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Thymosin β4 induces proliferation, invasion, and epithelial-to-mesenchymal transition of oral squamous cell carcinoma

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Abstract

Thymosin β4 induces proliferation, invasion, and epithelial-to-mesenchymal transition of oral squamous cell carcinoma

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Objectives: Epithelial-to-mesenchymal transition (EMT) is an important process in the invasion and metastasis of epithelial tumor including oral squamous cell carcinoma (OSCC) and modulated by cytoskeleton remodeling. However, the functional role of Tβ4 in the process of EMT and Tβ4 expression in patients with OSCCs have not yet been studied. Therefore, this study was examined whether Tβ4 expression could induce in an EMT-like phenotype and affects invasiveness and cell motility-associated signaling molecules in OSCC cells. In addition, Tβ4 expression was evaluated the correlation between its expression based on immunohistochemical analysis and clinicopathological features and investigated the relationship between Tβ4 expression and the grade of tumor budding in 59 patients with OSCCs.

Methods: To examine cell proliferation, a WST-1 assay was used and for testing ancorage-independent growth, a soft agar colony forming assay was performed. Using real time PCR and western blot, expression of Tβ4 and EMT marker was detected and immunocytochemistry was carried out observation of EMT-like phenotype. To ascertain invasiveness of cancer cells, Matrigel assay was performed and MMP enzyme activity was analyzed by gelatin zymography. Also, the expression of cell motility-associated signaling molecules was confirmed by western blot and to address the potential molecular mechanism underlying Tβ4-overexpressing, phosphokinase antibody array was quantified expression of phosphorylation protein. In addition, using the immunohistochemistry, Tβ4 expression was evaluated the correlation of clinicopathological features and tumor budding and overall survival in 59 patients with OSCCs.

Results: Tβ4-overexpressing OSCC cells, SCC-15_Tβ4 and SCC-25_Tβ4, enhanced cell proliferation and colony formation. Tβ4-overexpression induced an EMT-like phenotype, accompanied by a decrease in expression of the epithelial cell marker E-cadherin and an increase in expression of mesenchymal cell markers vimentin and N-cadherin. Also, the expression level of Twist, an EMT-inducing transcription factor, was significantly enhanced in SCC-15_Tβ4 and SCC-25_Tβ4 cells. Tβ4-overexpression augmented *in vitro* invasion and MMP-2 activity and enhanced the phosphorylation of paxillin and cortactin and expression of LIMK1. Moreover, in patient with OSCC, Tβ4 expression is significantly related with tumor budding, implicated in lymph node metastasis and overall survival in OSCC patients.

Conclusion: Taken together, these results suggest that Tβ4-overexpression could be one of the causes of tumorigenesis and progression in OSCCs. Further investigation on the Tβ4 molecule would encourage the development of specific targets for cancer treatment.

Keywords : Thymosin β4, oral squamous cell carcinoma, epithelial-to-mesenchymal transition, proliferation, invasion

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CONTENTS

I. Introduction

Behaviors such as tobacco smoking and excess alcohol intake are major causes of oral squamous cell carcinomas (OSCCs), which are a common type of malignant tumors of the head and neck (1-3). OSCCs encompass at least 90% of all oral malignancies (4, 5) and are characterized by a high degree of local invasiveness and a high rate of metastasis to the cervical lymph nodes (6), with a 5-year overall survival rate below 50% (7). However, mechanisms related to the metastasis of OSCCs are complex and remain poorly understood.

Accumulating lines of evidence have shown the contribution of epithelial-to-mesenchymal transition (EMT) to tumor metastasis(8, 9). Undergoing EMT, epithelial cells lose their unique polarity and gain mesenchymal phenotypes (10). These phenotypic changes of EMT correlate with decreasing E-cadherin expression in epithelial cells by the transcriptional repressor Twist1 (11) and increasing expression of mesenchymal markers, such as vimentin and N-cadherin (12). As well as changes in gene expression, cytoskeleton reorganization is also required for mesenchymal phenotypes such as increased cellular motility and invasiveness (13).

Actin and its associated proteins are units for cell migration and cytoskeleton remodeling, and their assembly process is modulated by a large number of actin-binding proteins (ABPs) (14). One of the representative proteins of ABPs is thymosin β4 (Tβ4), a highly conserved acidic 5-kDα N-terminally acetylated peptide. It forms a 1:1 complex with G-actin which inhibits polymerization to F-actin (15). Recently, numerous studies have reported that Tβ4 has multiple biological activities to promote angiogenesis (16), chemotaxis (17), and wound healing (18). The induction of migration and invasion by Tβ4 has also been reported in various cancer cells including the uterine cervix (19), prostate (20), pancreas (21) and kidney (22). Apparently, human gastric cancer cells, such as SNU668, showing a higher expression level of Tβ4 have a higher migratory potential compared to SNU638 cells showing

relatively low expression of Tβ4 (23). Upregulation of Tβ4 expression has also been found in melanoma cell lines isolated from metastatic tumors and in clinical metastatic melanoma tissues (24). However, the functional role of Tβ4 in the process of EMT and Tβ4 expression by immunohistochemistry in OSCCs have not yet been studied. One report, though, has shown Tβ4 overexpression in metastatic OSCCs (25).

Tumor budding is defined as the presence of either single cells or small clusters of up to 5 tumor cells scattered in the stroma ahead at the invasive front (26). Tumor budding have two malignant features of cellular discohesion and active invasion, which may explain the more aggressive cancer characteristic (27-29). It can be histologically represent an EMT feature which has an infiltrating tumor growth pattern (30). For instance, expression of the EMT marker E-cadherin was decrease at the invasive front and up-regulation of vimentin was found in invasive front tumor cells (31-33). Additionally, tumor budding cells showed increased expression of Snail (34, 35), Twist1, Twist2 (36), a transcriptional repressor of E-cadherin, and β-catenin at the tumor invasive front (37, 38). This evidences suggest that these tumor budding cells have undergone an EMT. More recently, tumor budding has been found to be a valuable prognostic maker of adverse clinicopathological features such as tumor stage, metastasis of lymph node and poor patient outcome (29, 39-41). However, implication of tumor budding and clinicopathological features in OSCCs has been few investigated. Therefore, this study was investigated whether overexpression of Tβ4 could induce *in vitro* tumorigenesis and an EMT-like phenotype in OSCC cells. Moreover, this study was evaluated the correlation between Tβ4 expression based on immunohistochemical analysis and clinicopathological features and investigated the relationship between Tβ4 expression and the grade of tumor budding in OSCCs.

II. Materials and Methods

Cell culture

Human oral squamous cell carcinoma SCC-15 and SCC-25 cells were derived from the tongue and featured a rounded morphology, not stratify (42). SCC-15 and SCC-25 cells were purchased from American Type Culture Collection (Rockville, MD, USA). SCC-15 and SCC-25 cells were cultured in DMEM/F-12 medium containing 10% fetal bovine serum (FBS) under a humidified atmosphere with 5% CO_2 at 37℃.

Human Tβ4 cloning and viral infection

Total RNA from Ca9-22 cells (a gift from Prof. Takata, Hiroshima University) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, first-strand complementary DNA was synthesized from the total RNA using SuperScript III (Invitrogen) according to the manufacturer's instruction. The cDNA coding region of human Tβ4 was amplified by polymerase chain reaction (PCR) from the cDNA using amplification conditions at 94℃ for 30 sec, 61℃ for 50 sec, and 72℃ for 50 sec. The amplified product with forward (5'-tccgcaaccatgtctgacaaaccc-3') and reverse (5'-cgattcgcctgcttgcttct-3') primers was inserted into the pGEM-T vector (Promega, Madison, USA), and the orientation of the insert was determined by enzymatic digestion. DNA sequence analysis confirmed that the sequence of the cloned Tβ4 cDNA was identical to HUMTHYB4 (accession number M17733). The cloned product was digested with Not1 and Sal1 and inserted into the Not1 and Sal1 sites of the pLNCX2 retro viral vector (Clonetech, Mountain View, CA, USA). pLNCX2 containing human Tβ4 was transfected into a packaging cell line such as the RetroPackTM PT67 (Clonetech). The retroviral particles were concentrated with a Retro-X concentrator (Clonetech) and used for infection of SCC-15 and SCC-25 target cells. Tβ4-overexpressing OSCC cell clones were selected for neomycin (Sigma-Aldrich, St. Louis, MO, USA) resistance. Two clones were selected for the

experiments and named SCC-15_ Tβ4 and SCC-25_ Tβ4.

Cell proliferation assay

A WST-1 assay kit (Roche Diagnostics, GmbH, Germany) was used to analyze cell proliferation. Briefly, 1×10^3 cells were seeded in a 96-well plate with serum-free media and incubated for 24, 48, and 72h. Then, 10ml of WST-1 reagent was added to each well. The reaction proceeded for 4h at 37℃ with 5% CO2. The absorbance of samples at 450nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). This assay was performed intriplicate.

Soft agar colony-forming assay

For testing anchorage-independent growth, a soft agar colony forming assay was performed using standard protocols. Briefly, each well (35nm) of a six-well culture dish was coated with 2 ml of a bottom agar (Duchefa, Haarlem, Netherlands) mixture (DMEM/F12, 10% (v/v) FBS, 0.6% (w/v) agar). After the bottom layer was solidified, 2 ml of a top agar-medium mixture (DMEM/F12, 10% (v/v) FBS, 0.3% (w/v) agar) containing 1×10^4 cells was added. The dishes were then incubated at 3 7℃ in a 5% CO2 humidified atmosphere. Additional medium was overlaid onto the agar, and the cells were then allowed to grow undisturbed for 2 weeks. The total number of colonies having a diameter ≥100mm was counted over five fields per well for a total of 15 fields from triplicate experiments (43).

RNA extraction and qRT- PCR

mRNA was isolated from cells using Trizol reagent (Invitrogen) and treated using the TURBO DNA-freeTMkit (Ambion, Carlsbad, CA, USA) according to the manufacturer's recommended protocol. RNA concentration and purity were determined using a Nanodrop at 260 and 280nm, respectively. Samples showed an absorbance ratio (260/280) of ≥1.8. cDNA was synthesized using M-MuLV RT and oligo

(dT)-18primers (Invitrogen) according to the manufacturer's instructions. For real-time PCR, quantitative PCR (qPCR) was performed using the 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with the SYBR Select Master Mix kit (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal control. The cycling profile was as follows: 50℃ for 2 min, 95℃ for 2 min followed by 40 cycles of 95℃ for 3 sec and 60℃ for 30 sec. Primer sequences were designed as follows: Tβ4 forward, 5'-AAA CCC GAT ATG GCT GAG AT-3'; Tβ4 reverse, 5'-TGC TTC TCC TGT TCA ATC GT-3'; E-cadherin forward, 5'-GAACAG CAC GTA CAC AGC CCT-3'; E-cadherin reverse, 5'-GCA GAA GTG TCC CTG TTC CAG-3'; vimentin forward, 5'-GAT CGA TGT GGA TGT TTC CAA-3'; vimentin reverse, 5'-GTT GGC AGC CTC AGA GAG GT-3'; Twist 1 forward, 5'-TGT CCG CGT CCC ACT AGC-3'; Twist 1 reverse, 5'-TGT CCA TTT TCT CCT TCT CTG GA -3'; GAPDH forward, 5'-TGC ACC ACC AAC TGC TTA GC-3'; GAPDH reverse, 5'-GGC ATG GAC TGT GGT CAT GAG-3'. All reactions were performed in triplicate, and data were analyzed using the 2-ΔΔCT method (44). Statistical significance was determined using the Student's t-test with GAPDH-normalized 2-ΔΔCT values.

Immunofluorescent staining and confocal microscopy

Cells (5×10^4) cultivated on glass cover slips were fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton X-100, and blocked with 1% bovine serum albumin (BSA)/10% FBS in phosphate buffered saline (PBS). The cell samples were incubated with primary antibodies at 4℃ overnight, washed with PBS containing 0.1% BSA, and then reacted with FITC- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 40 min. After washing, the samples were rinsed with PBS containing 0.1% BSA, stained with 5 mg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma), and mounted. Confocal analyses were performed using an Olympus FC-300 Confocal Laser Scanning

Microscope (Center Valley, PA) equipped with FITC- and Cy3-channel filter systems. All images were converted to TIFF format and arranged using Photoshop 7.0 (Adobe, Seattle, WA, USA).

In vitro cell invasion assay

For the Matrigel invasion assay, 5×10^4 cells/well in serum-free medium were seeded on to a upper chamber (No. 354480, BD Bioscience, Bedford, MA, USA) filter that was pre-coated with Matrigel, and the lower chamber was filled with medium containing 10% FBS as a chemoattractant. The trans well chambers were incubated at 37℃ with 5% CO2. After 24h of incubation, the cells on the upper surface of the filter were wiped off with a cottons wab, and the filter was removed from the chamber and stained with the Diff-Quick stain set (Fisher, Pittsburgh, PA, USA). Cell migration was determined by counting the number of cells that migrated through the pores to the lower side of the filter under a microscope at 100 × magnification. We performed the assay three times, and three randomly selected fields were counted for each assay.

Gelatin zymography

To analyze MMP-2 activity in Tβ4-overexpressing OSCC cells, the conditioned medium was collected and concentrated using Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA, USA). Equal amounts of protein (40 μg) were loaded onto a gelatin-containing gel (8% acrylamide gel containing 1.5 mg/ml gelatin) and separated by electrophoresis. The gel was renatured in 2.5% Tween-20 solution with gentle agitation for 30 min at room temperature. Next, the gel was developed overnight at 37℃ in zymogram incubation buffer (50 mM Tris-HCl (pH 7.6) and 5 mM $CaCl_2$), stained with Coomassie Brilliant Blue R250, and destained with a solution of 50% methanol and 10% acetic acid until the part of membrane degraded by MMP-2 became clear.

Western blotting

Intracellular protein was prepared using the Nuclear/Cytosol fraction Kit (Biovision, CA, USA). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA). After addition of the sample loading buffer, total protein from cell lysates was separated by SDS-PAGE gel electrophoresis (Bio-Rad), transferred onto a polyvinylidene difluoride membrane (Pall, Ann Arbor MI, USA), blocked with TBST buffer composed of 50 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20 supplemented with 5% fat-free milk at 4℃ for 1 h. Dilutions of primary antibodies, anti-Tβ4 (Santa Cruz, 1:500), anti-E-cadherin (Santa Cruz, 1:500), anti-N-cadherin (Upstate, 1:1000), anti-vimentin (Santa Cruz, 1:500), anti-Twst1 (Santa Cruz, 1:500), anti-MMP2 (Santa Cruz, 1:500), anti-phospho-paxillin (Tyr118) (Abcam, 1:1000), anti-paxillin (Santa Cruz, 1:1000), anti-phospho-cortactin (Tyr466) (Bioss, 1:1000), anti-cortactin (Santa Cruz, 1:1000), anti-vinculin (Santa Cruz, 1:1000), anti-LIMK1 (Cell Signaling, 1:1000), anti-tubulin (Santa Cruz, 1:1000), anti-Histone H3 (Santa Cruz, 1:500), and anti-GAPDH (Abcam, 1:1000) were made in TBST with 3% fat-free milk. Following three washes with TBST, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST with 3% fat-free milk for 1 h at room temperature. The blots were washed again three times in TBST buffer, and signals were developed using an enhanced chemiluminescence kit (Amersham Biosciences, UK) for 1 min according to the manufacturer's instructions. The Image Saver 5 (ATTO, Tokyo, Japan) was used for signal detection.

Human phospho-kinase antibody array

To determine levels of phospho-kinases at thymosin β4-overexpressing cells, cells were lysed using lysis buffer of the Human phospho-kinase array kit (Proteome ProfilerTM, R&D Systems, Minneapolis, MN, USA). Experiments were performed according the protocol of manufacturer. Briefly, membranes with spotted 46 capture

antibodies were incubated with diluted whole cell lysates at overnight. Thereafter, the membranes were incubated with a cocktail of biotinylated detection antibodies and streptavidin-HRP. Next, ECL chemiluminescent system was used for phosphorylated protein detection. To quantify expression levels, the Image Saver5 (ATTO, Tokyo, Japan) was used for spot detection and the density of each spot was measured using CS Analyzer (ATTO, Tokyo, Japen).

Patients and tissue specimen

Fifty-nine OSCC specimens were collected 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 investigated through the medical records of the patients. Tumor grading was done based on the World Health Organization (WHO) Classification of Tumours (45). Tumors were staged according to the TNM system recommended by the American Joint Committee on Cancer (AJCC)(46). This study protocol was approved by the Institutional Review Board (IRB) of Seoul National University Dental Hospital (IRB approval number: CRI14001). Data collection and analysis were carried out according to the institutional guidelines. The clinicopathological features of 59 OSCC patients are summarized in Table 1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded OSCC specimens were sectioned at 4-μm thickness. The sections were deparaffinized in xylene, and rehydrated through graded alcohol solutions. The slides were incubated with 3% H_2O_2 solution for 10minutes to quench endogenous peroxidase activity. For antigen retrieval, the slides were immersed in 0.01M citrate buffer (pH6.0) and heated in the microwave for 10 minutes. Then, 10% normal goat serum was used to reduce non-specific staining. The slides were subsequently incubated with rabbit polyclonal antihuman Tβ4 (1:400, clone sc-67114,

Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hour. Immunoreaction was performed using the REAL^{TM}EnVision$^{TM/HRP}$kit (Dako), and the reaction was visualized with diaminobenzidine (Dako). All slides were counterstained with Mayer's hematoxylin, dehydrated and mounted. Coloncancer tissue was used as a positive control. For negative control, phosphate buffer saline was used instead of primary antibody.

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 on each slide at ×400 magnification. Tumor cells showing cytoplasmic staining were considered as positive cells, and the evaluation of each stained section was performed in regard to the staining intensity and the percentage of positive cells, respectively. Based on a previous study performed by Can et al. (47) granulocytes were used as an internal positive control to assess the staining intensity. The staining intensity was scored as follows: 0, negative staining; 1, weak staining (lightly stained compared to granulocytes); 2, strong staining (similarly or deeply stained compared to granulocytes). The 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 6. For the statistical analysis, the final score ≥ 3 was defined as positive Thymosin β4 expression, and the score < 3 as negative Thymosin β4 expression.

Evaluation of tumor budding

All hematoxylin and eosin-stained slides of 59 OSCC patients were reviewed by two

independent pathologists. Tumor budding was defined as a single tumor cell or a cluster consisting of fewer than five tumor cells at the invasive tumor front. A microscopic field with the greatest number of tumor budding was selected for evaluation of each case, and the number of tumor budding was counted at ×200 magnification. For the statistical analysis, the number of tumor budding ≥ 3 was considered as positive, and the number < 3 as negative (48).

Statistical analysis

Statistical Package for the Social Science (SPSS) software (IBM SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. *In vitro* study, The Student's *t* test was used to determine the statistical significance of differences between experimental and control groups. $P < 0.05$ and $P < 0.01$ were considered statistically significant. Concerned with study of OSCC patients, the Pearson chi-square test or the Fisher's exact test was performed to evaluate the relationship between thymosin β4 expression or tumor budding and clinicopathological factors. The correlation between thymosin β4 expression and tumor budding was analyzed by the Spearman rank correlation test as well as the Pearson chi-square test. Cumulative survival rates were calculated by the Kaplan-Meier method, and the differences between the curves were analyzed by the log-rank test. *P* value less than 0.05 was considered to be statistically significant.

III. Results

Thymosin β4 overexpression enhances cell proliferation and colony formation

To investigate the role of Tβ4 in OSCCs, SCC-15 and SCC-25 were generated stable Tβ4-overexpressing OSCC cell clones through retroviral-mediated transduction. As shown in Figure 1a and b, two Tβ4-overexpressing OSCC cells, SCC-15_Tβ4 and SCC-25_Tβ4, displayed elevated Tβ4 expression by real-time PCR and Western blot analysis. Tβ4-overexpressing OSCC cells showed increased proliferative activity (Fig. 1c and d). SCC-15_Tβ4 cells proliferated at 118.5%, 121.5%, and 119% of the rate of control vector-infected cells after 24, 48, and 72 h, respectively (P<0.05). SCC-25_Tβ4 cells showed 330.1%, 406.7%, and 456.1% of control cells, respectively (P<0.05). In addition, Tβ4-overexpressing OSCC cells resulted in increased ability of soft agar colony formation (Fig. 1e and f). SCC-15_Tβ4 and SCC-25_Tβ4 cells showed 316.1-fold and 16.8-fold higher colony formation, within 2 weeks after seeding the cells, than control vector-infected cells (P<0.01). These data suggest that overexpression of Tβ4 promotes in vitro tumorigenicity of OSCC cells.

Thymosin β4 overexpression induces an EMT program in OSCCs.

To determine whether Tβ4 promotes EMT in OSCC cells, real-time PCR (Fig. 2a-c) and Western blotting (Fig. 2d) were utilized to assess changes in protein and mRNA levels of EMT-specific markers. These results were observed that Tβ4 overexpression in OSCC cells converted the predominant epithelial phenotype to a mesenchymal phenotype, accompanied by a decrease in expression of the epithelial cell marker E-cadherin and an increase in expression of mesenchymal cell markers vimentin and

N-cadherin. Immunofluorescence microscopy showed that the two Tβ4-overexpressing OSCC cell lines, SCC-15_Tβ4 and SCC-25_Tβ4, had a complete loss of E-cadherin at cell-to-cell junctions; meanwhile, mesenchymal markers N-cadherin and vimentin were upregulated (Fig. 3). To elucidate the possible interaction between Tβ4 and other EMT-inducing transcription factors, the expression of known EMT inducers were examined by using real-time PCR and Western blotting in SCC-15_Tβ4 and SCC-25_Tβ4 cells. These results were found that mRNA and protein levels of Twist1 were significantly enhanced in these cells (Figs. 2 and 3). Together, these results indicate that Tβ4 is an inducer of EMT and its function can be associated with Twist1 signaling.

Thymosin β4 overexpression promotes invasiveness of OSCC cells.

Since the EMT phenotype is usually accompanied by acquisition of greater migratory and invasive ability, this study was evaluated whether Tβ4 was involved in the migration and invasion of OSCC cells. Tβ4-overexpressing OSCC cells showed increased invasiveness (Fig. 4a). SCC-15_Tβ4 and SCC-25_Tβ4 cells showed 13.6-fold and 10.7-fold higher invasiveness, respectively, compared with control vector-infected cells ($P<0.01$). To further explore the mechanism, detection of the MMP-2 acivity was performed by gelatin zymography. As shown in Fig. 4B, the activity of MMP-2 in Tβ4-overexpressing cells was higher than that of control cells. Moreover, this result was shown enhanced protein levels of MMP-2 in SCC-15_Tβ4 and SCC-25_Tβ4 cells by Western blotting (Fig. 4b). These results indicate that Tβ4 is a strong stimulator of cell motility and has invasive behavior in OSCC cells.

Thymosin β4 enhances phosphorylation of paxillin and cortactin and expression of LIMK1 in OSCC cells.

To understand the mechanism underlying the action of Tβ4 to stimulate the invasion of OSCC cells, this study was evaluated the expression levels of proteins related to both actin reorganization and cancer invasion. To distinguish whether activation or upregulation of these molecules is due to Tβ4 *per se* or FBS-mediated nutritional stimulation, Western blot analysis was performed under conditions with both 0.1% and 10% FBS. Tβ4-overexpressing OSCCs increased phosphorylation of paxillin at Y118 and cortactin at Y466 compared to control cells (Fig. 5). Interestingly, phosphorylation of paxillin and cortactin induced by Tβ4 overexpression was not altered by nutritional stimulation. On the other hand, both Tβ4 overexpression and FBS-mediated stimulation did not affect the expression of vinculin. In order to assess whether Tβ4 overexpression affected upstream signaling molecules that regulate actin binding proteins, this study was further investigated the expression level of LIMK1 (Fig. 5). SCC-15_Tβ4 and SCC-25_Tβ4 cells showed increased expression levels of LIMK1 regardless of FBS-mediated stimulation. These results indicate that Tβ4 induces enhanced cell motility through the phosphorylation of cortactin and paxillin and overexpression of LIMK1 in OSCC cells.

Evaluating of Thymosin β4 expression in OSCC specimens

To understand the role of Tβ4 expression in OSCC patients, 59 specimens were verified Tβ4 expression by immunohistochemistry. Immunohistochemical scores of Tβ4 expression are classified in Table 2. Five of 59 cases (8%) showed no positive cells. Thirty-eight cases (64%) were weakly stained, whereas 16 cases (27%) showed strong intensity (Fig. 7). In terms of the percentage of positive cells, more than half of the cases (31/59, 53%) were categorized as percentage score 3. Subsequently, 21 (36%)

and two (3%) cases were scored, respectively, as 2 and 1. According to the criteria of the final immunohistochemical score, 38 cases (64%) were classified as positive Tβ4 expression, and the rest (21/59, 36%) as negative Tβ4 expression.

Relationship between Thymosin β4 expression or tumor budding and clinicopathological factors in OSCC patients

To examine the correlation of Tβ4 expression and clinicopathological factors in OSCC patients, the Pearson chi-square test was performed. There was no significant relationship between Tβ4 expression and clinicopathological factors (Table 3). Since tumor budding has been regard as the morphological correlation of EMT process, this study was counted the tumor budding and investigated relation between tumor budding and clinicopathological factors. In this results, positive tumor budding showed statistically significant association with positive lymph node metastasis (P=0.011). However, no significant relationship was observed between tumor budding and the other clinicopathological factors (Table 4).

Correlation between Thymosin β4 expression and tumor budding in OSCC patients

In vitro study, two Tβ4-overexpressing OSCC cells, SCC-15_Tβ4 and SCC-25_Tβ4, did exhibit the EMT-liked phenotype. Therefore, to validate the correlation of Tβ4 expression and tumor budding in OSCC patients, the Pearson chi-square test and Spearman rank correlation were performed. There was statistically significant association between Tβ4 and tumor budding (Table 5, P=0.002). In addition, Spearman rank correlation test showed moderate positive correlation between two variables (r=0.405, P=0.001). The association between Tβ4 expression and tumor budding in two representative cases is shown histologically in Figure 8.

Correlation between tumor budding and overall survival in OSCC patients

To investigate the cumulative survival rates, 59 OSCC patients were calculated by the Kaplan-Meier method, and the differences between the curves were analyzed by the log-rank test. Of 59 OSCC patients, 35 patients had died (59%), and 5-year survival rate was 44% (26/59). There was no significant relationship between Tβ4 expression and overall survival (*P*=0.901, Fig. 9). On the other hand, positive tumor budding was strongly associated with shorter overall survival (*P*=0.006, Fig. 10).

IV. Discussion

Here, this study was investigated the functional role of Tβ4 overexpression in OSCCs; the mechanism underlying induced proliferation, invasion, and EMT by Tβ4 overexpression. In the present study, Tβ4 overexpression enhanced the proliferation of OSCC cells. Consistently, recombinant human Tβ4 has been shown to promote lymphocyte proliferation (49), and its treatment also enhances endothelial cell proliferation (50). Reversely, shRNA-mediated knockdown of Tβ4 significantly suppresses growth of the small intestine in rats, colorectal cancer stem cells, and bladder cancer cells (51, 52). Thus, Tβ4 might play diverse roles under different conditions and its significance in cell proliferation could vary according to the origin of cells.

The loss of dependence on cell adhesion is often regarded as an indicator of increased metastatic capacity in tumor cells (53). In accordance with our results, Tβ 4-overexpressing colon cancer cells have been shown to promote anchorage-independent growth via the soft agar colony formation assay (54). In the multistep process of metastasis, anchorage-independent growth of cancer cells is necessary for survival without cell-substratum interaction. In addition, these results were found that Tβ4 overexpression induced an EMT-like phenotype in OSCC cells accompanied by downregulation of E-cadherin and upregulation of mesenchymal molecular markers such as N-cadherin and vimentin, which are known to induce EMT (9). Also, Tβ4-overexpressing OSCC cells were showed an elongated, scattered, and fibroblastic morphology compared to the cobblestone-like morphology of the vector-control cells (55, 56).

Consistent with these results, fibroblastic morphology and loss of E-cadherin have been observed in Tβ4-overexpressing colon (56). Similarly, Tβ4 knockdown in colorectal cancer cells increases E-cadherin expression and decreases N-cadherin and Vimentin expression (57). Twist1, an important transcriptional repressor that regulates

the expression of E-cadherin, is also induced in cancer cells during the EMT process (11, 58). In the present study, Tβ4 overexpression in OSCC cells induced upregulation of Twist1 expression. To our knowledge, this is the first report to show the induction of Twist1 by Tβ4 overexpression in cancer cells.

After the acquisition of EMT features, cells are endowed with migratory and invasive properties leading to cancer metastasis (10). Basically, in order to degrade and invade adjacent tissues, cancer cells generate a number of matrix metalloproteinases (MMPs) (59). Here, Tβ4 overexpression strongly increased the invasiveness of OSCC cells and induced the activation of MMP-2. This is consistent with a report by Nemolato et al. (60), showing upregulation of Tβ4 expression in invading tumor cells with features of EMT. Tβ4-upregulated colorectal cancer cells are more invasive, and Tβ 4-downregulated cells show decreased MMP-2 expression (57). In glioma cells, Tβ4 silence inhibits invasion and MMP activity (61). Interestingly, latrunculin A, which inhibits the binding of Tβ4 to actin, blocks the invasion of fibroblasts in 3D Matrigel (24). These data indicate that Tβ4 contributes to the invasion of cancer cells.

Cancer invasion and metastasis are highly linked to the reorganization of the actin cytoskeleton (62, 63). Reorganization of the actin cytoskeleton is controlled by a number of proteins, including actin and actin regulatory proteins, adhesion molecules, membrane remodeling and signaling proteins, and matrix degradation enzymes (64). In this study, Tβ4 overexpression in OSCCs increased the phosphorylation of cortactin and paxillin, but not the expression of vinculin, suggesting that the activation of cortactin and paxillin could be linked to Tβ4-induced cancer cell invasion. Interestingly, Tβ4 overexpression-induced phosphorylation of cortactin and paxillin was not changed by nutritional stimulation in OSCCs. The actin-binding and scaffolding protein cortactin plays a crucial role in the regulation of the actin cytoskeleton and is commonly upregulated in multiple cancer types (65, 66). Colocalization of cortactin and phosphotyrosine has been suggested to identify active invadopodia in human breast cancer cells; and blocking of cortactin tyrosine phosphorylation was shown to

dramatically inhibit tumor cell invasion (67, 68). Paxillin is one of the scaffold proteins that plays a role in tumorigenesis and metastasis (69). Changes in tyrosine phosphorylation of paxillin have been linked to invasion (70, 71). Recently, Tβ4 activation has been shown to induce the phosphorylation of paxillin in human dental pulp cells (72).

Several intracellular signaling pathways trigger cancer invasion and metastasis by regulating reorganization of the actin cytoskeleton (62, 63). The Ser/Thr Kinase LIMK1, a key regulator of actin dynamics, has been associated with protein expression in all embryonic and adult tissues (73). In MDA-MB-231 breast cancer cells, the forced overexpression of LIMK1 results in increased levels of phospho-cofilin, phospho-paxillin, phospho-AKT, and phospho-ERK1/2 (74). Overexpression of constitutively active LIMK1 results in increased invasion of prostate cancer cells and secretion of MMP-2/9 (75). Overexpression of LIMK1 also causes increased invasiveness of cancer cells (76). Consistent with these studies, Tβ4 overexpression upregulated LIMK1 expression in OSCC cells in the present study.

To address the potential molecular mechanism underlying Tβ4-overexpressing OSCC cell lines, this study was quantified expression of a panel of phospho-kinases using an antibody-based array. This technique is a powerful mechanism for gaining a global view of changes in signal transduction events within cells. In this study, Phospho-kinase assay and Western blot analysis indicate the higher phosphorylated level of WNK (with-no-lysine[K] or lysine deficient protein kinase) isoform 1 in Tβ4 -overexpressing OSCCs cells than the control cells (Figure 6). WNKs are basically known as serine-threonine protein kinases in ion balance regulation, cell signaling, survival and proliferation (77), and recently, its roles in various signaling cascades linked to tumorigenesis have been reviewed (78). Interestingly, in the condition of Akt activation, the phosphorylation of endogenous WNK1 at T60 has been detected, but not in the condition of Akt inactivation. These results suggest that WNK1 could be phosphorylated by Akt as its substrate (79). Furthermore, the involvement of

WNK3 in glioma cell invasion has been reported (80), and Tβ4-induced phosphorylation of Akt at S473 in human colon cancer cells and invasion/ migration of human colorectal cancer cells via Akt signaling pathway have been reported (56, 57).

Since WNK1 has been shown to be phosphorylated by Akt (79), Tβ4-overexpressing cells were evaluated the levels of p-Akt. In these results, Akt expression is induced by Tβ4-overexpressing cells, but Akt phosphorylation was not likely to relate to the level of Tβ4 expression (Figure 6). Considering these results showing the Tβ4 overexpression-induced migration/invasion and the expression of Akt and phosphorylated WNK1 in OSCCs, Tβ4 could control the expression of Akt and its downstream signaling such as WNK1 for migration and invasion of cancer cells.

Regarding Tβ4-binding partners, several proteins have been identified. Tβ4 interacts with hMLH1 in colon cancer (81), and Ku80 is related in signaling initiated by Tβ4 that leads to activation of PAI-1 gene expression (82). Also, Tβ4 interacts with the cytoplasmic domain of stabilin-2 and overexpression of Tβ4 increased the phagocytic activity of stabilin-2 (83). Recently, novel evidence demonstrates that Tβ4 directly targets the NF-κB RelA/p65 subunit after TNF-α stimulation and inhibits the sensitizing effects of its intracellular binding partners, PINCH-1 and ILK (84). Therefore, it is needed that additional studies should elucidate interaction of Tβ4 and several binding partner in OSCCs.

In this study, to understand the role of Tβ4 expression in OSCC patients, 59 OSCC specimens were evaluated the expression of Tβ4 and for the first time analyzed its relation to series of clinicopathological parameters. Tβ4 expression was observed in 38 out of 59 tumor samples in contrast to negative Tβ4 expression. Consistently, Tβ4 has been shown to be widely expressed in breast cancer (85), gastrointestinal stromal tumor (47) and colorectal cancer (60) and is mostly localized in the cytoplasm of tumor cells. However, there is also a contrary report that Tβ4 expression was down-regulated in metastatic colorectal carcinoma (86). Interestingly, immunoreactivity

for Tβ4 was detected in the perinuclear area of colorectal adenocarcinomas, giving rise to spot-like reactivity highly suggestive of localization to the trans-Golgi Network (87). On the basis of these data, Tβ4 may play diverse roles in tumor cells and cytoplasmic reactivity of Tβ4 could reflect the primary physiological role such as the maintenance of actin cytoskeleton integrity, on which cell morphogenesis and motility depend (88).

Using microarray analysis, Tß4 was revealed overexpression in metastatic oral squamous cell carcinoma (OSCC) cell lines derived from patient donors (25). In gastrointestinal stromal tumors (GISTs), Tβ4 expression was related with tumor size, local recurrence and/or metastasis (47). Kang et al described that the expression levels of Tβ4 were significantly up regulated in the recurrent colorectal cancers (89). However, Kim et al reported that the level of Tβ4 was first increased at the early cancer stages but decreased at advanced stages and in metastatic cervical tumors (90). More recently, Tβ4 activity has been implicated in lymph node metastasis but not with several other clinicopathological parameters including age, histological grade, tumor size, estrogen receptor, progesterone receptor, c-erbB2, and p53 in human breast carcinoma (85), which is similar to the correlation found in a report on colon cancer (56). In accordance with this study, even though expression of Tβ4 was not related with clinicopathological parameters and overall survival in OSCC patients, Tβ4 expression was shown a significant correlation to tumor budding by applying the Spearman's rank test.

Tumor budding was first described in colorectal carcinoma and defined as the presence of single tumor cells or small groups of up to five tumor cells at the invasive tumor front (26). It is regard as the morphological correlation of local invasion and cancer cell dissemination having undergone EMT (91, 92). Using the digital image analysis, another report was showed tumor budding cells were highly enriched TGFβ signaling, a well-known for EMT signaling mediator, compared with cells from the central parts of the tumor in OSCC specimens (93). High-grade tumor budding have adverse malignant features including vascular invasion, lymph node, and

distant metastases and is detrimental to overall and disease-free survival (94). Although tumor budding has been associated with adverse prognostic factor in several cancer types, its application as a prognostic tool is lack of supporting evidences in OSCC. Like as some of the latest reports, we showed that tumor budding was correlated with lymph node metastasis (95) and survival rates in OSCC patients (96). To this study knowledge, it is the first report to show significant correlation of Tβ4 expression and tumor budding. Taking into account the result of this study, tumor budding has a feasible option to be included as OSCC prognostic factor in routine clinicopathology reporting. Given the demonstrated prognostic and predictive value of tumour budding in various cancers, a detailed molecular basis about tumor budding should be the first step in development of target drugs that inhibit this process; the Tβ4 pathway seems particularly promising.

V. Conclusion

In summary, Tβ4-overexpressing OSCC cells, SCC-15_Tβ4 and SCC-25_Tβ4, enhanced cell proliferation and colony formation and induced an EMT-like phenotype. Moreover, the expression level of Twist1, an EMT-inducing transcription factor, was significantly enhanced in SCC-15_Tβ4 and SCC-25_Tβ4 cells. Tβ4-overexpression augmented in vitro invasion and MMP-2 activity and enhanced the cell motility-associated signaling molecules. In this study, OSCCs patients with the Tβ4 expression showed tumor budding suggesting that tumor budding is significantly associated with lymph node metastasis and poor overall survival. Taken together, these results suggest that Tβ4-overexpression could be one of the causes of tumorigenesis and progression in OSCCs. Further investigation of the Tβ4 molecule would encourage the development of specific targets for cancer treatment.

VI. References

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Table 1. Clinicopathological features of 59 OSCC patients

Variable	*No. of cases (%)*
Age (years; mean, 58.1)	
20-29	1 (2)
30-39	3 (5)
40-49	12 (20)
50-59	16 (27)
60-69	15 (25)
70-79	12 (20)
Gender	
Male	50 (85)
Female	9 (15)
Differentiated status	
Well	44 (75)
Moderately	13 (22)
Poorly	2 (3)
T classification	
T1	8 (14)
T2	27 (46)
T3	3 (5)
T4	21 (36)
N classification	
N0	21 (36)
N1	16 (27)
N2	22 (37)
M classification	
M0	57 (97)
M1	2 (3)
Stage	
I	5 (8)
II	6 (10)
III	13 (22)
IV	35 (59)
Recurrence	
No	43 (73)
Yes	16 (27)

Table 2. Immunohistochemical analysis of Thymosin β4 expression in 59 OSCC patients

*Intensity score**	*x*	*Percentage score***	=	*Final score****	*No. of cases (%)*
0		0		0	5 (8)
1		1		1	2 (3)
1		2		2	14 (24)
1		3		3	22 (37)
2		2		4	7 (12)
2		*3*		*6*	*9 (15)*

* 0, negative; 1, weak; 2, strong

** 0, 0%; 1, 1-10%; 2, 11-50%; 3, 51-100%

*** <3, negative Thymosin β4 expression; ≥3, positive Thymosin β4 expression

Table 3. Relationship between Thymosin β4 expression and clinicopathological factors in OSCC patients

Variable	*No. of cases (N=59)*	*Thymosin β4*		*P-value*
		Negative (N=21)	*Positive (N=38)*	
Age				
<59	*31*	*11*	*20*	*0.985*
≥59	*28*	*10*	*18*	
Gender				
Male	*50*	*18*	*32*	*0.878*
Female	*9*	*3*	*6*	
Differentiated status				
Well	*44*	*14*	*30*	0.300
Moderately to poorly	*15*	*7*	*8*	
Tumor size				
T1+T2	*34*	*12*	*22*	*0.955*
T3+T4	*25*	*9*	*16*	
Lymph node metastasis				
Negative	*21*	*7*	*14*	*0.788*
Positive	*38*	*14*	*24*	
Distant metastasis				
Negative	*57*	*21*	*36*	*0.534*
Positive	*2*	*0*	*2*	
Stage				
I+II+III	*24*	*8*	*16*	*0.764*
IV	*35*	*13*	*22*	
Recurrence				
No	*43*	*14*	*29*	*0.425*
Yes	16	7	9	

Table 4. Relationship between tumor budding and clinicopathological factors in OSCC patients

Variable	*No. of cases (N=59)*	*Tumor budding*		*P-value*
		Negative (N=38)	*Positive (N=21)*	
Age				
<59	*31*	*19*	*12*	*0.599*
≥59	*28*	*19*	*9*	
Gender				
Male	*50*	*33*	*17*	*0.547*
Female	*9*	*5*	*4*	
Differentiated status				
Well	*44*	*27*	*17*	0.403
Moderately to poorly	*15*	*11*	*4*	
Tumor size				
T1+T2	*34*	*20*	*14*	*0.296*
T3+T4	*25*	*18*	*7*	
Lymph node metastasis				
Negative	*21*	*18*	*3*	*0.011*
Positive	*38*	*20*	*18*	
Distant metastasis				
Negative	*57*	*37*	*20*	1.000
Positive	*2*	*1*	*1*	
Stage				
I+II+III	*24*	*18*	*6*	*0.159*
IV	*35*	*20*	*15*	
Recurrence				
No	*43*	*28*	*15*	*0.852*
Yes	16	10	6	

Table 5. Correlation between Thymosin β4 expression and tumor budding

Tumor budding	*No. of cases (N=59)*	*Thymosin ß4*		*P-value*
		Negative (N=21)	*Positive (N=38)*	
Negative	*38*	*19*	*19*	*0.002*
Positive	21	2	19	

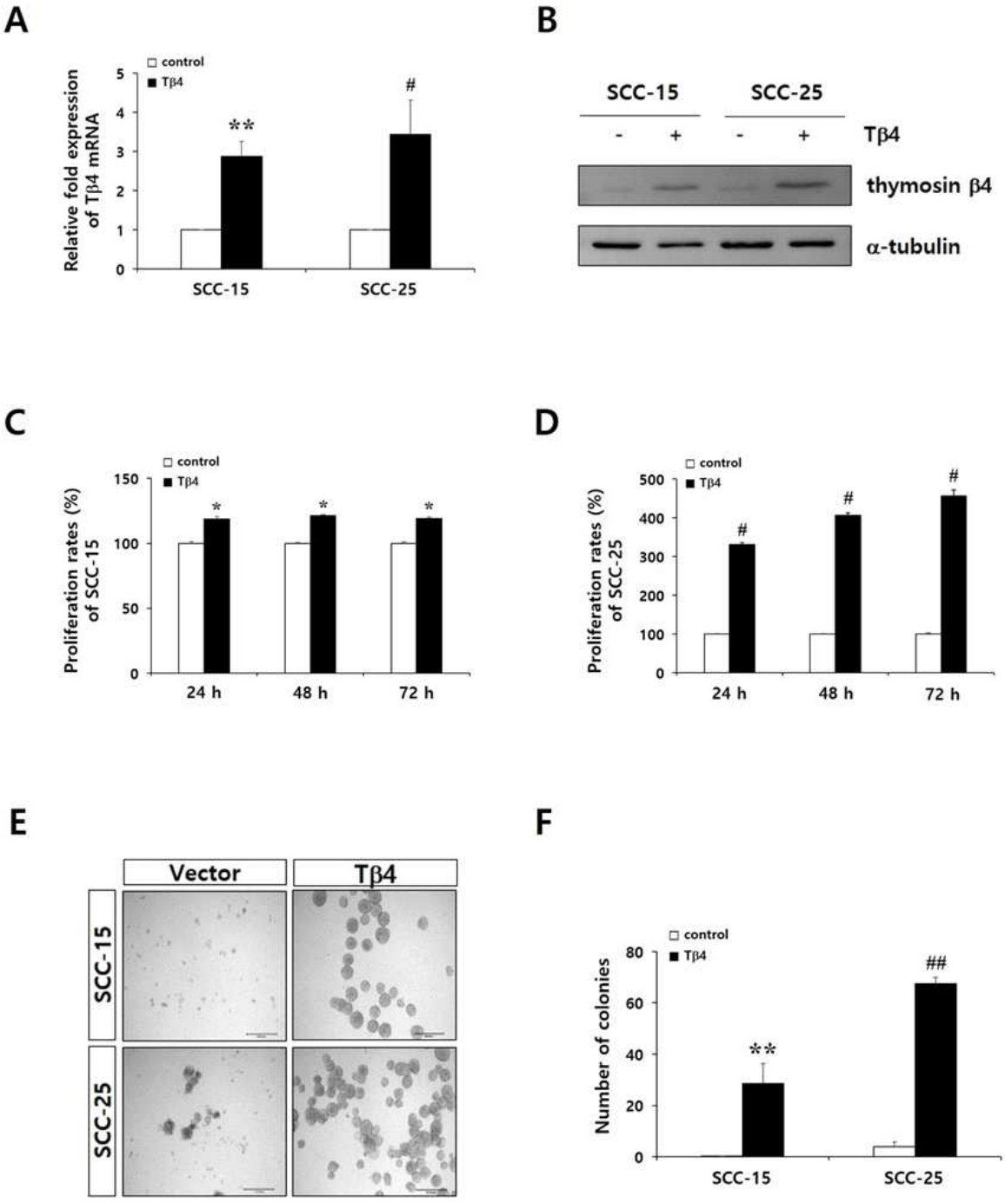
A
control
Tβ4
Relative fold expression of Tβ4 mRNA
SCC-15
SCC-25
B
SCC-15
SCC-25
Tβ4
thymosin β4
α-tubulin
C
Proliferation rates (%) of SCC-15
24 h
48 h
72 h
D
Proliferation rates (%) of SCC-25
E
Vector
Tβ4
SCC-15
SCC-25
F
Number of colonies

Fig. 1. Overexpression of Thymosin β4 in OSCC cells enhances *in vitro* tumorigenic properties. Thymosin β4 mRNA (a) and protein (b) were efficiently upregulated in two thymosin β4-overexpressing OSCC cell lines, SCC-15_Tβ4 and SCC-25_Tβ4, as measured by real-time RT-PCR and Western blot analysis. Thymosin β4 overexpression enhanced proliferative activity (c and d) and anchorage-independent growth (e and f). The data shown are the means ± SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus control SCC-15 cells; # $P < 0.05$, ## $P < 0.01$ compared with control SCC-25 cells. Scale bar, 200μm.

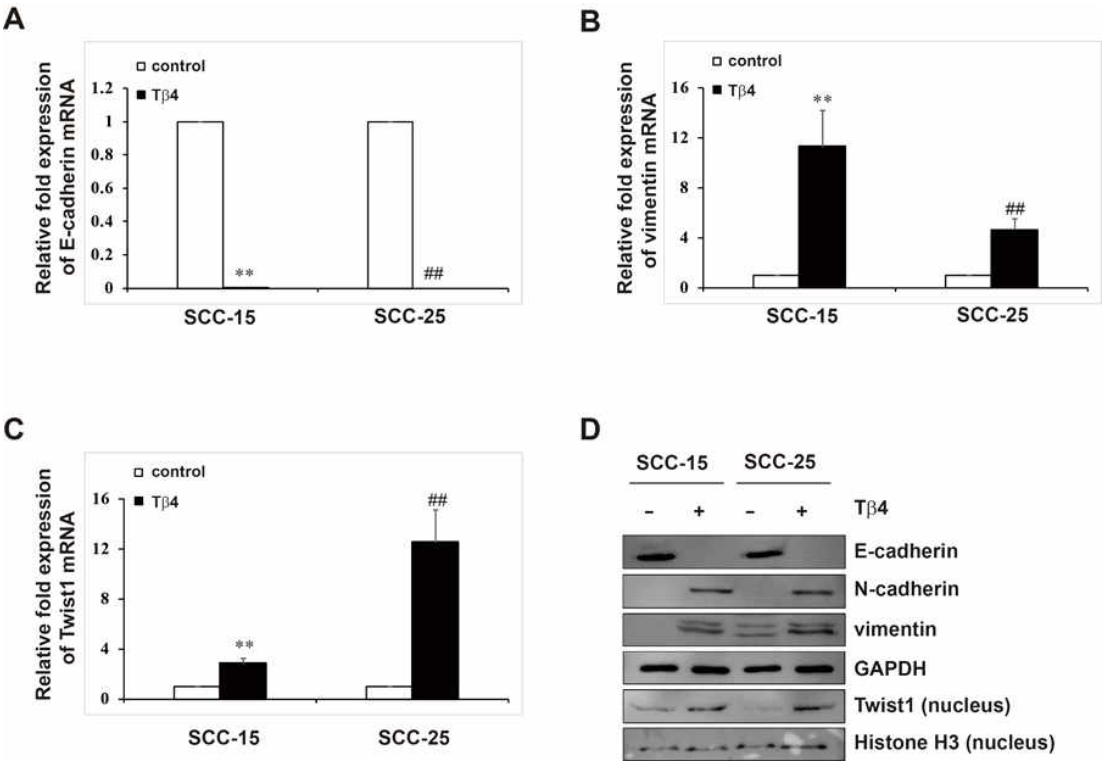

Fig. 2. Thymosin β4 induces the EMT process in OSCC cells. Real-time RT-PCR (a-c) and Western blot (d) analysis showed that thymosin β4 overexpression induced a decrease in expression of the epithelial cell marker E-cadherin an increase in mesenchymal cell markers vimentin and N-cadherin, and an increase in the transcriptional repressor of E-cadherin, Twist. Each data point represents the mean ± SD of three independent repetitions of the experiment. ** $P < 0.01$ versus control SCC-15 cells; ## $P < 0.01$ compared with control SCC-25 cells.

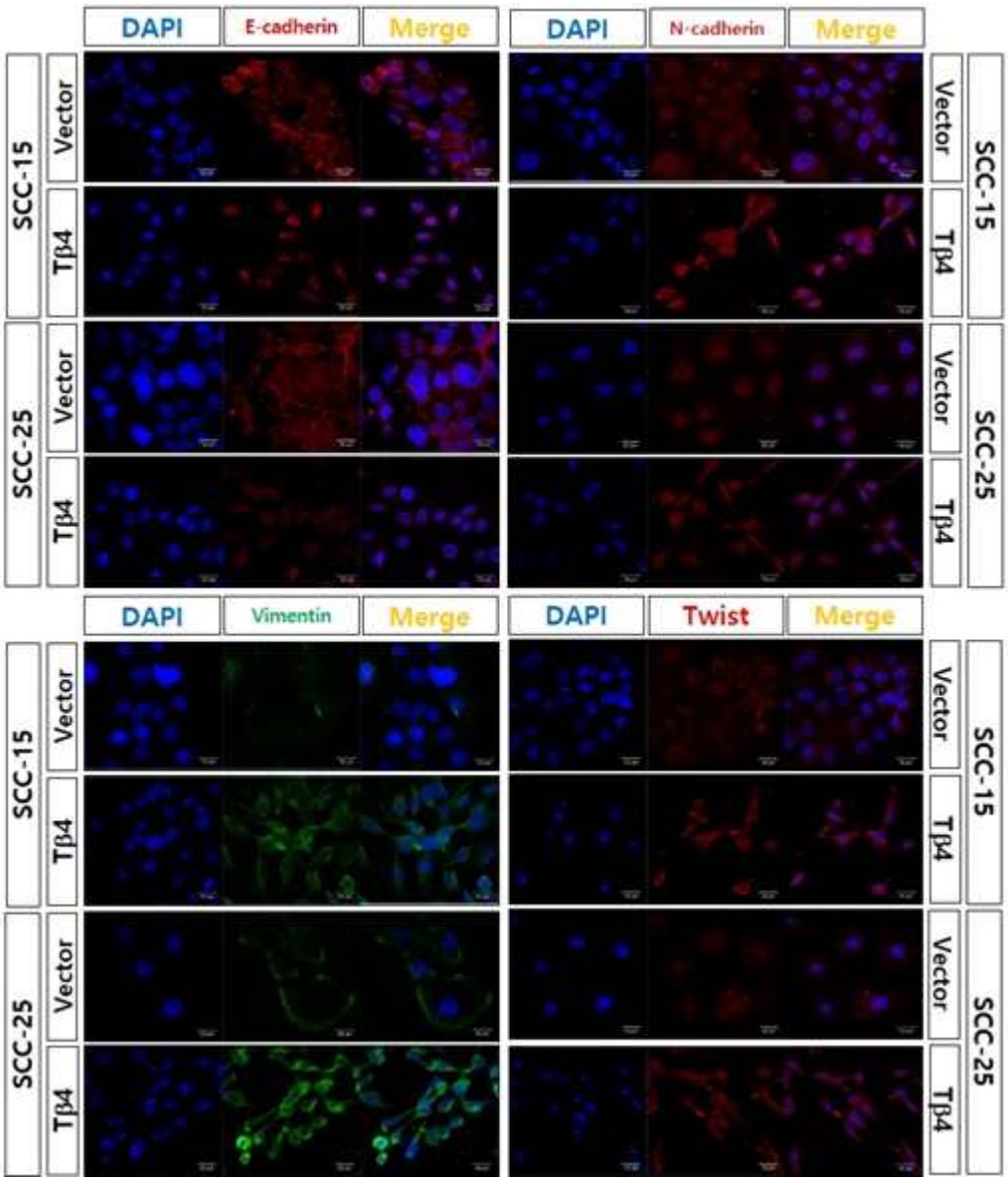

Fig. 3. Thymosin β4 induces an EMT-like phenotype in OSCC cells. Immunofluorescence staining analysis revealed that thymosin β4 overexpression induced a decrease in expression of the epithelial cell marker E-cadherin, an increase in mesenchymal cell markers N-cadherin and Vimentin, and an increase in the transcriptional repressor of E-cadherin, Twist. Original magnification, × 40. Scale bar, 20μm.

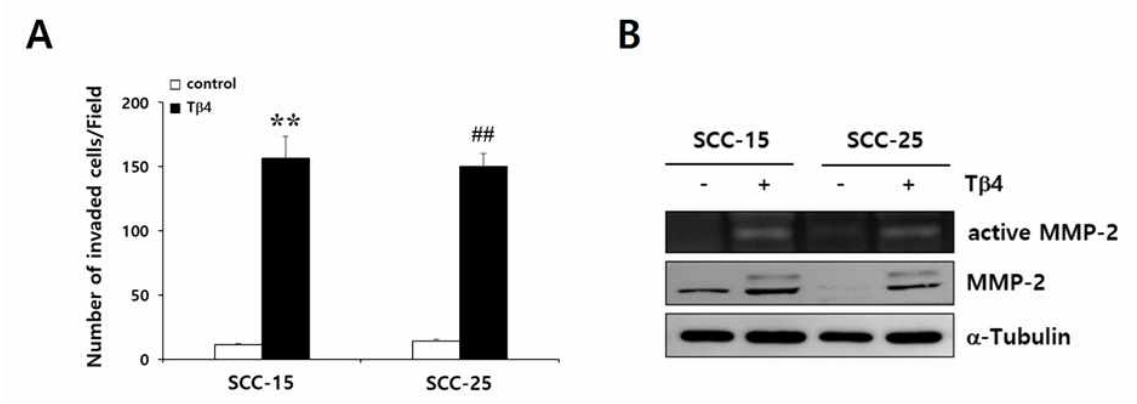

Fig. 4. Thymosin β4 overexpression enhances invasiveness in OSCC cells. (a) The Matrigel invasion assay showed that thymosin β4-overexpressing OSCC cells had significantly higher invasiveness than control cells. The data shown represent the mean ± SD of three independent trials. ** $P < 0.01$ versus control SCC-15 cells; ## $P < 0.01$ compared with control SCC-25 cells. (b) Thymosin β4 overexpression enhanced the activity and expression of MMP-2 in OSCC cells on gelatin zymography and Western blotting.

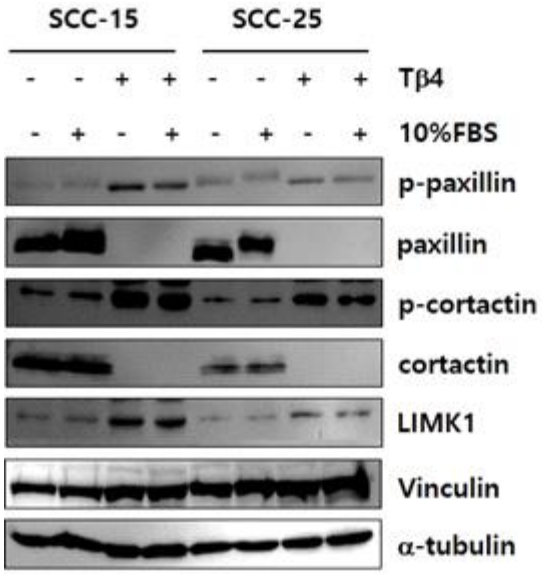

Fig. 5. Thymosin β4 enhanced phosphorylation of paxillin and cortactin and expression of LIMK1 in OSCC cells. Western blot analysis indicated that thymosin β4-overexpressing OSCCs increased phosphorylation of paxillin at Y118 and cortactin at Y466 and LIMK1 expression compared to control OSCCs.

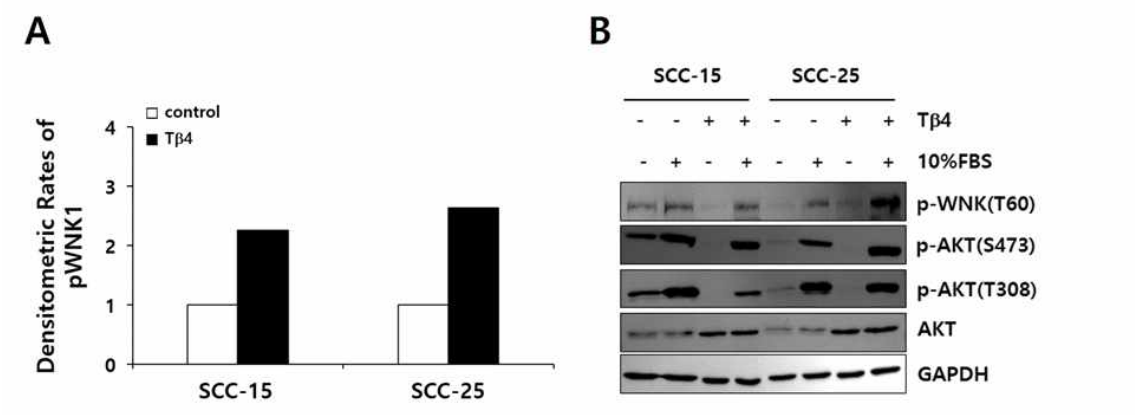

Fig. 6. Phospho-kinase array in two Thymosin β4-overexpressing OSCC cell lines, SCC-15_Tβ4 and SCC-25_Tβ4. Human phospho-kinase array analysis was performed following the manufacture's instruction. The intensity for p-WNK1 antibody resulting spots was showed that thymosin β4-overexpressing cells induced higher expression than control cells (A). Western blot (B) analysis confirmed that thymosin β4 overexpression enhanced the expression of p-WNK1 in OSCC cells.

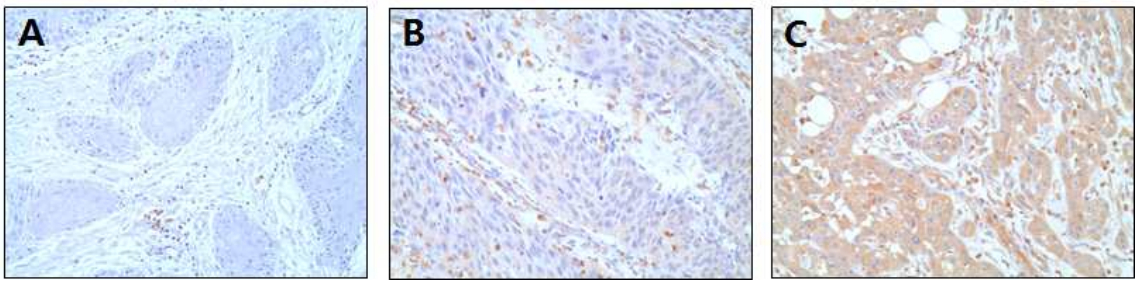

Fig. 7. Immunohistochemical staining of Thymosin β4 expression in oral squamous cell carcinoma (OSCC) samples. (A), showing immunoreactivity was negative expression of thymosin β4 compared to granulocytes. (B), showing immunoreactivity was present weak and lightly staining of thymosin β4 compared to granulocytes. (C), showing immunoreactivity was present similarly or deeply staining of thymosin β4 expression compare to internal control (granulocyte). Thymosin β4 expression was mostly localized in the cytoplasm of tumor cells (A-C, ×200).

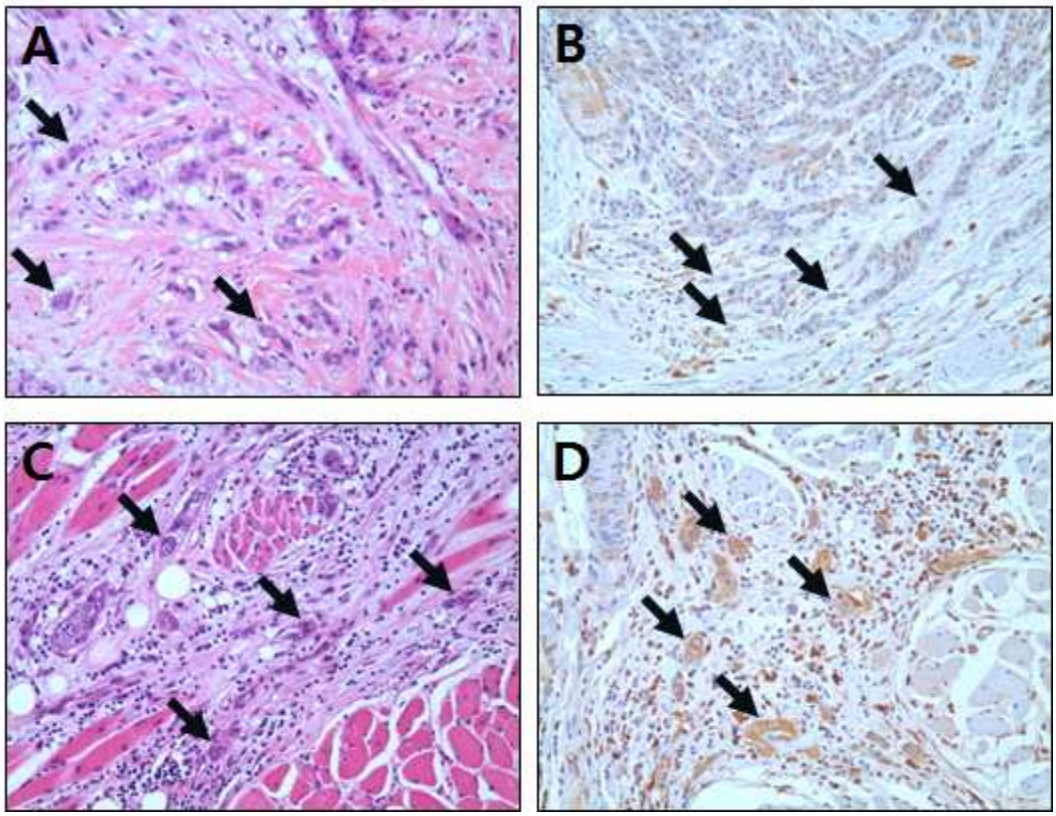

Fig. 8. Tumor budding in oral squamous cell carcinoma (OSCC) samples. (A) H & E staining and (B) weak expression of thymosin β4, cancer tissue was showed high-intensity tumor budding at the invasive front (≥5 tumor buds in one × 200 power field). (C) H & E staining and (D) strong exprssion of thymosin β4 in the cytoplasm of tumor cells, arrows were indicated tumor buddings at the invasive front (A-D, × 200).

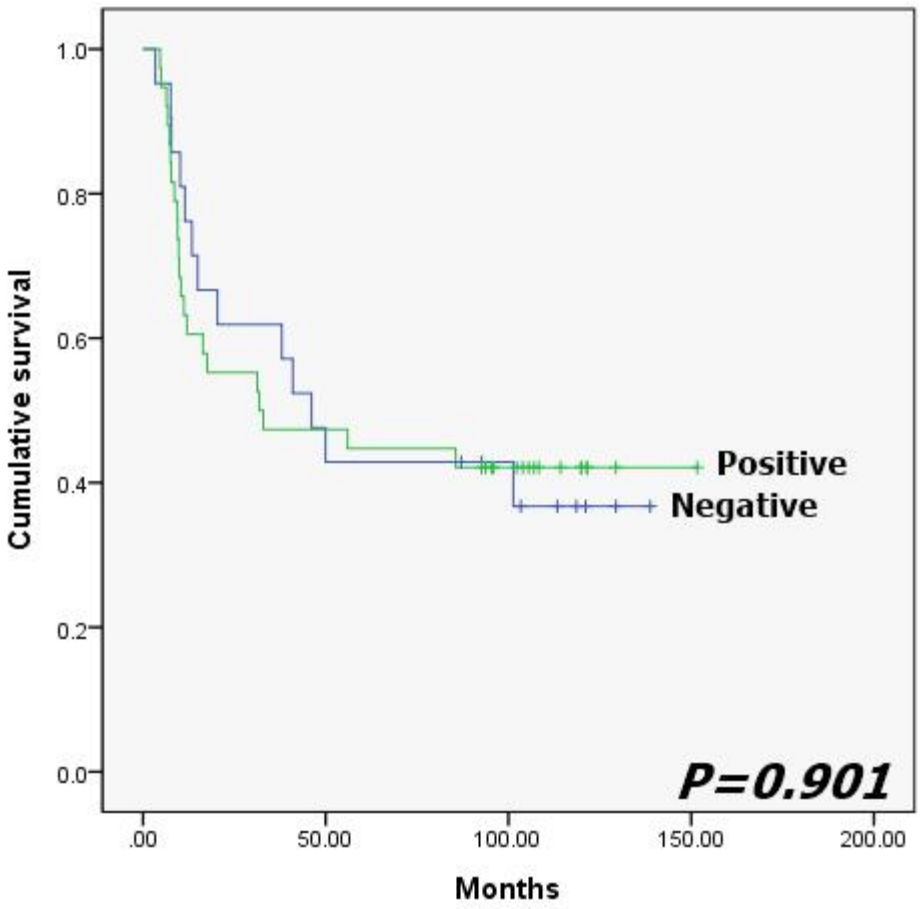

Fig. 9. Association between Thymosin β4 expression and overall survival in OSCC patients. Survival curves of patients with thymosin β4 positive expression (n=38) and negative expression (n=21). Kaplan-Meier plots with subsequent long-rank test revealed that OSCC patients with thymosin β4 expression were not associated with overall survival compared to patients with negative thymosin β4 expression (*P*=0.901).

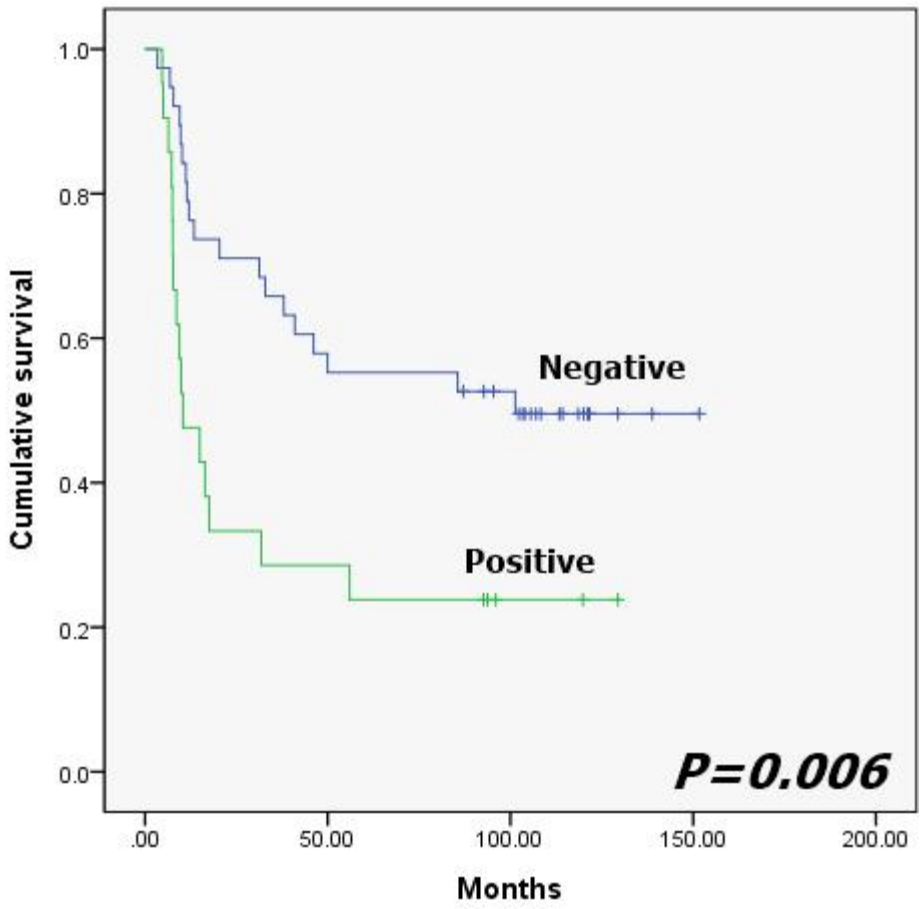

Fig. 10. Association between Tumor budding and overall survival in OSCC patients. Survival curves of patients with tumor budding positive (n=21) and negative (n=38). Kaplan-Meier plots with subsequent long-rank test revealed that tumor budding in OSCC was significantly related with overall survival compared to patients with negative tumor budding (*P = 0.006*).

국문초록

구강평편세포암의 증식과 침습 및 상피-간엽 전환 과정에서 Thymosin β4의 역할

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1. 목 적: 상피-간엽 전환은 구강 편평세포암종을 포함하여 많은 암종에서 침습과 전이과정에 중요한 역할을 하고 있다. 그러나 구강 편평세포암종에서는 상피-간엽 전환과정에 actin 중합 억제제로 알려진 thymosin β4의 영향에 관하여서는 보고된 바가 없다. 그러므로, thymosin β4를 구강 편평세포암종 세포주에 과발현시켜 세포증식과 상피-간엽 전환의 표현형의 변화를 관찰하며, thymosin β4가 암세포의 침습을 유도하고 세포 운동성 관련 신호전달분자의 발현에 영향을 미치는지 확인하고자 한다. 또한, 59명의 구강 편평세포암종 환자들에게서 thymosin β4의 발현을 조사하여 임상병리학적 지표와의 상관관계를 분석하고자 한다.

2. 방 법: WST-1 분석을 통해 구강 편평세포암종 세포주의 증식을 확인하고, 연한천분석을 이용하여 암세포의 집락형성을 관찰하며, 실시간 역전사 중합효소연쇄반응을 이용하여 상피-간엽 전환 표지자의 mRNA발현의 변화를 확인하고자 하였다. 다음으로, 면역세포화학 염색을 통해 상피-간엽 전환의 표현형 변화를 관

찰하고, 암세포의 침습을 확인하기 위해 마트리겔 침습분석 검사를 실시하였고, 침습에 관여하는 MMP 발현을 확인하기 위해 Zymography를 이용하였다. Western blot을 통하여 세포 운동성 관련 신호전달 분자의 단백질발현 여부를 비교하였고, 인산화 효소 항체배열 분석을 통해 thymosin β4의 과발현으로 인한 대표적인 신호전달 분자의 인산화 변화를 알아보았다. 또한, 59명의 구강 편평세포암종 환자조직을 이용하여 면역조직화학 염색를 실시하여 thymosin β4의 발현과 임상병리학적 지표와 비교하였다.

3. 결　과: thymosin β4를 과발현시킨 SCC-15_Tβ4와 SCC-25_Tβ4 구강 편평세포암종 세포주에서는 세포증식과 집락형성이 증가하였다. 상피세포 표지자인 E-cadherin의 발현은 감소하였고 간엽세포 표지자인 vimentin과 N-cadherin의 발현은 증가하였으며, 상피-간엽 전환의 유도 전사인자인, Twist-1의 발현 또한 증가하였다. thymosin β4의 과발현은 암세포의 시험관 침습과 MMP-2의 활성을 증가시키며 paxillin과 cortactin의 인산화와 LIMK1의 발현을 유도하였다. 또한 구강 편평세포암종 환자에서 thymosin β4의 발현은 상피-간엽 전환 관련 종양 발아와 상관관계가 있음을 확인하였으며, 이는 Kaplan-Meier 생존분석 상 유의한 결과를 보였다.

4. 결　론: 이 결과를 통하여 thymosin β4의 발현은 구강 편평세포암종에서 종양 의형성과 진행요인으로 작용할 수 있음을 알 수 있다. 더 나아가 thymosin β4에 대한 연구의 확대는 암 치료에 대한 표적물질을 발전시키는데 가치가 있을 것으로 기대된다.

주요어 : thymosin β4, 구강 편평세포암종, 상피-간엽 전환, 세포증식, 침습

학　번 : 2007-31006