치의과학 박사학위논문

Decreased expression of SOX7 induces cell proliferation and invasion, and correlates with poor prognosis in oral squamous cell carcinomas

구강편평세포암종의 세포 증식 및 침습과 환자의 예후에서의 SOX7 의 역할

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ABSTRACT

Decreased expression of SOX7 induces cell proliferation and invasion, and correlates with poor prognosis in oral squamous cell carcinomas

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SOX7, a member of the SOX family of transcription factors, acts as a tumor suppressor in multiple cancers. Downregulation of SOX7 has been reported in advanced tumors, correlating with poor prognosis. The aims of the present study were to investigate the effects of SOX7 on cell proliferation, invasion, and colony formation in oral squamous cell carcinoma (OSCC) cells and to evaluate the effectiveness of SOX7 protein as a prognostic indicator in OSCC patients.

OSCC cell lines were treated with SOX7 small interfering RNA (siRNA) or SOX7 peptide, and their effects on cell proliferation, invasiveness, and colony formation were investigated by WST-1, Matrigel invasion, and clonogenic assays. In addition, SOX7 protein expression in OSCC and normal oral mucosal tissues was examined by immunohistochemistry. The

association between SOX7 protein expression and clinicopathological parameters of OSCC patients was statistically analyzed.

SOX7 silencing induced cell proliferation and invasion in SCC-4 cells. SOX7 peptide treatment inhibited cell proliferation, colony formation, and invasion in SCC-9 and SCC-25 cells. The expression level of SOX7 protein was decreased in OSCC tissues, compared with normal oral mucosal tissues ($P < 0.001$). Negative SOX7 expression in OSCC patients was significantly associated with positive lymph node metastasis ($P = 0.041$), advanced TNM stage ($P = 0.024$), and poor prognosis ($P = 0.017$).

These results suggest that SOX7 inhibits cell proliferation, colony formation, and invasion in OSCC as a tumor suppressor and that negative SOX7 expression could be a poor prognostic indicator in OSCC patients.

Keywords: Cell proliferation, Invasion, Oral squamous cell carcinoma, Prognostic indicator, SOX7

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Ⅰ. Introduction

The Sex-determining region Y-box (SOX) family belongs to the high mobility group (HMG) superfamily of transcription factors (1,2). The founding member of SOX family is Sry, the principal determinant of male sex development (3). Sry contains a HMG domain highly conserved among SOX proteins in the same subfamily (1). Based on the degree of homology inside the HMG domain and the presence of conserved motifs outside the HMG box, SOX genes have been divided into nine subgroups (2). The SOX-F subfamily, one of these subgroups, comprises three SOX proteins (SOX7, SOX17, and SOX18) that have a pivotal role in cardiovascular development (4). In particular, SOX7 is also involved in a number of human cancers (1). SOX7 gene has been regarded as a tumor suppressor in multiple cancers, including colorectal cancer (5) and breast cancer (6). In addition, decreased expression of SOX7 has been reported in lung adenocarcinoma (2), endometrial cancer (7), ovarian cancer (8), and gastric cancer (9).

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, accounting for between 90 and 95% of all oral malignancies (10). OSCC is characterized by a considerable rate of early recurrence, frequent lymph node metastasis, and a high mortality rate due to the disease itself (11). Oral carcinogenesis is a multifactorial process involving numerous genetic processes in which the function of oncogenes and tumor suppressor genes can be altered (10). Genetic mutations and loss of gene

expression of *TP53* or *CDKN2A* suppressor genes are frequently observed in OSCC (11,12).

SOX7, as a tumor suppressor gene, has not been studied about its inhibitory effect on oral carcinogenesis. The aims of the present study were to investigate the effects of SOX7 on cell proliferation, invasion, and colony formation in OSCC cells and to evaluate the effectiveness of SOX7 protein as a prognostic indicator in OSCC patients.

Ⅱ. Background

Structure and function of SOX7

The human SOX7 gene is located in a region of chromosome 8p23.1 and is approximately 7.7 kilo base pairs (bps) in length. Two exons comprise the mRNA product with a coding region of 1,165 bps and a nearly 2 kilo bps long 3' untranslated region (UTR). The promoter region of the SOX7 gene contains a frequently methylated CpG island that plays a role in regulating SOX7 gene expression. The human SOX7 protein is 388 amino acids in length, characterized by a C-terminal transactivation domain and an N-terminal HMG domain (amino acids 44 - 122) (13). Previous studies suggested that phosphorylation within the HMG domain markedly reduces the DNA binding affinity of HMG domain-containing proteins (14, 15). Within the HMG domain of SOX7, there are four putative phosphorylation sites (S76, S82, S89 and T87) that may contribute to SOX7 binding to DNA. The SOX7 protein bears 70% and 51% homology to SOX17 and SOX18, respectively. Although SOX7 shares its DNA binding element with many other SOX proteins, it has little homology with them outside the HMG box region, with the highest homology (58%) to SOX17. This suggests that SOX7 may have interacting partners and biological functions distinct from other SOX proteins. Many SOX proteins have been shown to interact with β-catenin, and either inhibit or promote its transcriptional activity (16). SOX7 directly binds β-catenin and negatively regulates its activity. Guo et al. mapped a specific β-catenin binding site to the residues

D328RNEFDQY335 of SOX7 and suggested that E331 and F332 are essential to β−catenin binding (17). Interestingly, both SOX7 and SOX17 antagonize SOX4−mediated activation of β−catenin's transcriptional activity and inhibit Wnt signaling in endometrial carcinoma cells, likely through competitive binding to β−catenin (18, 19).

SOX7 possesses various functions in development, such as the initiation and maintenance of arterial identity (20). Additionally, the transcriptional activity of SOX7 plays a role in parietal endoderm differentiation through inducing the expression of the fibroblast growth factor 3 (*Fgf−3*), Gata−4, Gata−6, vascular endothelial (VE)−cadherin, and laminin−·1 (Lama1) genes (21−24). SOX7 modulates the balance between proliferation and differentiation in mesodermal precursors and its knockdown inhibits hematopoietic differentiation (25). Seguin et al demonstrated that ectopic SOX7 expression can transform human embryonic stem cells into stable endoderm progenitors, suggesting a critical role for SOX7 in lineage determination (26). SOX7 is integral to cardiogenesis, and mice with homozygous deletion of the SOX7 exon 2 ($SOX7^{\Delta ex2/\Delta ex2}$) showed embryonic lethality with features indicative of cardiovascular failure (20, 27). SOX7 also regulates angiogenesis and vasculogenesis through mechanisms that are redundant with those of SOX18, implicating its relevance to cancer (20, 28, 29). Consistent with its important role in normal development, SOX7 dysregulation has been implicated in different diseases. Wat et al. reported that mice with the homozygous deletion of SOX7's exon 2, comprising 79%

of its coding region, are embryonically lethal and those with the heterozygous mutation develop congenital diaphragmatic hernia (27). These data suggest that SOX7 haploinsufficiency may contribute to development of congenital diaphragmatic hernia in humans.

Previous studies on the role of SOX7 in human cancers

As many well-characterized tumor suppressors, SOX7 expression is reduced in human cancers compared with normal tissues. Most studies demonstrated frequent downregulation of SOX7 in a variety of human cancers, including breast, lung, colon, and prostate (5-7, 17, 30-32), while only one report suggested increased SOX7 transcript levels in several pancreatic, gastric, and esophageal cancer cell lines, and four cases of primary gastric cancer (30). Multiple mechanisms contribute to the suppression of gene expression, including allele deletion, promoter methylation, histone modifications, mRNA translation or stability, and protein turnover rates (33). SOX7 downregulation has predominantly been attributed to the hypermethylation of its promoter that correlates with poor prognosis in myelodysplastic syndrome (MDS) and lung cancer patients (2, 34). Recently, we demonstrated that shRNA-mediated silencing of DNA methyltransferase 1 (DNMT1), the key enzyme that maintains DNA methylation patterns during cell division, results in elevated SOX7 mRNA levels in MCF-7 and MDA-MB-231 breast cancer cells (6). Consistently, SOX7 mRNA expression is restored upon pharmacological inhibition of

DNMTs by 5−aza−2'−deoxycytidine in multiple cancer cell lines (5, 6, 17), indicating that SOX7 is a common target of aberrant tumor epigenetics. However, while SOX7 protein levels are consistently increased in prostate and colon cancer cells upon DNMT inhibition, we were unable to detect concomitant protein increase in breast cancer cell lines, suggesting that additional mechanisms are involved in downregulating SOX7 expression (6).

The SOX7 gene is deleted (both homozygously and hemizygously) in multiple non−small cell lung cancer (NSCLC) cell lines (32). Additionally, loss of heterozygosity (LOH) at the 8p22-23 locus that houses the SOX7 gene was found in 36% of ductal carcinoma *in situ* breast cancer patients (35). Collectively, these reports indicate that several human cancers preferentially downregulate SOX7 through epigenetic and genetic mechanisms.

The effects of overexpressing SOX7 on cancer cells *in vitro* have been extensively studied by several groups. In prostate and colon cancer cells, forced SOX7 expression inhibits proliferation and colony formation (5, 17), and induces apoptosis in NSCLC, colon, endometrial, and prostate cancer cells (5, 7, 32) We demonstrated that ectopic SOX7 expression through a Doxycycline (Dox)−inducible system significantly reduces MDA−MB−231 breast cancer cell proliferation and their xenograft tumor growth in athymic nude mice (6). Importantly, SOX7 silencing conferred a growth advantage to HEK293 cells (5) and significantly increased proliferation, migration and invasion of nontumorigenic breast cells (6). The proliferative effects of

SOX7 depletion provide strong support to its tumor suppressive role, because these studies can truly recapitulate the scenario of SOX7 downregulation during tumorigenesis.

Despite the growing body of evidence for SOX7's tumor suppressive role in many cancers, the precise molecular mechanism(s) by which it achieves these effects remains unclear. Currently, SOX7 has been demonstrated to activate the expression of FGF3, GATA4, GATA6, LAMA1, VE-Cadherin and SOX4 (19, 21-24). Thus, the target genes of the transcription factor SOX7 are highly understudied. To date, only one report is relevant to SOX7-mediated gene expression in a cancer-relevant setting (19); most SOX7-target genes were identified through developmental studies. SOX7 activates expression of the basement membrane component Lama1, a large glycoprotein that participates in regulating cell migration and other processes through its interactions with various receptors, such as integrins (36-38). SOX7 activates Lama1 through binding its enhancer, and acts synergistically with other transcription actors (SP1/SP3, NF-Y, and SOX17) to mediate Lama1 expression (23). Coexpression of SOX7 with the Fgf-3 promoter-driven luciferase reporter led to increased luciferase activity, and mutation of the SOX7 binding site significantly reduced this activation, suggesting an activating role for SOX7 in Fgf-3 transcription (22). Fgf-3 is critical to development (39-41) and suggested to act as an oncogene in mouse mammary tumors (42); thus, its activation by SOX7 in tumors would contradict the widely supported tumor suppressive function

of SOX7. These functional discrepancies are likely due to the dependency of SOX7-regulated gene expression on the cellular and molecular contexts of a particular cell type. Consistent with this prediction, SOX7 activates Lama1 in undifferentiated mouse F9 embryonal cells, but not HeLa cells (23).

SOX7 also targets vascular endothelial (VE)-cadherin (24), an adhesion protein essential for maintaining endothelial cell contacts (43). SOX7 binding to the VE-cadherin promoter led to its activation in a reporter assay in HEK293 cells, and the integrity of the SOX7 binding site proximal to the transcription start site is necessary for complete promoter activation in bEnd.3 endothelial cells (24). Interestingly, stimulation by vascular endothelial growth factor (VEGF) that is frequently overexpressed in human cancers downregulates SOX7 (24), while the same treatment also increases the permeability of endothelial cell monolayers (44). Thus, it is reasonable to hypothesize a feedback loop in human tumors with upregulated VEGF signaling and downregulated SOX7, in which VEGF signaling inhibits SOX7-mediated VEcadherin expression, resulting in reduced endothelial cell contacts and increased intravasation of tumor cells into the bloodstream. This predicted mechanism may explain the positive correlation between SOX7 expression and distant metastasis-free survival in prostate and breast cancers (6, 31). Notably, the effects of SOX7 on its currently discovered target genes are all activating (21-24). This is consistent with a previous study indicating that SOX7 possesses a

transactivation domain but lacks any welldefined transrepression domain (45).

Multiple studies indicate SOX7 antagonism of Wnt/β-catenin signaling (5, 7, 13, 17, 19), a commonly upregulated pathway in human cancers involved in the regulation of cell proliferation, survival, and migration (46). SOX7 was first found to inhibit TCF/LEF-β-catenin activation of the TCF/LEF-dependent TOPFLASH reporter in HEK293 cells (13), and this observation was later recapitulated in colon and prostate cancer cell lines (5, 17). Further, SOX7 colocalizes and physically interacts with β-catenin in endometrial cancer cells (7). As discussed above, the SOX7-β-catenin interaction is mediated by D_{328}RNEFDQY_{335} of SOX7 (17); however, the SOX7-binding region in β-catenin has yet to be determined. Functionally, SOX7 overexpression reduces mRNA and protein levels of the Wnt-signaling targets cyclin D1 and survivin in colon cancer cells (5), but whether this regulation is through SOX7-mediated expression of these two genes is unclear. Interestingly, a SOX7-related protein, SOX4, may function as either an oncogene or tumor suppressor, depending on cellular contexts (47). It acts as a positive regulator of β-catenin signaling through its upregulation of TCF4 transcription (19). SOX7 transcriptionally activates SOX4 expression in endometrial cancers, but also inhibits SOX4-mediated β-catenin/TCF4-driven transcription (19), indicating that a complex regulatory network likely exists in the tumor scenario among different SOX members. The well-characterized SOX7 inhibition of

Wnt/β−catenin signaling suggests that SOX7 may have a tumor suppressive role that is independent of its DNA−binding ability, in addition to its activity in regulating gene transcription.

Ⅲ. Materials and Methods

Cell culture

Four human OSCC cell lines (SCC−4, SCC−9, SCC−15, and SCC−25) and MDA−MB−231 human breast cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA). SCC cells were cultured in DMEM/F−12 medium and MDA−MB−231 cells were cultured in RPMI 1640, containing 10 % fetal bovine serum (FBS) under a humidified atmosphere with 5 % CO_2 at 37 °C.

Transfection of small interfering RNA

For the interference of SOX7 mRNA and protein expression, SOX7 siRNA was purchased from Thermo Fisher Scientific (s224906, San Jose, CA, USA). SCC−4 cells were seeded in a 6−well plate at 2 x 10^5 cells. After 18 hours, cells were transfected with 50 nM of control siRNA or SOX7 siRNA, using the Lipofectamine RNAi Max reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. 48 hours after incubation at 37°C in a CO_2 incubator, cells were used to assays for western blot, cell proliferation and in vitro invasion.

Cell proliferation assay

Human SOX7 peptide was purchased from Abcam (Cambridge, UK). A WST-1 assay kit (Roche Diagnostics, GmbH, Germany) was used to analyze cell proliferation. Briefly, SCC-9 and SCC-25 cells were seeded in a 96-well plate at 1×10^3 cells. After 18 hours, serum-free media containing SOX7 peptide (50 or 100 ng/ml) were added to each well and incubated for 24, 48, and 72 hours. Then, 10 μl of WST-1 reagent was added to each well. The reaction proceeded for 4 hours at 37°C with 5% CO_2. The absorbance of samples at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). All assays were performed in triplicate.

Western blot analysis

Intracellular proteins were prepared using the Nuclear/Cytosol fraction Kit (Biovision, Carlsbad, CA, USA). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA). After addition of sample loading buffer, total protein from cell lysates was separated by SDS-PAGE gel electrophoresis (Bio-Rad), transferred onto a polyvinylidene difluoride membrane (Pall, AnnArbor MI, USA), and detected using the following antibodies: Sox-7 (Santa Cruz, sc-20093), GAPDH (Abcam, ab9484) and -Tubulin (Santa Cruz, sc-23948). Immunostaining was detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences,Westborough, MA, USA) according to the

manufacturer's instructions.

Clonogenic assay

The assay was performed as described previously (48). Briefly, SCC-9 and SCC-25 cells suspended in DMEM/F-12 medium with 10% FBS were seeded in a 6-well culture plate at 500 cells. After 18 hours, 100 ng/ml of SOX7 peptide was added to each well and the plate was re-incubated. Every 3 days, the medium was replaced with fresh medium containing SOX7 peptide. Two weeks later, colonies were stained by 0.5% crystal violet and counted. All the experiments were performed in triplicate.

In vitro invasion assay

For the Matrigel invasion assay, 5×10^4 cells/well in serum-free medium were seeded onto a upper chamber (No. 354480, BD Bioscience, Bedford, MA, USA) filter that was pre-coated with Matrigel, and the lower chamber was loaded with medium containing 10% FBS as a chemoattractant. The transwell chambers were incubated at 37 °C with 5 % CO_2. After 16 to 48 hours of incubation, the cells on the upper surface of the filter were wiped off with a cotton swab, and the filter was removed from the chamber and stained with the Diff-Quick stain set (Fisher, Pittsburgh, PA, USA). The cells that migrated to the undersurface of the membrane were counted using a light microscope. Ten microscopic fields at x100 magnification were randomly selected to count the cells for each assay.

Patients and tissue specimens

Fifty-seven OSCC specimens were retrieved from the patients who were diagnosed and surgically treated at the Department of Oral and Maxillofacial Surgery, Seoul National University Dental Hospital, between 1999 and 2005. The clinical features including age, gender, recurrence and survival were collected from medical records of the patients. Tumor grading was done based on the World Health Organization Classification of Tumours (49). Tumors were staged according to the TNM system recommended by the American Joint Committee on Cancer (50). The clinicopathological features of 57 OSCC patients are summarized in Table 1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of 57 OSCC and 12 adjacent normal oral mucosal tissues were sectioned at 4-μm thickness. The sections were deparaffinized in xylene, and rehydrated through graded alcohol solutions. Heat-induced antigen retrieval was done in a microwave oven for 10 minutes in Tris/EDTA buffer (pH 9.0). Endogenous peroxidase activity was inactivated by Peroxidase Blocking Reagent (Dako, Glostrup, Denmark) for 5 minutes. Sections were incubated with rabbit polyclonal antibody against SOX-7 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Immunoreaction was performed using REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) (Dako), and reactions were

visualized using REAL^{TM} DAB+ Chromogen with REAL^{TM} Substrate Buffer (Dako). All sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Normal oral mucosal tissues were used as positive controls, and primary antibodies were omitted for negative controls.

Evaluation of immunohistochemical staining

Immunohistochemically stained sections were evaluated semi-quantitatively by two independent pathologists. In cases of disagreement between two observers, the sections were reassessed to reach a consensus. Ten representative microscopic fields were assessed per slide at x400 magnification. Tumor cells showing nuclear staining were considered as positive, and evaluation of each stained section was performed for both staining intensity and percentage of positive cells as described previously with a slight modification (2, 8). Staining intensity was scored as follows: 0 for negative, 1 for mild, 2 for moderate, and 3 for strong staining. Percentage of positive cells was scored on following scales: 0 for 0%, 1 for 1-10%, 2 for 11-50%, and 3 for 51-100%. The final immunohistochemical score was established by multiplying the intensity score and the percentage score, ranging from 0 to 9. For statistical analysis, the final score $\geq$ 2 was defined as positive SOX7 expression, and the score $<$ 2 as negative SOX7 expression.

Statistical analysis

For in vitro study, the results were expressed as means ± standard deviations. Student's t-test was used to determine the significant difference between the experimental and control group. For immunohistochemical study, Pearson chi-square test and Fisher's exact test were performed to evaluate the relationship between SOX7 expression and clinicopathological factors. Cumulative survival rates were calculated by the Kaplan-Meier method, and the difference between the curves was analyzed by the log-rank test. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics Version 21 (IBM, Armonk, NY, USA).

Ⅳ. Results

SOX7 silencing induced cell proliferation and invasion in OSCC cell lines

SCC-4 cells showed most prominent SOX7 expression, and SCC-15 cells demonstrated relatively weak SOX7 expression. On the other hand, SCC-9 and SCC-25 cells demonstrated almost negative SOX7 expression (Figure 1A). SCC-4 cells, treated with SOX7 siRNA, showed decreased SOX7 expression (Figure 1B). WST-1 assays revealed that SOX7 downregulation enhanced proliferation of SCC-4 cells at 24, 48, and 72 hours, compared with control SCC-4 cells ($P < 0.05$; Figure 1C). Similarly, Matrigel assays showed that SOX7 downregulation induced cell invasion in SCC-4 cells ($P < 0.001$; Figure 1D).

SOX7 peptide inhibited cell proliferation, colony formation, and invasion in OSCC cell lines

In WST-1 assays, SCC-9 and SCC-25 cells, which were treated with SOX7 peptide, showed decreased rates of cell proliferation, compared with control cells ($P < 0.05$ at 72 hours; Figure 2A and 2B). In addition, SCC-9 and SCC-25 cells, treated with SOX7 peptide, showed decreased levels of colony formation ($P < 0.05$ and $P < 0.005$, respectively; Figure 2C and 2D). Matrigel assays revealed that SOX7 peptide treatment markedly decreased the number of invaded cells, compared with control cells ($P < 0.05$ and $P < 0.005$, respectively; Figure 2E and 2F).

The expression level of SOX7 protein was decreased in OSCC tissues

All normal oral mucosal tissues (12/12, 100%) showed nuclear staining for SOX7 protein (Figure 3A). Of 57 OSCC samples, however, 17 cases (29.8%) showed nuclear localization of SOX7 protein ($P < 0.001$, compared with normal oral mucosa; Figure 3B). In the other cases, SOX7 protein was negatively expressed (Figure 3C) or located solely in the cytoplasm (Figure 3D).

SOX7 protein expression correlated with clinicopathological factors in OSCC patients

Negative SOX7 expression in OSCC was significantly associated with positive lymph node metastasis ($P = 0.041$) and advanced TNM stage ($P = 0.024$). However, no correlation was observed between SOX7 expression and the other clinicopathological factors (Table 2).

Negative SOX7 protein expression correlated with poorer prognosis

Of 57 OSCC patients, 32 patients had died (56.1%), and 5-year survival rate was 47.4% (27/57). Negative nuclear SOX7 expression was strongly associated with poorer overall survival ($P = 0.017$; Figure 4).

Ⅴ. Discussion

Several studies have indicated that SOX7 antagonizes the Wnt/β-catenin signaling pathway, a commonly upregulated pathway in human cancers (1). Downregulation of SOX7 is associated with high expression of β-catenin and its downstream targets, cyclin D1 and FGF9, in endometrial cancer (7). Similarly, in epithelial ovarian cancer, SOX7 is negatively correlated with cyclin D1 and COX2, suggesting SOX7 inhibits the activity of Wnt target genes (8). Cui et al. (9) have demonstrated a negative correlation between SOX7 and β-catenin and hypothesized that the regulation of β-catenin signaling by upregulating SOX7 expression may prevent carcinogenesis in gastric cancer. Although the definite binding region has not been determined, SOX7 has been reported to directly bind to β-catenin and inhibit Wnt/β-catenin signaling (1, 8, 9).

Dysregulation of the Wnt signaling pathway can promote the progression of oral cancer and is significantly associated with prognosis in patients with OSCC (51). Epigenetic silencing by DNA methylation of Wnt antagonists is frequently detected in oral cancer and considered as a prognostic marker (52). Lee et al. (53) have showed a significant association between EGFR expression and aberrant accumulation of β-catenin in oral cancer, suggesting EGFR signaling regulates β-catenin localization and stability. In addition, LEF1, a transcriptional factor mediating Wnt/β-catenin signaling, is significantly associated with poor prognosis in OSCC (54). In

the present study, cell proliferation, colony formation, and invasion were suppressed by SOX7 peptide treatment in OSCC cells. Considering the aforementioned correlation between SOX7 and Wnt/β-catenin signaling, these findings may correlate with the inhibitory effect of SOX7 on the Wnt/β-catenin signaling pathway involving the development and progression of OSCC. Further studies are necessary to clarify the association of SOX7 expression with the activity of β-catenin and its targets in OSCC.

Genetic and epigenetic alterations of SOX7 have been found in human cancers (5, 32). Zhang et al. (5) discovered that SOX7 silencing in colorectal cancer is partially due to the aberrant DNA methylation of the SOX7 gene. In non-small cell lung cancer, a homozygous deletion was found at 8p23.1 where the SOX7 gene is located (32). Besides these alterations, Zheng et al. (55) have suggested that microRNA-452 directly inhibits the expression of SOX7 gene and promotes cancer stem cells by activating the Wnt/β-catenin signaling pathway. In the present study, decreased SOX7 expression was observed in OSCC cell lines and OSCC tissues. DNA methylation, genetic deletion, or microRNAs may be involved in the inactivation of the SOX7 tumor suppressor gene in OSCC. Further investigations are warranted to determine the mechanism of SOX7 downregulation in OSCC.

Decreased expression of SOX7 correlates with clinicopathological factors and prognosis in cancer patients (2,8,9). Reduced SOX7 expression is

associated with lymph node metastasis, advanced TNM stage, and poor survival in lung adenocarcinoma (2) and gastric cancer (9). Similarly, our results demonstrated that decreased SOX7 expression significantly correlated with positive lymph node metastasis, advanced TNM stage, and poor overall survival in OSCC patients. Taken together, the expression level of SOX7 can be a useful prognostic factor in cancer patients.

VI. Conclusion

These results suggest that SOX7 inhibits cell proliferation, colony formation, and invasion in OSCC as a tumor suppressor and that negative SOX7 expression could be a poor prognostic indicator in OSCC patients.

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Table 1. Clinicopathological features of 57 OSCC patients

Variable	No. of cases (%)
Age (years; mean, 55.9)	
20−29	2 (4)
30−39	3 (5)
40−49	14 (25)
50−59	16 (28)
60−69	12 (21)
70−79	10 (18)
Gender	
Male	48 (84)
Female	9 (16)
Differentiated status	
Well	41 (72)
Moderately	14 (25)
Poorly	2 (4)
T classification	
T1	7 (12)
T2	28 (49)
T3	3 (5)
T4	19 (33)
N classification	
N0	19 (33)
N1	17 (30)
N2	21 (37)
M classification	
M0	55 (96)
M1	2 (4)
Stage	
I	4 (7)
II	6 (11)
III	14 (25)
Ⅳ	33 (58)
Recurrence	
No	41 (72)
Yes	16 (28)

Table 2. Relationship between SOX7 expression and clinicopathological factors in OSCC patients

Variable	No. of cases (N=57)	SOX7 Negative (N=40)	SOX7 Positive (N=17)	*P* value
Age				
<56	25	17	8	0.751
≥56	32	23	9	
Gender				
Male	48	34	14	0.524
Female	9	6	3	
Differentiated status				
Well	41	28	13	0.438
Moderately to poorly	16	12	4	
Tumor size				
T1+T2	35	22	13	0.109
T3+T4	22	18	4	
Lymph node metastasis				
Negative	19	10	9	0.041
Positive	38	30	8	
Distant metastasis				
Negative	55	38	17	1.000
Positive	2	2	0	
Stage				
I+II+III	24	13	11	0.024
IV	33	27	6	
Recurrence				
No	41	28	13	0.438
Yes	16	12	4	

Figure 1. Silenced SOX7 enhanced cell proliferation and invasion in OSCC cell lines. (A) Western blot analysis of SOX7 expression in four OSCC cell lines (SCC-4, SCC-9, SCC-15 and SCC-25), MDA-MB-231 breast cancer cells (NC, negative control), and PC-3 cell lysate (PC, positive control; Santa Cruz, sc-2220). -tubulin was used as an internal control. (B) By transfection of siRNA, SOX7 expression was markedly decreased in SCC-4 cells. GAPDH was used as an internal control. By transfection of siRNA, the proliferation rate (C) and the number of invaded cells (D) were significantly increased in SCC-4 cells. *P < 0.05 and **P < 0.005.

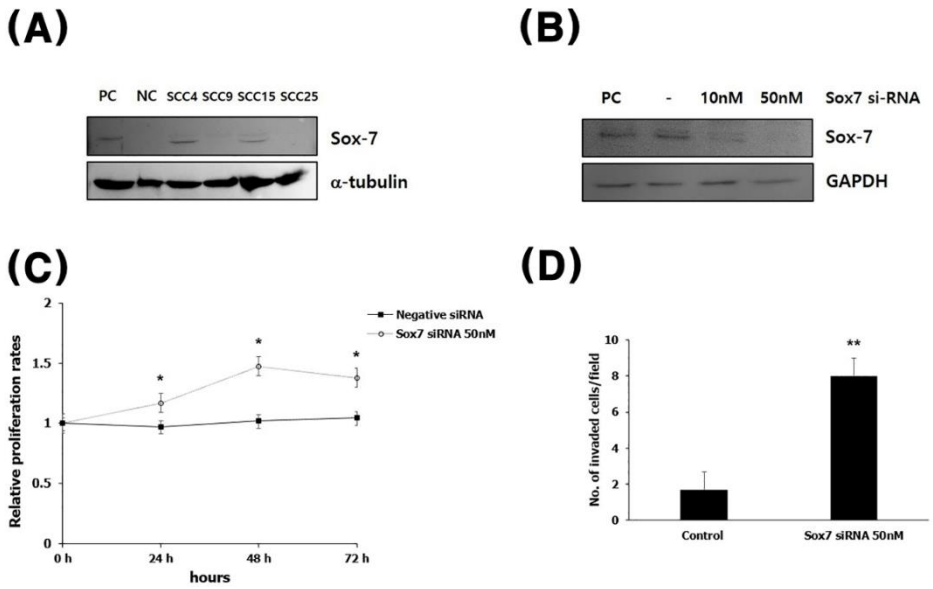


Figure 2. SOX7 showed tumor suppressive effect in OSCC cell lines. (A and B) Growth curves of SCC-9 and SCC-25 cells. SOX7 peptide treatment showed inhibition of proliferative activity. (C and D) Clonogenic assays revealed the inhibitory effect of SOX7 peptide treatment on colony formation in SCC-9 and SCC-25 cells. (E and F) The Matrigel invasion assay showed that SOX7 peptide treatment induced decreased invasiveness in SCC-9 and SCC-25 cells. *P < 0.05 and **P < 0.005.

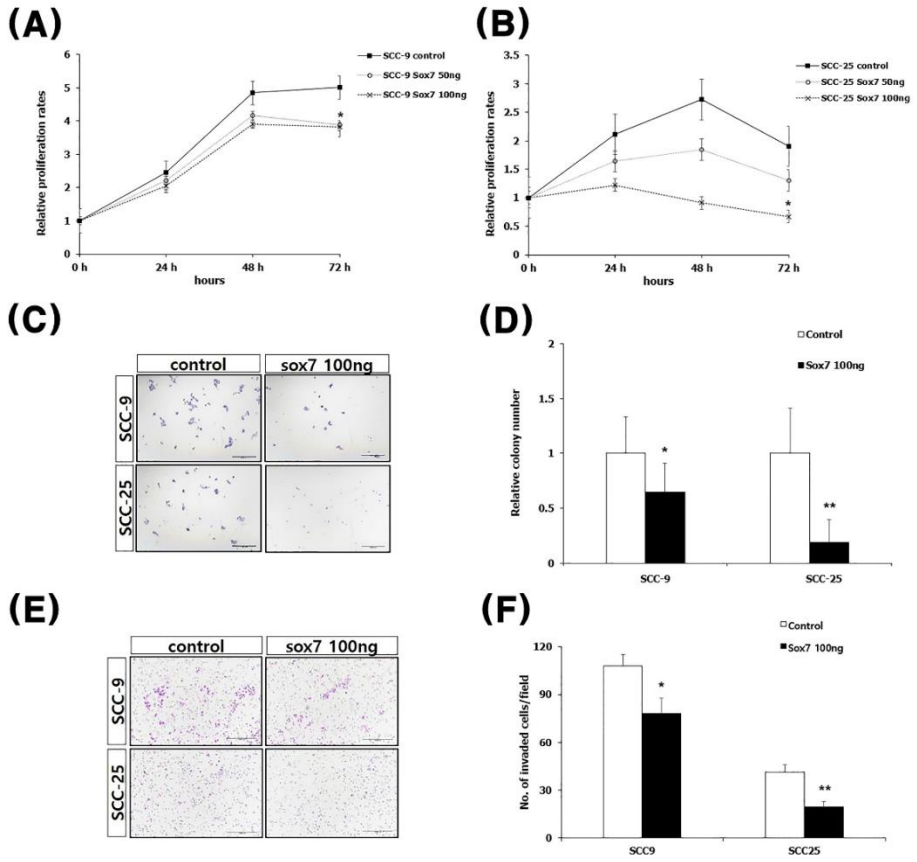


Figure 3. Immunohistochemical staining of SOX7 protein. (A) Strong nuclear expression in a normal oral mucosal tissue (original magnification x200). (B) Strong nuclear expression in a OSCC tissue (original magnification x400). (C) Negative SOX7 staining in a metastatic lymph node. (original magnification x200). (D) Focal cytoplasmic staining which was finally defined as negative SOX7 expression (original magnification x400).

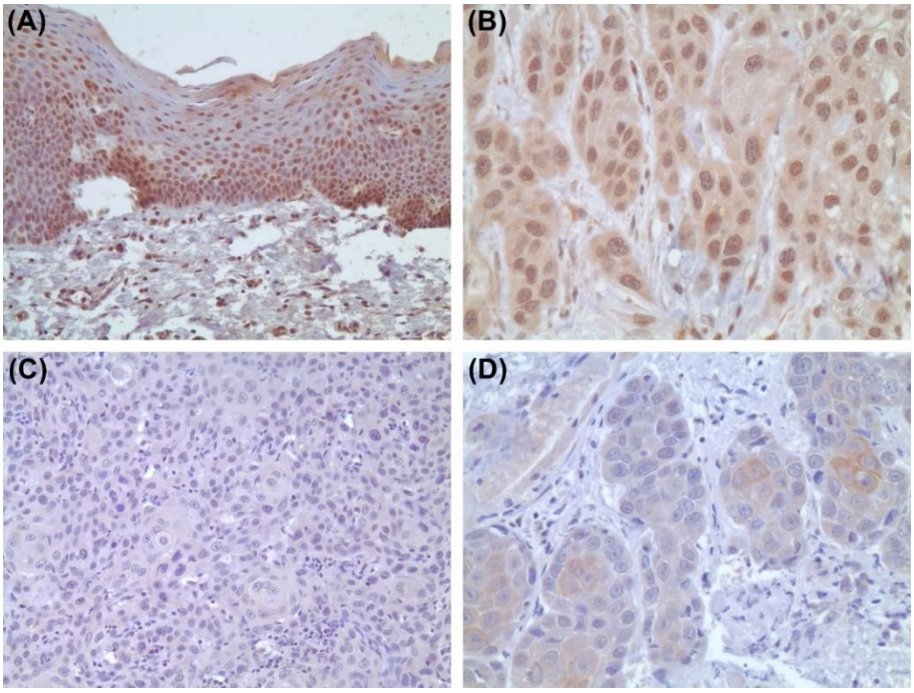
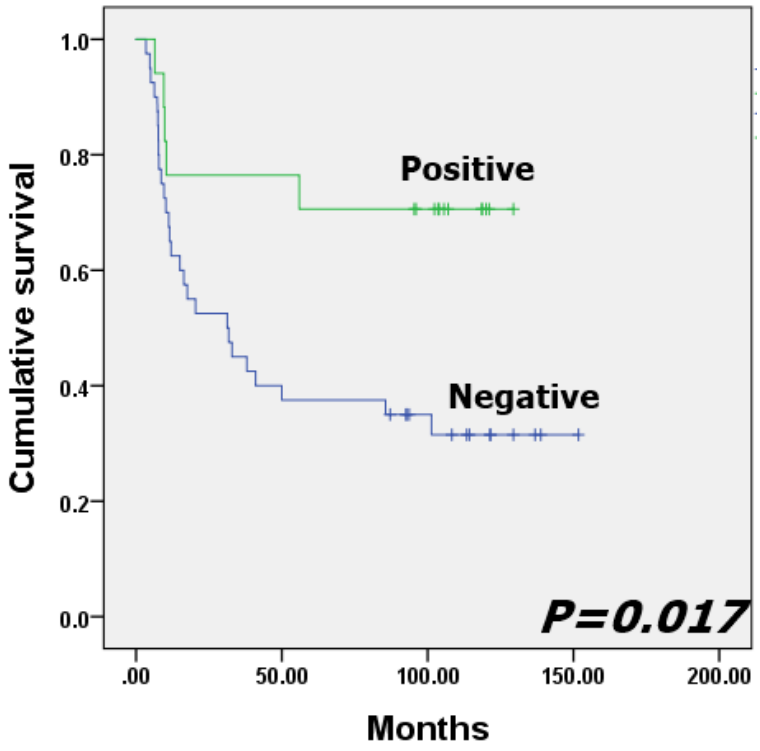


Figure 4. Kaplan-Meier survival analysis in OSCC patients (N=57). Patients with negative SOX7 expression showed significantly poor survival.



국문초록

구강편평세포암종의 세포 증식 및 침습과 환자의 예후에서의 SOX7 의 역할

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SOX family의 전사인자 중 하나인 SOX7은 다수의 암에서 종양 억제자로서 작용하는 것으로 알려져 있다. 몇몇 진행된 종양에서 SOX7의 발현 억제가 보고되고 있으며, 이러한 저발현은 불량한 예후와 관련이 있는 것으로 알려져 있다. 본 연구에서는 구강편평상피세포암종 (OSCC) 세포주에서의 SOX7의 항종양 작용에 대해 알아보고자 하였다. 또한, OSCC 조직에서의 SOX7 단백질의 발현을 살펴보고, 그 발현과 예후와의 상관성을

분석하고자 하였다. OSCC 세포주에서 SOX7에 대한 small interfering RNA (siRNA) 혹은 SOX7 펩타이드 처리에 따른 세포 증식능, 침윤능 및 군집형성능의 변화를 살펴보기 위해, WST-1, Matrigel 그리고 clonogenic assay 분석를 시행하였다. 또한, 면역조직화학적 염색을 통하여, OSCC 및 정상 구강점막 조직에서의 SOX7 단백질 발현 양상을 평가하였고, SOX7 단백질 발현 정도와 여러 임상병리학적 요소들 간의 상관관계에 대해 분석하였다. SCC-4 세포주에서 SOX7 발현 억제에 따른 세포 증식능 및 침윤능의 증가가 관찰되었다. SCC-9 및 SCC-25 세포주에서 SOX7 펩타이드 처리에 따른 세포 증식능, 침윤능 및 군집형성능의 억제가 관찰되었다. 정상 구강점막과 비교 시, OSCC 조직에서 SOX7 단백질의 저발현이 관찰되었다 ($P < 0.001$). OSCC 환자에서 SOX7의 음성 발현은 림프절전이 ($P = 0.041$), 진행된 TNM 병기 ($P = 0.024$) 및 불량한 예후 ($P = 0.017$)와 유의한 상관성을 보였다. 결론적으로, OSCC에서 SOX7이 종양 억제자로 작용할 수 있고, OSCC 환자에서의 SOX7 음성 발현은 불량한 예후인자임을 제안한다.

주요어 : SOX7, 구강편평상피세포암종, 예후인자, 항종양 작용

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