significant increases of cell proliferation, migration, and invasion compared to the control groups without treatment (all $P < 0.001$, respectively). Contrariwise, when L1CAM expression was suppressed by anti-L1CAM siRNA in HSC-4 and HN22 cell lines showing relatively high L1CAM expression, there were significant decreases of cell proliferation, migration, and invasion compared to the control group (all $P < 0.001$, respectively). Western blot analysis demonstrated that downregulation of L1CAM induced a significant increase of E-cadherin expression and decrease of expression of N-cadherin, vimentin, Snail, Slug, and Twist, reversing the EMT process. In addition, p-PI3K, p-AKT, and p-ERK1/2 expressions were reduced in L1CAM knockdown cell lines. In OSCC tissue samples, PNI was detected in 40.0% of cases and showed the significant association with tumor size ($P = 0.001$), depth of invasion ($P = 0.000$), lymph node (LN) metastasis ($P = 0.001$), and TNM stage ($P = 0.001$). L1CAM expression was positive in 32.5% of 80 cases and significantly correlated with moderate differentiation ($P = 0.013$),

deep DOI ($P = 0.009$), positive LN metastasis ($P = 0.002$), PNI ($P = 0.000$), and advanced stage ($P = 0.004$).

Conclusions: L1CAM seems to be involved in the progression of OSCC by increasing the proliferation, migration, and invasion of OSCC cells, promoting EMT activity, and stimulating PI3K/AKT and MAPK signaling pathways. Expression of L1CAM in OSCC tissues significantly correlates with LN metastasis and advanced clinical stage as well as presence of PNI in OSCC. These findings suggest that L1CAM play an important role in tumor progression and metastasis of OSCC and could be a reliable biomarker for predicting clinical outcome and a promising therapeutic target in OSCC.

Keywords: L1CAM, oral squamous cell carcinoma, perineural invasion, epithelial-mesenchymal transition, prognosis

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I. Introduction

Oral cancer including lips and oral cavity is the sixteenth common malignant tumor in the world and more than 370,000 oral cancer patients occur every year. Also, more than 177,000 people died because of oral cancer (the 16th leading cause of death worldwide).[1] Especially, squamous cell carcinoma (SCC) accounts for more than 90% of head and neck areas.[2] For the past 20 years, many studies have been conducted for revealing the mechanisms of oral squamous cell carcinoma (OSCC) and many molecules have been developed as a target for cancer therapy, but the patient's survival rate still does not exceed 50%.[3] In the majority of oral cancer patients, frequent cervical lymph node metastasis and advanced clinical stage due to the late detection of cancer get worse the patient's prognosis. Therefore, finding optimal biomarkers that influence cancer progression is important to improve clinical outcomes of OSCC.

L1 cell adhesion molecule (L1CAM) is a 200-220 kDa transmembrane glycoprotein of the immunoglobulin (Ig) superfamily composed of six Ig-like domains and five fibronectin type III repeats followed by a transmembrane region and a highly conserved cytoplasmic tail.[4] L1CAM can be released from the cell surface by a disintegrin and metalloproteinase (ADAM) family of proteinases proximal to the membrane, resulting in soluble L1.[5, 6] During the last 20 years, new functions of L1CAM in cancer have been found in addition to the physiologic roles in the neural development. L1CAM overexpression has been reported in melanoma,[7] ovarian cancer,[8] gastric cancer,[9] and pancreatic cancer.[10] L1CAM seems to be involved in tumor progression, metastasis, poor prognosis, and short survival rate in many human organs.[11] In addition, a couple of studies showed that the association might exist between perineural invasion and L1CAM

in extrahepatic cholangiocarcinoma and pancreatic ductal adenocarcinoma.[12, 13] However, few studies have been found about the relation between L1CAM and OSCC.

Perineural invasion (PNI) is the process of invasion of malignant cells through nerves. PNI was reported first in mid-1800s, in relation to the cancer development of the head and neck areas.[14, 15] PNI has been studied as one of important routes of cancer metastasis in several cancers, including pancreatic, prostate, colorectal and gastric cancers.[16-19] These studies revealed that PNI significantly correlated with LN metastasis, regional recurrence, and worse overall and disease-free survival rates. Head and neck SCC also shows PNI with various incidence rates from 6 – 82 %.[20-27] According to the recent studies, the presence of PNI has been considered an independent predictor for cervical LN metastasis at the time of diagnosis, post-operative neck recurrence, and poor survival in T1/2 OSCC.[23, 28] Thus, cN0 patients showing PNI may have the occult metastasis in the neck, indicating the necessity of adjuvant radiotherapy or elective neck dissection. Although there has been some debate, the 2014 guideline of head and neck cancer treatment in National Comprehensive Cancer Network (NCCN) described the presence of PNI as an indication for adjuvant radiotherapy.[29] However, PNI detection under a microscope is not a simple procedure when it is determined only by H&E stained images and MR imaging may fails to detect microscopic PNI foci, resulting in underestimation of perineural spread.[30] Therefore, additional biomarkers could be helpful for detecting PNI presence and predicting LN metastasis in OSCC.

Epithelial-mesenchymal transition (EMT) is a cellular event which is essential for invasion and metastasis of carcinoma. Although EMT is a physiologic process occurring in embryogenesis and normal regeneration such as wound healing, it can be also observed during cancer initiation and progression. During EMT process, cancer cells lose their

epithelial characteristics and gain the features of mesenchymal cells, leading to invasion, plasticity, and metastasis.[31] L1CAM has been known to be involved in EMT development via various cell signaling cascades such as PI3K/AKT and MAPK signaling pathways, which are essential for cell proliferation, migration, and survival.[32, 33] L1CAM also appears to play a role in EMT of OSCC and consequently promotes invasion and metastasis of OSCC cells.[34]

In this study, *in vitro* functional assays and signaling pathway analyses were performed to elucidate how L1CAM affects the behavior of OSCC cells to enhance the tumor progression. Next, the correlations between L1CAM expression and clinicopathological parameters including presence of PNI in OSCC tissues were assessed by the immunohistochemical study. Eventually, we determined if L1CAM could be a potential prognostic biomarker or a therapeutic target for cancer treatment.

II. Review of Literature

History and structure of L1CAM

The cell-surface antigen L1 (now called L1CAM) was found in the cerebellar cortex of the mouse during neuron formation and development.[35] In the central nerve system, L1CAM has functions of differentiation, outgrowth and guidance of axons and dendrites,[36] and subsequently it was found in some cancer cells.[37, 38] L1CAM is suggested with potential tumor development on many tumor cell lines including melanoma and carcinomas.[39, 40] Initially, L1 antigen isolated from cultured cerebellar cells of mouses consisted of a band in the 140 (faintly) – 200 (mainly) kd range, but now L1CAM is known as a 200 – 220 kDa transmembrane glycoprotein and a member of the Ig-like superfamily.[41] It consists of six Ig-like domains and five fibronectin type III repeats, a transmembrane region and cytoplasmic domains. To understand the roles of L1CAM, it is necessary to know its structure of each domain.

Several reviews were published to better understand complex L1CAM structures.[42-44] The six Ig domains linked to one another except the link between Ig2 – Ig3. There are seven – residue peptide sequences (ATNSMID) between Ig2 – Ig3 region, which are hydrophilic and flexible leading to horseshoe-shape and zipper-like binding of L1CAM.[45] In addition, Ig2 domain has the ability of L1-L1 homophilic binding interaction.[46] Also, there is only one Arg-Gly-Asp (RGD) sequence in the Ig6 domain of L1CAM[47] and this RGD sequence of L1CAM is related to β1-integrin heterophilic binding.[48] Not only Ig domains are involved in L1CAM binding, but also fibronectin type III repeats are related to L1CAM *cis*- or *trans*- interaction.[49] There are protein-

carbohydrate and/or carbohydrate-carbohydrates binding interactions between fibronectin repeats.[50]

Signaling mechanisms of L1CAM

L1CAM does not have the ability of direct intrinsic kinase activity by itself, but it is associated with various adapter molecules such as integrins, protein kinases, or growth factor receptors.[51] Because of L1CAM structure which has a long ectodomain and relatively short cytoplasmic domain, different functions are present depending on binding sites and corresponding partners. L1CAM ectodomains can bind integrins, neurocan and L1CAM *cis*- or *trans*- forms with diverse interactions and cytoplasmic domains can interact with ezrin, AP2, Ankryin.[52-57]

So far, three L1CAM-related signaling pathways have been discovered. At first, mitogen-activated protein kinases (MAPK) pathways have been found to be involved in L1CAM signaling. L1CAM physically activates MAPK cascade components Raf-1 and initiate the sequence of Rac, MEK and MAPK via the Src tyrosine kinase.[33, 58] ERK pathway is considered as one of major determinants in the cellular activities such as proliferation and motility.[59] L1CAM induces ERK activity resulting in promoting motility and invasion-related transcription genes such as integrin β_3-subunit and the outcomes are increasing migration and invasiveness in NIH-3T3 cells.[60] It is reported for L1CAM to activate ERK in various areas such as gastric cancer,[61] ovarian carcinoma,[62] and pancreatic cancer.[63]

NF-κB signaling pathway is another important role of L1CAM-mediated cell growth and metastasis. IL-1β has been known to activate inflammation that increases

carcinogenesis and activates downstream NF-κB signaling cascade.[64] L1CAM-integrin binding promotes IL-1β and secreted IL-1β stimulates NF-κB pathway in pancreatic PT45-P1 cancer cells.[65] The cytoplasmic domain of L1CAM binds directly to the cytoskeleton protein ezrin.[55] Gavert et al. showed that overexpression of NF-κB p65 subunits are present to increase cell proliferation and motility in colorectal cancer (CRC), L1CAM-ezrin-activated NF-κB colocalize at the invasive front of human CRC tissue, so they concluded that L1CAM-ezrin binding is essential for metastasis.[66]

The other pathway is the proteolytic cleavage of L1CAM structure. L1CAM can be cleaved by the metalloproteinase ADAM 10 [67] and ADAM 17 [68] and released ectodomain L1CAM becomes 200 kDa soluble active L1CAM which promotes cell motility and integrin-mediated migration.[69] Shed soluble L1CAM can be accumulated in the ECM and increases the chance of autocrine/paracrine binding $\alpha_v\beta_5$ integrins, which provokes the phosphorylation of ERK (p-ERK).[70, 71] Remaining 32 kDa cytoplasmic L1CAM is cleaved by γ-secretase/presenilin complex, resulting in the L1-intercellular domain (ICD).[72] This L1-ICD translocates into the nucleus and takes part in gene regulation which is involved in tumor progression and invasion.[73]

Epithelial-Mesenchymal Transition (EMT)

The EMT is a reversible cellular process which transforms epithelial cells into mesenchymal cells firstly described by Greenberg and Hay.[74] EMT occurs in normal embryogenesis,[75] wound healing,[76] and cancer progression.[77] Normal epithelial cells have apical-basal polarity and tightly attached to adjacent cells and basement membranes by tight junctions and adherence junctions which are related to epithelial cadherin (E-cadherin), occludins and claudins. During the development of EMT, E-cadherin is

suppressed, which leads to the loss of polygonal morphology of epithelial cells. The cells change into spindle-shaped mesenchymal cells and express molecular markers such as neural cadherin (N-cadherin), vimentin and fibronectin. These mesenchymal phenotype cells degrade the basement membrane and can initiate invasion and metastasis to other sites.[78]

There are numerable markers which are related to EMT process. E-cadherin is one of the most remarkable markers routinely used in cancer diagnosis, because E-cadherin is the key player of making cell polarity and organizing epitheliums.[79] Typically, carcinoma cell lines which lost E-cadherin expression showed invasiveness and dedifferentiation.[80] Moreover, transfection with E-cadherin can change highly mesenchymal and invasive cells into well-differentiated epithelial-like cells.[80-82] N-cadherin, which is the opposite side of E-cadherin, have also been studied. Blocking of N-cadherin shows the ability of reducing cell-migration and tumor progression.[83, 84] Vimentin, a 57kDa intermediate filament protein, is expressed mainly in connective tissue mesenchymal cells of adults,[85] so it is considered as one of standard markers in EMT.[86] Downregulation of vimentin can make cancer cells inhibit growth, local recurrence and metastasis.[87]

Besides EMT effectors such as E-cadherin and vimentin, transcription factors play a key role as EMT regulators. The roles of transcription factors are proliferation, invasion and migration of epithelial cancer cells. Snail/Slug is a zinc finger-containing transcription factor required for embryogenesis [88] and it triggers epithelial to mesenchymal transitions.[89, 90] This tumorigenic function of Snail/Slug is related with downregulation of E-cadherin and occludin expression.[91-93] Twist also suppresses E-cadherin expression by binding directly to E-boxes on the E-cadherin promoter and induces the expression of

mesenchymal markers, such as N-cadherin and fibronectin.[94] ZEB1/2 has similar functions by inhibiting epithelial polarity, differentiation and cell-to-cell adhesion.[95, 96]

EMT is induced by a variety of signaling pathways. TGFβ has an important role in not only wound healing and fibrosis, but also the acquisition of a mesenchymal phenotype and the promotion of invasion and metastasis.[97, 98] TGFβ binds TGFβ-type I receptors and induces EMT via both the SMAD3 and SMAD1/5 pathway.[99] TGFβ-induced SMAD complexes activate EMT transcription factors (Snail, Slug, Twist, and Zeb1/2), which suppress E-cadherin and express TGFβ receptors, enabling to maintain EMT.[100] WNT signaling (WNT-Frizzed-LRP5/6) is also related to EMT program by accumulating β-catenin which binds to the transcription factors such as TCF (T cell factor) and LEF (lymphoid enhancer-binding factor) for activating EMT.[101, 102] The NOTCH receptors can bind the Delta-like or Jagged family, which triggers proteolytic cleavage and NOTCH is changed to intracellular fragment of NOTCH (NOTCH-ICD).[103] The NOTCH-ICD translocates to the nucleus and leads to expression of Snail and Slug.[104] In addition, there are PI3K-AKT pathway, p38 MAPK pathway, the RAS-RAF-MEK-ERK pathway and JAKs-STATs pathway involved in EMT phenomenon (Please see a recent review by Dongre and Weinberg [105]).

Since EMT was discovered at the invasive front in the colorectal carcinomas by histopathological analysis,[106] EMT has been found in OSCC, too. WNT family is involved in one of the EMT signaling pathways and Uraguchi et al. showed that OSCC cell lines expressed 11 of 19 WNT family members and WNT3 expression and nuclear localization of β-catenin were predominant at the invasive front using immunohistochemical analysis in 28/42 (57%) specimens.[107] The Src family of proteins shows downregulation of E-cadherin and upregulation of vimentin in OSCC cell lines

and there is a significant correlation between Src expression and aggressive tumor characteristics such as invasiveness and lymph node metastasis.[108]

Perineural Invasion (PNI)

Perineural invasion (PNI) is the process of cancer cell invasion through nerves. Although this phenomenon has been found in the extensive literature, the definition of PNI is subjective. In 1985, Batsakis suggested the first, but broad definition of PNI which is neurotropism by carcinomas referring to invasion in, around, and through the nerves.[109] The current accepted definition of PNI proposed by Liebig et al. is tumor in close proximity to nerve and involving at least 33% of its circumference or tumor cells within any of the 3 layers of the nerve sheath.[110] However, this current definition is also criticized, because "close proximity "can be interpreted differently and "33%" is an empirical number.[111, 112]

Generally, PNI is associated with poor clinical outcomes in cancers of many organs and several meta-analysis reviews have been published recently. Zhao et al. showed that there were strong relationships between PNI and a series of unfavorable clinicopathological factors such as lymphatic invasion (OR: 7.00, $P < 0.001$), vascular invasion (OR: 5.79, $P = 0.008$), deeper tumor invasion (OR: 4.79, $P < 0.001$), lymph node metastasis (OR: 3.60, $P < 0.001$) and worse survival outcome (HR: 1.69, $P < 0.001$) in gastric cancer.[113] In pancreatic cancer, PNI appears to be an independent prognostic factor for overall survival rate (HR 1.68, $P < 0.00001$), disease-free survival rate (HR 2.53, $P = 0.0001$).[114] Also, In multivariate meta-analysis of colorectal cancer, PNI is an independent prognostic factor for 5-year disease-free survival rate, 5-year cancer-specific survival rate and 5-year overall survival rate (HR 2.35, HR 1.91 and HR 1.85, respectively).[115]

PNI also seems to be associated with oral cancers especially squamous cell carcinomas (SCC). Numerous studies showed that PNI was correlated with poor clinical outcomes [112] and a predictive factor of cervical metastases.[116, 117] In a large retrospective study on 1524 OSCC patients, PNI is significantly correlated with disease-free survival (HR 1.84) and overall survival (HR 1.7).[118] According to a recent meta-analysis on head and neck SCC, PNI shows significant associations with overall survival (HR 2.8), disease-free survival (HR 2.42) and disease-specific survival (HR 2.60).[119]

III. Materials and Methods

Cell lines and culture conditions

The Ca9.22, HSC-2, HSC-3, HSC-4, and SAS cell lines were provided from Hokkaido University (Hokkaido, Japan). The HN22 cell line was generously provided from School of Dentistry, DanKook University (Cheonan, Korea). All OSCC cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (Welgene, Gyeongsan-si, Korea) containing 10% fetal bovine serum (FBS; Welgene, Gyeongsan-si, Korea) and 100 U/ml penicillin and streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Real time RT-PCR

Total mRNA was extracted from above mentioned cell lines using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. For quantitative real-time RT-PCR, Applied Biosystems StepOne Plus Real-time PCR System (Applied Biosystems, CA, USA) was used for analyzing L1CAM expression level in two independent experiments on triplicate. The primer sequences to perform q RT-PCR were:

L1CAM Forward-5'ACGAGGGATGGTGTCCACTTCAAA,

L1CAM Reverse-5'TTATTGCTGGCAAAGCAGCGGTAG

L1CAM mRNA expression were measured using a SYBR Premix Ex Taq™ kit (Takara, Japan). β-actin expression was used as a reference. The relative level of L1CAM mRNA was normalized to that of β-actin and calculated by the $2^{-\triangle\triangle Ct}$ method.

Western blotting

Whole cells were retrieved and lysed in RIPA buffer (Merck Millipore, USA), separated by SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. The membranes were incubated with the following 1:1,000 dilution primary antibodies: L1CAM (SC-53386), E-cadherin (BD610181), N-cadherin (BD610920), vimentin (BD550513), Snail (CST3879), Slug (CST9585), Twist (CST3879), p-PI3K (AB182651), PI3K (AB40776), p-Akt (CST9271), Akt (CST9272), p-ERK 1/2 (CST9101), ERK (CST9102), and ß-actin (SC-47778). The proteins were identified by SuperSignal West Pico Chemiluminescent Substrate (SC-2048; Santa Cruz Biotechnology), and immunoreactive bands were visualized using ImageQuant LAS 500 (GE Healthcare Life Sciences). Densitometric analysis of western blots was carried out using ImageJ software (Version 1.51 k, NIH).

Cell proliferation assay

Cells are seeded in 6-well plates and incubated overnight prior to the treatment of rhL1CAM or siL1CAM. After treatment, cells were incubated for 24 hours and stained with 0.4% trypan blue solution (Gibco, Paisley, UK) and viable cells were counted using a hemocytometer.

Wound healing assay

For wound healing assay, 3 x 10^5 cells of Ca9.22, HSC-2, HSC-4 and HN22 were

seeded in 6-well plates. 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 during 12 h to enable wound healing with the treatment of rhL1CAM or siL1CAM. The wound dimensions were measured at 0 h and 12 h using ImageJ software (National Institute of Health, USA).

Transwell migration and invasion assays

For transwell migration or invasion assays, Ca9.22, HSC-2, HSC-4 and HN22 cells in serum-free medium were seeded in the upper chamber with Matrigel-coated transwell inserts (BD Bioscience, Bedford, MA, USA). Lower chambers were filled with the medium containing 10% FBS as a chemoattractant. After incubation of 24 h, the non-migratory or non-invaded cells in upper chamber were removed with a cotton swab and the migratory cells on the lower surface of the filter were fixed with 100% methanol for 2 min and stained with hematoxylin and eosin solution. The migratory or invaded cells was photographed under a light microscope (Nikon, Tokyo, Japan) and the number of migratory or invaded cells in randomly selected areas counted in 3 different microscopic fields (x100 magnification).

Patients and tissue samples

Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from 80 patients with tongue OSCC who were surgically treated at the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital between 2008 and 2012. Tumors were staged according to the TNM system recommended by the American

Joint Committee on Cancer.[120, 121] The clinicopathological characteristics of the patients including age, gender, differentiation, tumor size (pT), depth of invasion (DOI), LN metastasis (pN), PNI, TNM stage, and recurrence are shown in Table 1. All procedures followed in this study were in accordance with the guidelines of the Institutional Review Board of the Seoul National University Dental Hospital (#CRI 20003).

Immunohistochemistry

Immunohistochemical staining of L1CAM was performed in a total of 80 OSCC cases using a mouse monoclonal anti-human L1CAM antibody (clone 14.10; BioLegend). Tissue specimens were sectioned at 4-μm thickness, deparaffinized in Neo-clear (Merck, Darmstadt, Germany) for 25 min, and rehydrated with graded alcohol solutions for 25 min. Endogenous peroxidase was inactivated by incubation in 3% H_2O_2 solution for 10 min. Heat-induced epitope retrieval was performed in citrate buffer (pH 9.0) for 10 min using a microwave. The immunoreactions were performed using REAL^{TM} EnVision/HRP, Rabbit/Mouse (ENV) and visualized using REAL^{TM} DAB+ Chromogen (Dako, Glostrup, Denmark). All sections were counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemical staining

All stained samples with anti-L1CAM antibody were evaluated by two independent oral pathologists (K-Y.O. and H-J.Y.). 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 mentioned earlier.[122]

Statistical analysis

Statistical analyses were performed using a software package (SPSS version 26.0; IBM, Armonk, NY, USA). Correlations between L1CAM expression and clinicopathological parameters were assessed with Pearson's chi-square test or Fisher's exact test. The data from in vitro experiments are expressed as mean ± standard deviation from the three independent experiments. Statistical differences were determined using student's *t*-test. For all analyses, *p* values less than 0.05 were considered statistically significant.

IV. Results

OSCC cell lines show different levels of L1CAM expression

To evaluate the expression of L1CAM in OSCC cell lines, six different cell lines were cultured *in vitro*. The mRNA and protein levels of L1CAM were examined using qPCR and western blot analysis respectively (Fig. 1). Ca9.22 and HSC-2 showed a significantly low level of L1CAM, whereas HSC-4 and HN-22 showed a relatively high level of L1CAM compared to other cell lines. Therefore, Ca9.22 and HSC-2 were used for analyzing the effect of L1CAM up-regulation with the treatment of rhL1CAM (100 ng/ml). On the other hand, HSC-4 and HN-22 were used for analyzing the effect of L1CAM down-regulation with the treatment of siL1CAM (100 ng/ml).

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, Ca9.22 and HSC-2 cell lines were treated with rhL1CAM to overexpress L1CAM. In the proliferation assay, cell viability of Ca9.22 cell line increased proportionally when the rhL1CAM concentration increased up to 400 ng/ml. Cell viability of HSC-2 cell line also increased gradually when rhL1CAM concentration increased up to 100 ng/ml, but it showed a plateau more than 200 ng/ml of rhL1CAM concentration (Fig. 2A). The number of Ca9.22 and HSC-2 cells increased with the passage of time at 100 ng/ml concentration of rhL1CAM (Fig. 2B). Next, wound healing assay was used to assess the role of L1CAM

of cell proliferation and migration. Wound areas in the rhL1CAM groups of Ca9.22 and HSC-2 cells closed more than twice as much as that of control groups (Fig. 3). Lastly, transwell migration and invasion assay also revealed that rhL1CAM-treated groups have significantly increased migration and invasion ability compared to the control groups (Fig. 4 and 5). These results indicated that overexpression of L1CAM promote the cell proliferation, migration, and invasion, suggesting its roles in the progression of OSCC.

Downregulation of L1CAM inhibits the cell proliferation, migration, and invasion of OSCC cells *in vitro*

The HSC-4 and HN22 cell lines were treated with siL1CAM. In the proliferation assay, cell viability of HSC-4 and HN22 cell lines decreased proportionally when the siL1CAM concentration increased up to 100 ng/ml. (Fig 6A). The number of HSC-4 and HN22 cells decreased with the passage of time (Fig 6B). Next, wound healing assay was performed to assess the effect of L1CAM knockdown on the cell migration. In Figure 7A, wound areas in the siL1CAM groups of HSC-4 and HN22 cell lines closed lesser than that of control groups. Quantitative data showed that the ability of wound closure in siL1CAM-treated cell lines is about one third lower than non-treated groups (Fig. 7B) and there was a significant difference between two groups on triplicate experiments ($P < 0.001$). Lastly, transwell migration and invasion assays also revealed that siL1CAM-treated groups have significantly decreased migration and invasion ability compared to the control groups (Fig. 8 and 9). From the triplicate data, there was a significant difference between two groups ($P < 0.001$). These results indicated that knockdown of L1CAM can inhibit the cell proliferation, migration, and invasion of OSCC cells, suggesting the possibility of L1CAM as a therapeutic target.

L1CAM is involved in the EMT of OSCC cells and the PI3K/AKT and MAPK signaling pathways

In order to identify the role of L1CAM in the EMT of OSCC cells, changes in the expression of EMT-associated markers during the knockdown of L1CAM were examined by western blotting. As shown in Fig. 10, the epithelial cell marker E-cadherin was significantly increased after siL1CAM treatment. Also, there were decreases of mesenchymal markers such as N-cadherin, vimentin, Snail, Slug, and Twist. Therefore, it was found that L1CAM is involved in the EMT process of OSCC cells. After the treatment of siL1CAM, expression of PI3K/AKT and ERK1/2 did not change in the HSC-4 and HN22 cell lines, but there were remarkably decreasing changes of p-PI3K, p-AKT and p-ERK1/2 levels. Therefore, L1CAM seems to regulate the oncogenic functions through the PI3K/AKT and the MAPK signaling pathways. Taken together, L1CAM seems to promote the cell proliferation, migration, invasion, and EMT in OSCC via the PI3K/AKT and ERK pathways, which have been mainly implicated in the pathogenesis of diverse human cancers.

L1CAM expression is significantly correlated with the adverse clinicopathological features, especially PNI

L1CAM expression was not detected in the normal oral mucosa, but strongly expressed in the peripheral nerve tissue (Fig. 11A and B). In OSCC tissue samples, the extent of L1CAM expression in tumor cells varied within the same tissues. OSCC cells showed the membranous pattern of expression of L1CAM. Of the 80 patients, 26 (32.5%) were

positive for L1CAM. Tumor cells showing moderate to strong expression were frequently found at the invasive border of tumors or in the less-differentiated and sometimes spindle-shaped tumor cells (Fig. 11E and F). In the PNI area, tumor cells expressing L1CAM were found in the perineural tissues or even inside the nerves (Fig. 11G and H)

PNI was determined by the commonly accepted definition which is 1) tumor in close proximity to nerve and involving at least 33% of its perimeter or 2) tumor cells within any of the 3 layers of the nerve sheath according to Liebig et al.[110] PNI was detected in 40.0% of 80 OSCC samples. Correlations between PNI and clinicopathological parameters are shown in Table 2. PNI significantly correlated with tumor size ($P = 0.001$), DOI ($P = 0.000$), presence of LN metastasis at the time of diagnosis ($P = 0.001$), and advanced clinical stage ($P = 0.001$). These results confirmed the well-known value of PNI as a strong prognostic factor in OSCC.

Additionally, to verify the prognostic value of L1CAM in OSCC, correlations between L1CAM expression and clinicopathological parameters are analyzed (Table 3). L1CAM expression significantly correlated with moderate differentiation ($P = 0.013$), deep DOI ($P = 0.009$), positive LN metastasis (pN1/2/3) ($P = 0.002$), presence of PNI ($P = 0.000$), and advanced stage (III+IV) ($P = 0.004$). These findings suggested the possibility of L1CAM as a reliable prognostic marker for clinical outcome and an auxiliary diagnostic marker for detecting PNI.

V. Discussion

Immunohistochemical study demonstrated that L1CAM expression was observed with high frequency at the invasive front or less differentiated area of the tumor than in other areas.[12, 13] Moreover, L1CAM expression significantly related with depth of invasion and presence of metastasis. Therefore, in order to reveal how L1CAM affect the invasion and metastasis of OSCC cells during the tumor progression, *in vitro* functional assays were performed. L1CAM expressions of these 6 different OSCC cell lines were not evaluated before. Variable levels of L1CAM expression were detected between the different cell lines. There has been only one study exploring the effect of L1CAM on OSCC cell lines. Hung et al. showed that there were different levels of L1CAM expression in four OSCC cell lines and higher-L1CAM cell lines showed more invasive ability compared to lower-L1CAM cell lines.[34] The current study also agreed that OSCC cell lines had their different levels of L1CAM expression and overexpression of L1CAM increased cell proliferation and migration of OSCC cells. Therefore, it can be concluded that L1CAM play important roles in the development of OSCC.

Based on the result showing the promoting effect of L1CAM on the migration and invasion of OSCC cells, L1CAM may be related to EMT process. EMT is a reversible cellular process which epithelial cells lose adhesion to each other and change into mesenchymal-like cells for metastasis. There are several studies which show the relationship between L1CAM and EMT. Generally, overexpression of L1CAM augments EMT signaling. For the first time, Shtutman et al. examined the functions of L1CAM during EMT process. They found that nonneuronal L1CAM expression increased the

scattering of epithelial cells from their colonies by the disruption of adherens junctions and elevation of β-catenin transcriptional activity in MCF7 breast carcinoma cells.[123] In some cases of aggressive endometrial carcinomas, L1CAM was upregulated at the invasive front of tumor and E-cadherin expression was downregulated at the same area.[124] Although the direct mechanisms of L1CAM on EMT was not fully understood, once expressed L1CAM provokes cancer cell motility, invasion, and metastasis.[43] Also, western blot analysis of our study verifies that HSC-4 and HN-22 cell lines treated by siL1CAM show increasing levels of E-cadherin which is one of key epithelial markers and decreasing levels of N-cadherin, Snail, Slug and Twist which are representative markers of mesenchymal characteristics. According to these results, L1CAM appears to be one of influential factors in the EMT development.

The exact mechanism of L1CAM has not been known yet, but there are several pathways that get involved in the EMT process. A few steps such as PI3K/AKT and MAPK pathways overlap between L1CAM and EMT activation. As it was mentioned in the review of literature, L1CAM activates MAPK cascade components Raf-1 via the Src tyrosine kinase.[33] Silletti et al. showed that L1CAM promoted ERK activity which results in increasing cell mobility and invasion by integrin $\alpha_v\beta_3$, rac-1, cathepsins-L and -B.[60] Also, Chen et al. found that PI3K/AKT pathway of gastric cancer cell lines were influenced by L1CAM expression.[32] In our study, downregulation of L1CAM could suppress the phosphorylation of PI3K/AKT and MAPK consistently with the previous studies, suggesting that L1CAM can induce both migration and invasion of OSCC cells, via PI3K/AKT and/or MAPK signaling pathways.

Initially, PNI was underestimated as just one of lymphatic spread of tumor into nerves, but nowadays PNI is considered as one of major routes of metastasis in several types of

cancers including head and neck SCC. PNI is found in OSCCs arising from any intraoral sites, but with respect to the location of primary tumor, PNI was more frequently seen in tumors located at the tongue.[125, 126] Therefore, in order to explore the clinical significance of PNI and the PNI-related molecules, tissue samples of the tongue OSCC were exclusively investigated in the present study. The incidence of PNI in this study was 40%. This incidence is within the scope of results from most studies, however, there is a large variation in the frequency of PNI detection, ranging from 6% up to even 82% with additional S100 staining.[22, 25-27] These variations may come from non-standardized biopsy techniques, different specimen preparation, and the number of histological sections examined.[127] A recent large cohort study showed that PNI is one of independent prognostic factor for overall survival in OSCC.[128] In addition, most investigations demonstrated that the presence of PNI is corelated with tumor differentiation, depth of invasion, and LN metastasis, even though Wallwork et al. did not find any statistically significant association between PNI and the presence of LN metastases.[129, 130] The present study also showed that presence of PNI had a significant association with tumor size, LN metastasis, depth of invasion, and TNM stage. Because of the above results, it has been suggested that either the early stage or cN0 tumor with PNI should be treated with more aggressive procedures such as adjuvant radiation therapy or elective neck dissection.[23, 127] Therefore, more objective and reproducible methods of identifying PNI by pathologists is critical for predicting clinical outcomes and guiding the optimal therapeutic strategies.

L1CAM overexpression has been found in many human cancers and considered a useful prognostic marker. The current study also showed the prognostic value of L1CAM in tongue OSCC, especially with the significant correlation to the presence of PNI (P = 0.000). Among the L1CAM-positive cases, 69.2% (18/26) show PNI. There are many

intra- or extracellular molecules which are involved in PNI phenomenon. L1CAM is one of key signaling molecules of PNI, which is abundant in Schwann cells and known as having pivotal roles of neurogenesis. There are only a few studies which researched the relation between PNI and L1CAM. In extrahepatic cholangiocarcinoma, high L1CAM expression cells are present in the invasive front of tumors and tumors with high L1CAM are more frequently accompanied with perineural invasion.[12] Also, positive L1CAM expression is strongly associated with perineural invasion in 94 cases of pancreatic ductal adenocarcinoma on a tissue microarray analysis.[13] Nonmyelinated neuronal L1CAM is involved in adhesion between tumor epithelial cells from human primary colon adenocarcinomas and enteric neurons via heterophilic interactions and their relations are verified with decreasing attachment of tumor epithelial cells by blocking L1CAM.[131] Recently, Na'ara et al. demonstrated that L1CAM released from Schwann cells could be a strong chemoattractant to pancreatic cancer cells.[132] Although the molecular mechanisms of PNI in OSCC have not been fully understood, immunohistochemical results of the present study suggested that L1CAM may be one of involving molecules in PNI development and a candidate of the diagnostic marker for PNI in OSCC. Thus, further investigation is needed to elucidate the direct function of L1CAM during the PNI.

Based on published data and our present results, L1CAM enhanced motility and invasiveness of tumor cells and could be a promising molecular target for the treatment of OSCC. Accordingly, inhibition of L1CAM significantly reduced cell proliferation and migration/invasion of OSCC cells *in vitro*, so there is a possibility of L1CAM as a molecular target in anticancer treatments. Arlt et al. investigated the effects of anti-L1CAM monoclonal antibodies on proliferation and migration of various L1CAM-positive tumor cell lines *in vitro* and on ovarian tumor growth *in vivo*. They showed that L1CAM-directed antibodies significantly inhibited the proliferation of tumor cell lines

and reduced tumor size of ovarian carcinoma-bearing mice.[133] Afterward, several studies using xenograft models have demonstrated that anti-L1CAM treatment reduced tumor progression in various tumor cells.[134, 135] Therefore, anti-L1CAM therapy could be considered a promising therapeutic strategy for OSCC.

VI. Conclusions

According to the results of *in vitro* study, L1CAM seems to play important roles in the progression of OSCC by promoting the proliferation, migration, invasion, and EMT of OSCC cells via PI3K/AKT and/or MAPK signaling pathways. Expression of L1CAM in OSCC tissues significantly correlates with depth of invasion, LN metastasis, and advanced clinical stage, as well as presence of PNI in OSCC. Taken together, it is suggested that L1CAM play an important role in tumor progression and metastasis of OSCC and could be a reliable biomarker for predicting clinical outcome and a promising therapeutic target in OSCC.

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Table 1. Clinicopathologic characteristics of 80 patients with OSCC

Variable	Number of cases
Age at diagnosis (mean)	56 years
Range	27-83 years
Gender	
Male	48
Female	32
Histological differentiation	
Well	60
Moderately	20
PNI	
No	48
Yes	32
pT	
T1	21
T2	38
T3	16
T4	5
pN	
N0	50
N1	6
N2	10
N3	14
TNM stage	
I	19
II	24
III	11
IV	26

Table 2. Correlation of PNI with clinicopathologic parameters in OSCC patients.

Variables	Case	Perineural invasion (PNI)		*P*-value
		Negative (%)	Positive (%)	
	n = 80	n = 48	n = 32	
Age				
< 56	39	26 (66.7)	13 (33.3)	0.235[b]
≥ 56	41	22 (53.7)	19 (46.3)	
Gender				
Male	48	26 (54.2)	22 (45.8)	0.192[b]
Female	32	22 (68.8)	10 (31.3)	
Differentiation				
Well	60	38 (63.3)	22 (36.7)	0.292[b]
Moderately	20	10 (50.0)	10 (50.0)	
Tumor size (pT)				
T1+T2	59	42 (71.2)	17 (28.8)	0.001[b]*
T3+T4	21	6 (28.6)	15 (71.4)	
Depth of invasion				
≤ 0.5cm	30	28 (93.3)	2 (6.7)	0.000[b]*
0.5cm< ≤1.0cm	25	15 (60.0)	10 (40.0)	
> 1.0cm	25	5 (20.0)	20 (80.0)	
LN metastasis (pN)				
N0	50	37 (74.0)	13 (26.0)	0.001[b]*
N1+N2+N3	30	11 (36.7)	19 (63.3)	
TNM stage				

I+II	43	33 (76.7)	10 (23.3)	0.001[b]*
III+IV	37	15 (40.5)	22 (59.5)	
Local recurrence				
No	71	42 (59.2)	29 (40.8)	0.734[a]
Yes	9	6 (66.7)	3 (33.3)	

a; Fisher's exact test, b; Pearson's chi-square test, asterisk (*) means the clinically statistical difference ($P < 0.001$).

Table 3. Correlation of L1CAM expression with clinicopathologic parameters in OSCC patients.

Variables	Case	L1CAM		*P*-value
		Negative (%)	Positive (%)	
	n = 80	n = 54	n = 26	
Age				
< 57	39	27 (69.2)	12 (30.8)	0.747[b]
≥ 57	41	27 (65.9)	14 (34.1)	
Gender				
Male	48	36 (75.0)	12 (25.0)	0.079[b]
Female	32	18 (56.3)	14 (43.8)	
Differentiation				
Well	60	45 (75.0)	15 (25.0)	0.013[b]*
Moderately	20	9 (45.0)	11 (55.0)	
Tumor size (pT)				
T1+T2	59	42 (71.2)	17 (28.8)	0.238[b]
T3+T4	21	12 (57.1)	9 (42.9)	
Depth of invasion				
≤ 0.5cm	30	26 (86.7)	4 (13.3)	0.009[b]*
0.5cm< ≤1.0cm	25	16 (64.0)	9 (36.0)	
> 1.0cm	25	12 (48.0)	13 (52.0)	
LN metastasis (pN)				
N0	50	40 (80.0)	10 (20.0)	0.002[b]*
N1+N2+N3	30	14 (46.7)	16 (53.3)	

PNI				
No	48	40 (83.3)	8 (16.7)	0.000[b]*
Yes	32	14 (43.8)	18 (56.3)	
TNM stage (pStage)				
I+II	43	35 (81.4)	8 (18.6)	0.004[b]*
III+IV	37	19 (51.4)	18 (48.6)	
Local recurrence				
No	71	47 (66.2)	24 (33.8)	0.710[a]
Yes	9	7 (77.8)	2 (22.2)	

a; Fisher's exact test, b; Pearson's chi-square test, asterisk (*) means the clinically statistical difference ($P < 0.05$).

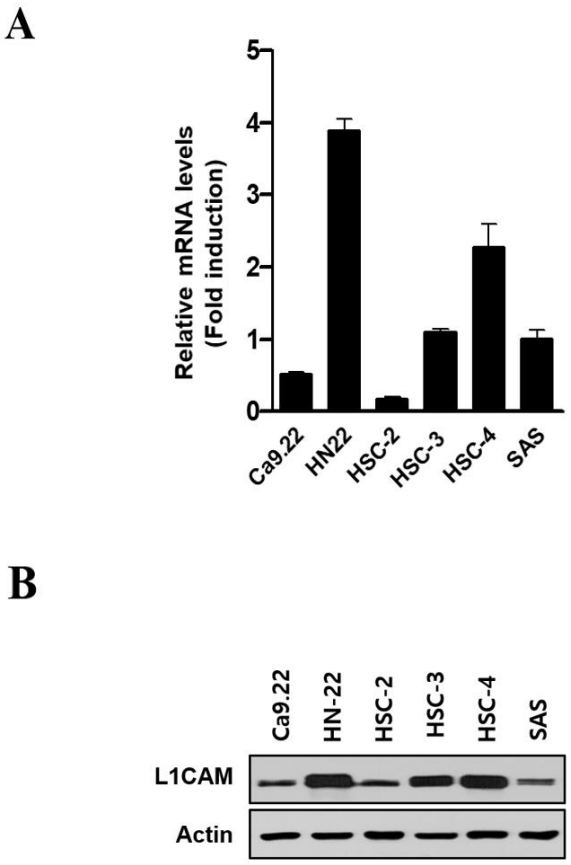

Figure 1. L1CAM expression levels of different OSCC cell lines. (A) mRNA expression of L1CAM in 6 OSCC cell lines was examined by real-time PCR. GAPDH was used as the housekeeping gene. The bar graphs are the mean ± SD of triplicates. (B) Protein expression levels of L1CAM in 6 OSCC cell lines. β-actin was used as the loading control.

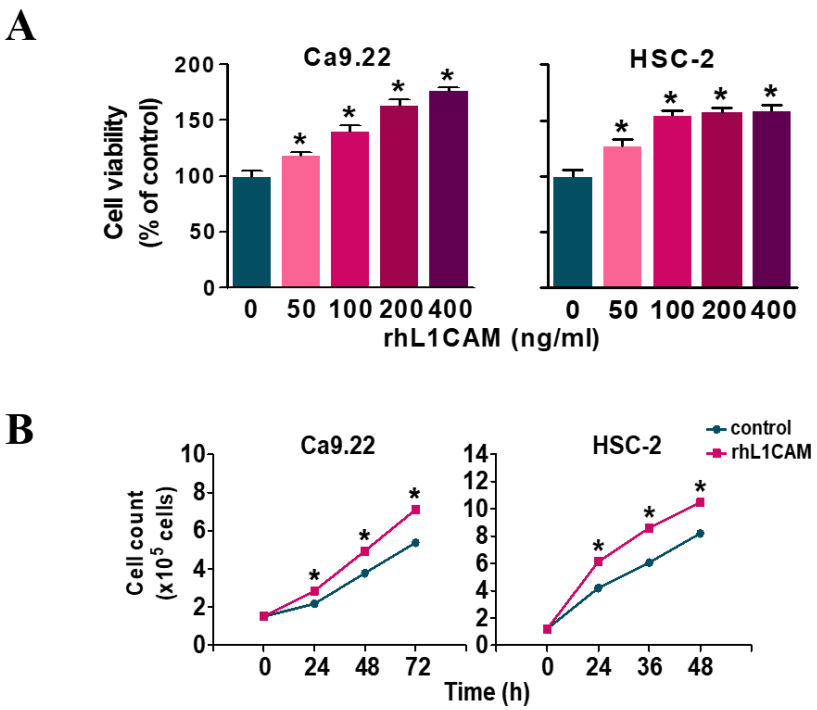

Figure 2. Effects of L1CAM overexpression on proliferation assay. (A) The Ca9.22 and HSC-2 cells were cultured at the indicated concentration of rhL1CAM. After culturing for 24 hours, the number of viable cells in each cell line was measured by a hemocytometer. Results were expressed as the mean and ± SD. (B) The proliferation of rhL1CAM (100 ng/ml)-treated Ca9.22 and HSC-2 cells were monitored for 0, 24, 48 and 72 hours, respectively. The number of viable cells in Ca9.22 and HSC-4 cells were counted. Asterisk (*) means the statistical difference ($P < 0.001$) based on the student's *t*-test.

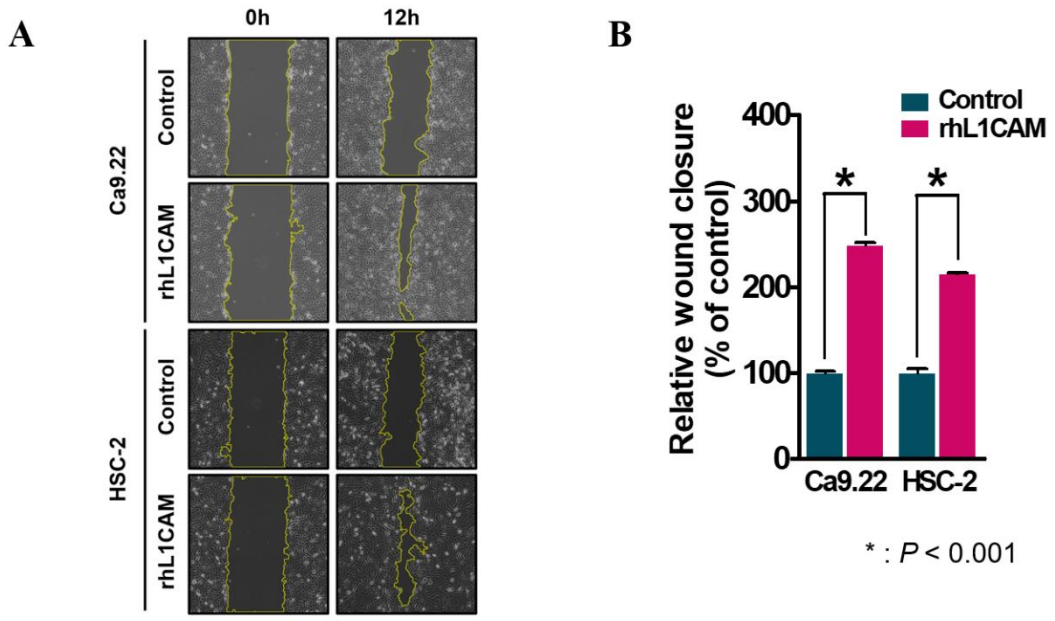

Figure 3. Effects of L1CAM overexpression on wound healing assay. (A) For the wound healing assays, Ca9.22 and HSC-2 cells were seeded in 6-well plates. The wounds were created with 10-µl pipette tip and the size of wound closure was measured after 12 hours. The wounded areas were examined under x100 magnification and the representative images of cell growths were taken after 12 hours. (B) The size of wound closure was calculated with using ImageJ software (National Institute of Health, USA). The data were shown as the mean and ± SD in three independent experiments. Asterisk (*) shows the statistical difference ($P < 0.001$) between the control and rhL1CAM-treated groups.

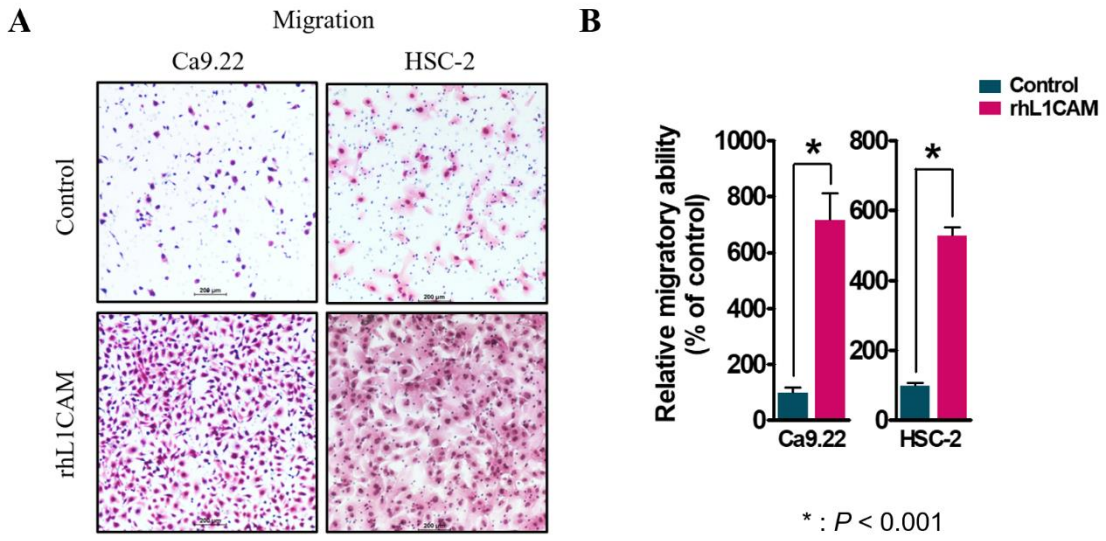

Figure 4. Effects of L1CAM overexpression on transwell migration assay. (A) Transwell migration assays were conducted in Ca9.22 and HSC-2 cells. After 24-hour incubation, the migrated cells were stained and observed under microscope (x100 magnification). The control groups are untreated, and the experiment groups were treated with rhL1CAM (100 ng/ml). The representative areas of three independent experiments were shown. (B) The relative migratory ability was measured by counting the number of the stained cells. The data were shown as mean and ± SD of three independent experiment fields compared to the control groups. Asterisk (*) shows the statistical difference ($P < 0.001$) between control and rhL1CAM-treated groups.

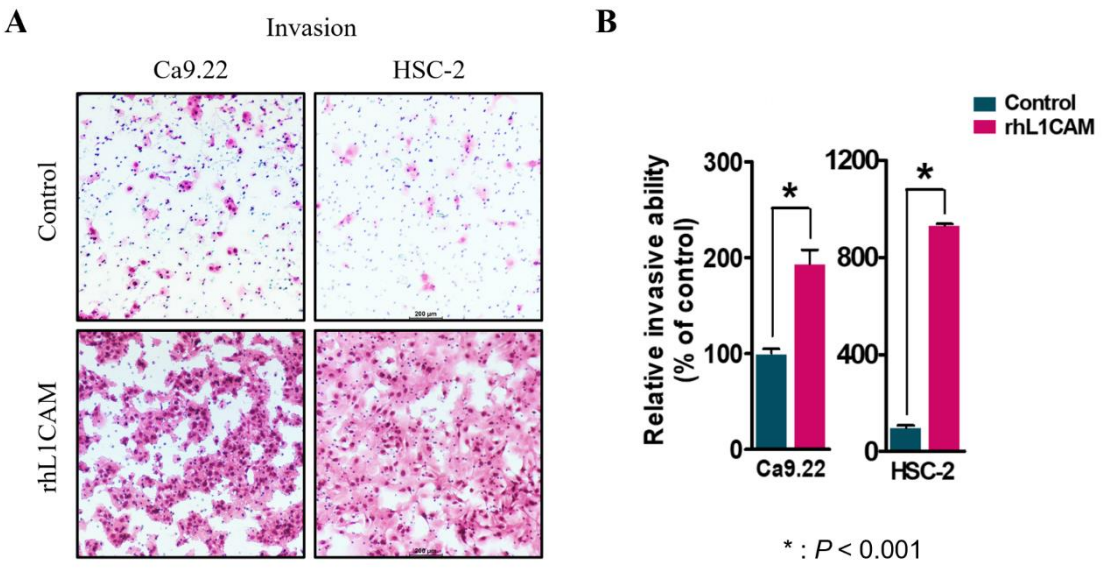

Figure 5. Effects of L1CAM overexpression on transwell invasion assay. (A) For the transwell invasion assays, Ca9.22 and HSC-2 cells were incubated into the Matrigel-coated upper chamber of a 24-well format transwell plates for 24 hours. HSC-4 and HN22 cells were untreated for the control groups and treated with rhL1CAM 100 ng/ml for the experiment groups. (B) The relative invasive ability was measured by counting the number of stained cells. The data were shown as the mean ± SD of the three independent experiments. Asterisk (*) shows the statistical difference ($P < 0.001$) between control and rhL1CAM-treated groups.

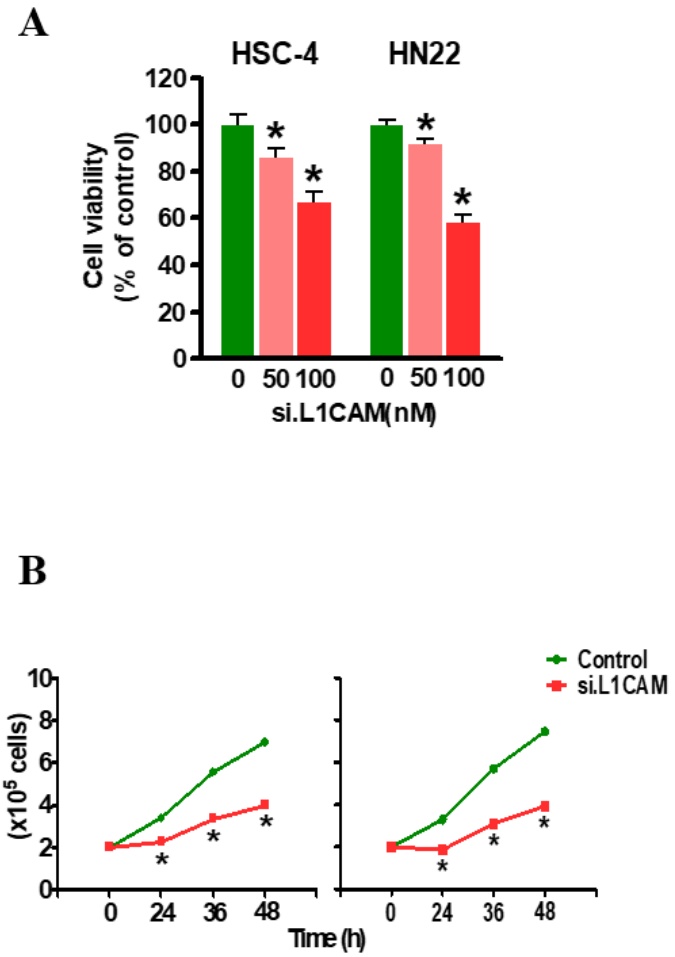

Figure 6. Effects of L1CAM downregulation on proliferation assay. (A) The HSC-4 and HN22 cell lines were cultured in triplicate at the indicated concentration of siL1CAM. After culturing for 24 hours, cell viability of each cell line was measured by a hemocytometer. Results were expressed as the mean and SD. (B) The proliferation of siL1CAM (100 ng/ml)-treated HSC-4 and HN22 cells were monitored for 0, 24, 36 and 48 hours, respectively. The number of viable cells in HSC-4 and HN22 cell lines were counted by a hemocytometer. Asterisk (*) means the statistical difference ($P < 0.001$) based on the Student's *t*-test.

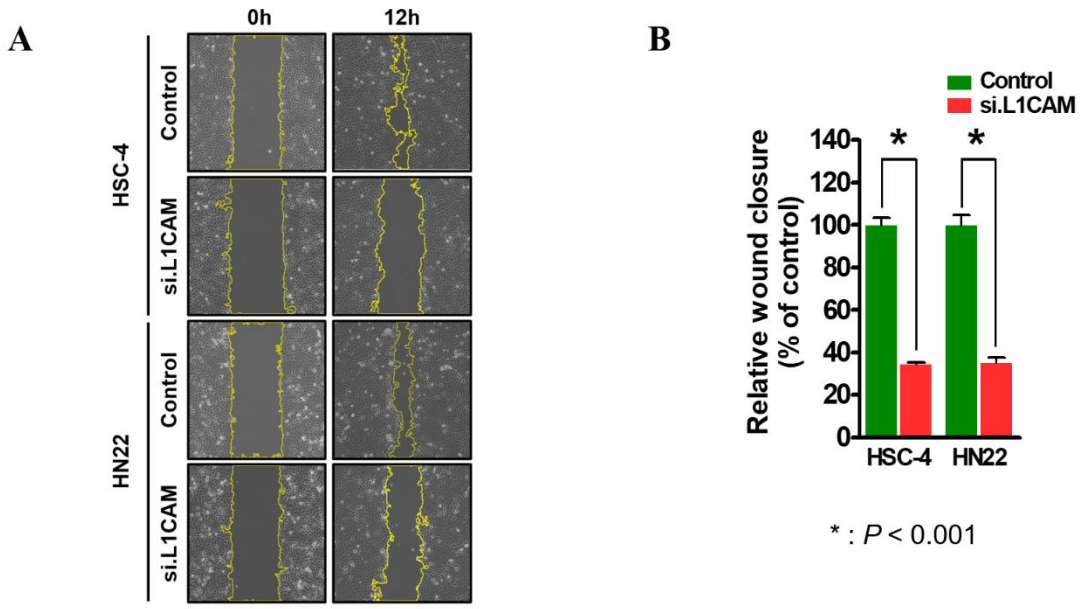

Figure 7. Effects of L1CAM downregulation on wound healing assay. (A) For the wound healing assays, HSC-4 and HN22 cells were seeded in 6-well plates. The wounds were created with 100-μl pipette tip and the size of wound closure was measured after 12 hours. (B) The size of wound closure was calculated with ImageJ software (National Institute of Health, USA) and Asterisk (*) shows the statistical difference ($P < 0.001$) between control and siL1CAM-treated groups.

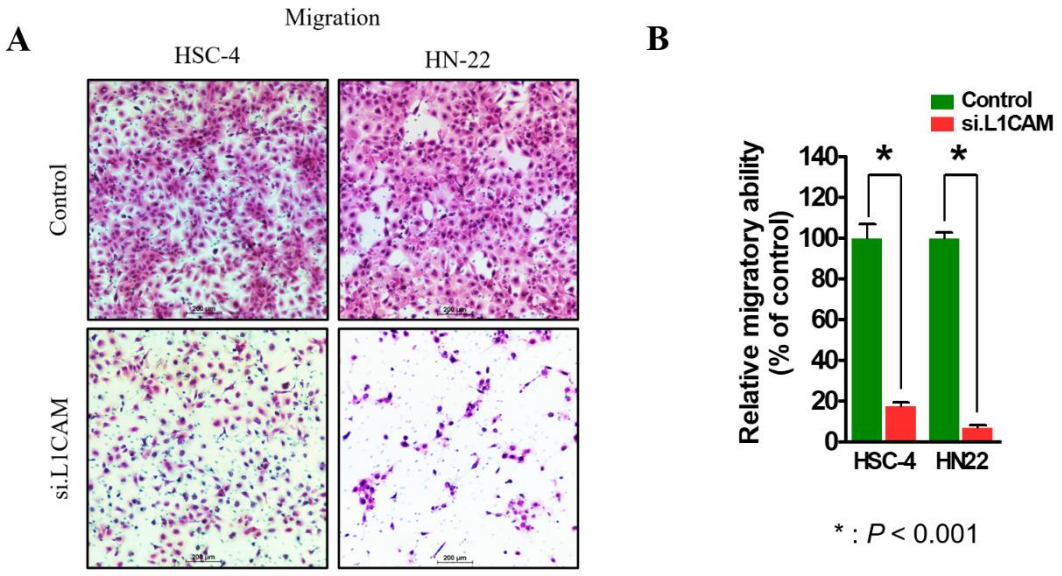

Figure 8. Effects of L1CAM downregulation on transwell migration assay. (A) Transwell migration assays were conducted in HSC-4 and HN22 cells. After 24-hour incubation, the migrated cells were stained and observed under microscope. The control groups are untreated, and the experiment groups were treated with siL1CAM (100 ng/ml). The representative areas of three independent experiments were shown. (B) The relative migratory ability was measured by counting the number of the stained cells. The data were shown as mean and ± SD of three independent experiment fields compared to the control groups. Asterisk (*) shows the statistical difference ($P < 0.001$) between control and siL1CAM-treated groups.

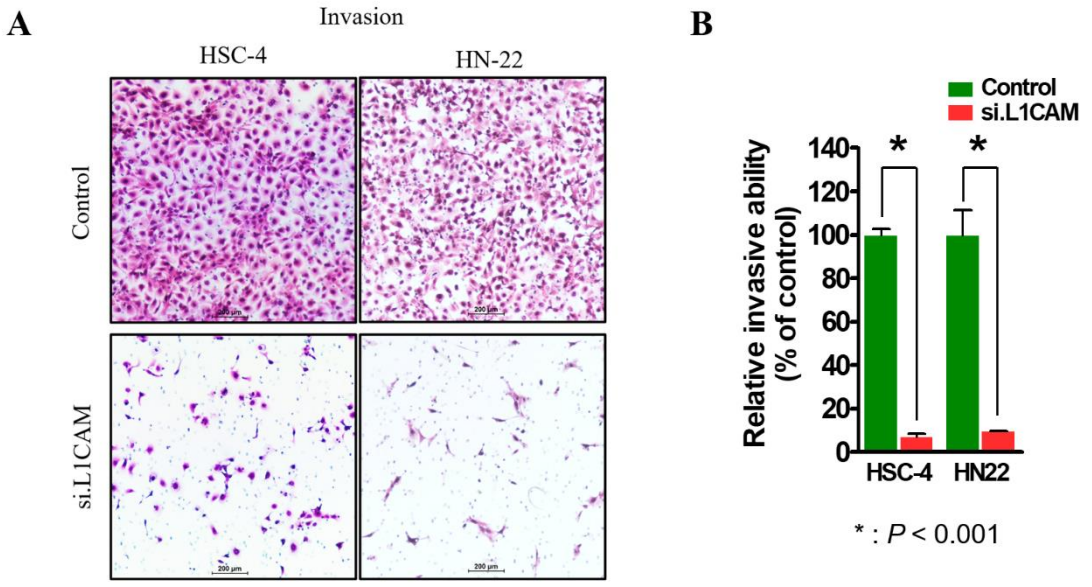

Figure 9. Effects of L1CAM downregulation on transwell invasion assay. (A) For the transwell invasion assays, HSC-4 and HN22 cells were incubated into the Matrigel-coated upper chamber of a 24-well format transwell plate for 24 hours. HSC-4 and HN22 cells were untreated for the control groups and treated with siL1CAM 100 ng/ml for the experiment groups. (B) The relative invasive ability was measured by counting the number of stained cells. The data were shown as the mean ± SD of the three independent experiments. Asterisk (*) shows the statistical difference ($P < 0.001$) between control and siL1CAM-treated groups.

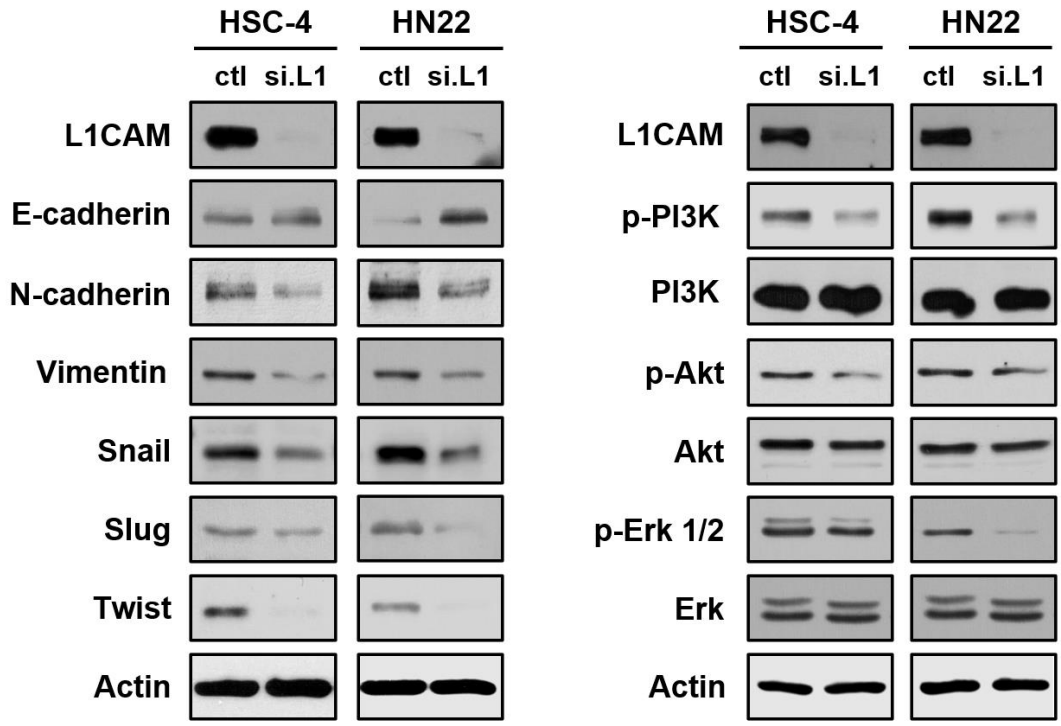

Figure 10. Western blot analysis of whole-cell lysates from HSC-4 and HN22 cells expressing control or siL1CAM 24 h after transfection with various antibodies to L1CAM, E-cadherin, N-cadherin, vimentin, Snail, Slug, Twist, p-PI3K, PI3K, p-AKT, AKT, p-ERK 1/2, ERK, and β-actin.

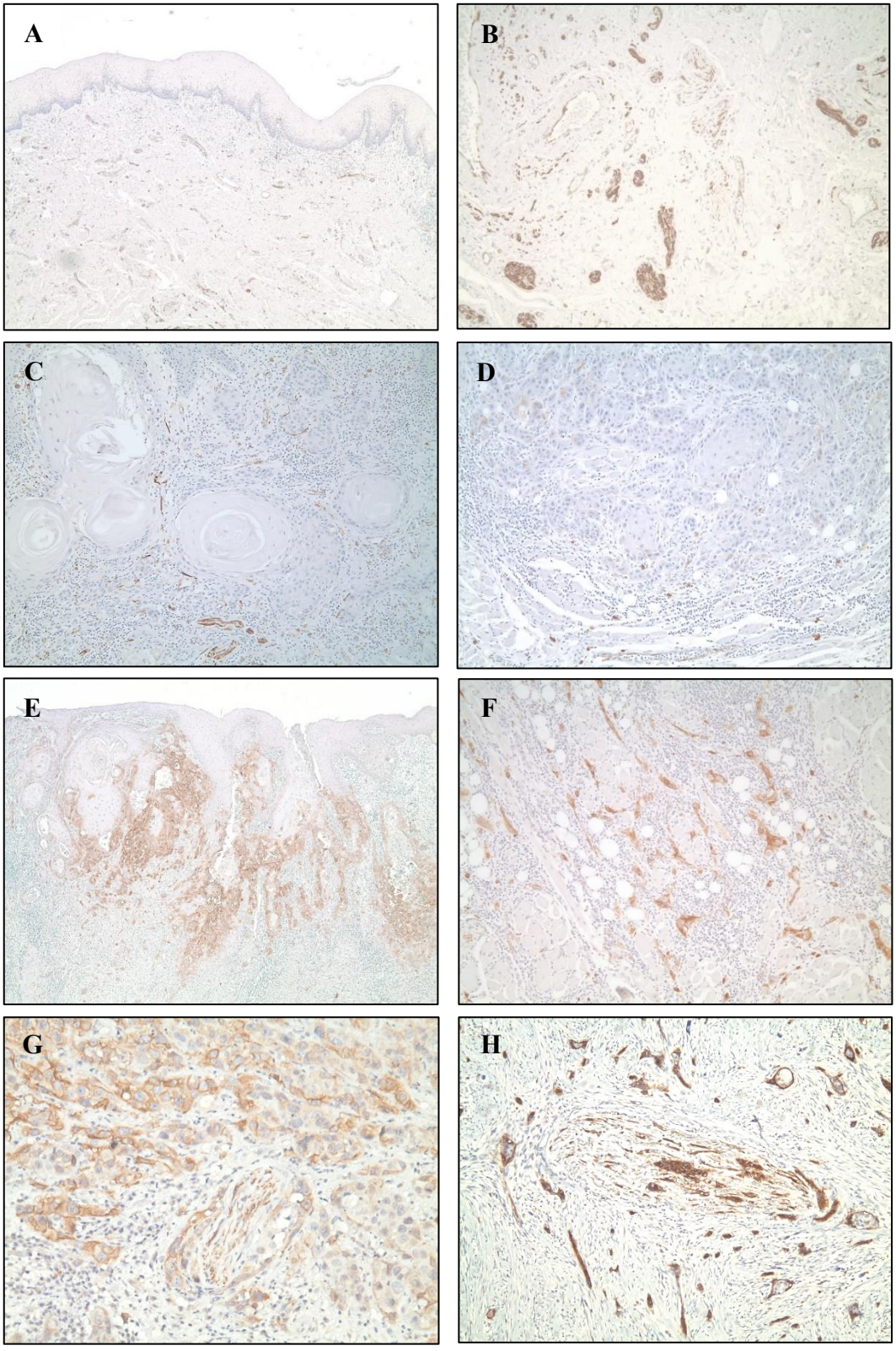
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Fig 11. L1CAM expression in normal mucosa and tongue OSCC tissues. (A) Negative expression in normal oral mucosa. (x40) (B) L1CAM expression can be detected in the vascular endothelial cells and nerve tissues (x100). (C, D) Negative L1CAM expression in more differentiated, keratinizing OSCC (x100) (E) Strong L1CAM expression at the area of invasion, but very week or negative expression in the upper well-differentiated cells (x 40) (F) Strong expression of invading tumor cells into the muscular tissue (x100). (G, H) L1CAM expression of tumor cells in the perineural and/or neural invasion area (x200).

국문 초록

구강편평세포암종에서 L1CAM의 역할 및 임상적 가치 규명에 관한 연구

구강병리학 전공 이 광 원 (지도교수: 윤 혜 정)

1. 목 적

L1CAM (L1 Cell adhesion molecule) 은 세포막 내, 외부에 걸쳐 존재하는 세포막 관통성 당단백질로서 신경세포의 발달에 필요한 물질로만 알려져 왔으나, 최근에는 L1CAM이 다양한 암에서 암의 진행과 전이에 관여한다고 알려졌다. 본 연구에서는 구강편평세포암종 세포주들을 이용하여 암세포주에서 L1CAM 발현을 분석하고, recombinant L1CAM (rhL1CAM)을 이용하여 과발현을 시키거나, 반대로 small interfering RNA (siL1CAM)를 이용해 발현을 억제시켜, L1CAM이 구강편평세포암종 세포주의 증식, 이동, 침습 및 상피간엽이행에 미치는 영향을 평가하고자 한다. 또한, L1CAM의 기능과 PI3K/AKT 및 ERK 신호전달 경로와의 관련성을 확인하려고 한다. 그리고, 구강편평세포암종 조직에서 L1CAM 발현을 평가하고 L1CAM 발현과 또 다른 형태의 전이 방법인 신경 주위 침습 (perineural invasion: PNI)을 포함한 여러 임상병리학적 지표들과의 연관성 및 예후 인자로서의 가치를 확인하고자 한다.

2. 방 법

L1CAM의 생물학적 기능을 확인하기 위해 상대적으로 L1CAM 발현이 낮은 구강편평세포암종 세포주인 Ca9.22와 HSC-2 세포주에 rhL1CAM을 처리하여 L1CAM을 과발현시켜 세포 증식, 이동, 침습 실험을 시행하였다. 역으로, L1CAM 발현도가 상대적으로 높은 구강편평세포암종 세포주인 HSC-4와 HN22에 siL1CAM을 처리하여 L1CAM 발현을 억제시켜 상기의 실험들을 진행하였다. 또한, L1CAM 발현 억제 시 세포주들의 상피간엽이행에 변화가 생기는지 확인하기 위해, Western blot 분석법을 이용하여 EMT 표지자의 발현 변화를 관찰하고, PI3K/AKT 및 ERK 신호전달 경로의 변화도 확인하였다. 또한, 혀에 생긴 구강편평세포암종 80개의 조직에서 L1CAM 발현과 임상병리학적 지표들과의

관련성을 분석하였다.

3. 결 과

구강편평세포암종 세포주 중 Ca9.22와 HSC-2 세포주에서 rhL1CAM을 이용하여 L1CAM을 과발현시킨 결과, 암세포의 증식, 이동, 및 침습이 대조군에 비해 모든 실험에서 통계학적 유의한 증가를 보였다 (각각 모두 $P < 0.001$). 또한, HSC-4 와 HN22 세포주를 siL1CAM을 이용하여 L1CAM 발현을 억제한 결과, L1CAM이 저하된 세포들이 대조군에 비해 암세포의 증식, 이동 및 침습이 모두 통계학적으로 유의한 감소를 보였다 (각각 모두 $P < 0.001$). Western blot 분석법을 통한 상피간엽이행 표지자 분석에서는 L1CAM이 저하된 세포주에서 상피 기원 표지자인 E-cadherin의 증가 및 vimentin을 포함한 간엽 기원 표지자들의 감소가 관찰되었다. 또한, L1CAM의 발현 억제 시, p-PI3K/p-AKT 및 p-ERK1/2의 발현 감소가 확인되었다. PNI는 전체 증례의 40%에서 관찰되었고, 종양의 크기, 침습 깊이, 경부 림프절 전이, 높은 임상 병기가 현저히 관련성을 보였다. 면역조직화학염색 분석 결과, L1CAM은 32.5%의 OSCC 증례에서 발현되었고, L1CAM의 발현은 분화도, 침습 깊이, 경부 림프절 전이, 신경 주위 침습, 임상병기 등과 유의성 있는 상관관계를 보였다.

4. 결 론

L1CAM의 발현은 PI3K/AKT 및 MAPK 신호전달 경로를 통해 구강편평세포암종 세포의 증식과 이동/침습을 증가시키고, 상피간엽이행을 촉진함으로써 구강편평세포암종의 진행과 전이에 관여함을 알 수 있었다. 또한, 구강편평세포암종 세포주에서 L1CAM의 발현을 억제할 경우, 암세포의 증식, 이동/침습이 현저히 억제됨을 확인하였고, 이는 L1CAM 발현 억제가 OSCC 환자의 새로운 치료전략이 될 수 있음을 시사한다. 환자 암 조직 내 L1CAM 과발현은 OSCC의 중요한 예후 인자로 알려진 국소 림프절 전이 및 신경 주위 침습을 포함한 부정적 임상병리학적 지표와 높은 상관관계를 보여, 구강편평세포암종에서 L1CAM의 예후 인자로서의 가치를 제시하였다. 따라서, 본 연구를 통해 구강편평세포암종의 진행에서 L1CAM의 중요한 역할, 예후 인자로서의 가치, 그리고 표적 항암 치료제로서 가능성을 확인할 수 있었다.

주요어: 구강편평세포암종, L1CAM, 신경 주위 침습, 상피간엽이행, 예후

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