치의학박사학위논문

The role of Cyr61 (CCN1) on oral squamous cell carcinoma progression

구강편평상피암종의 진행 과정에서
Cyr61 (CCN1)의 역할

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박 현 도

ABSTRACT

The role of Cyr61 (CCN1) on oral squamous cell carcinoma progression

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Introduction: CCN1 (Cyr61) has been shown to regulate angiogenesis, cell proliferation, adhesion, migration, and differentiation. An increasing body of evidence indicates that abnormal expression of the CCN proteins is associated with tumorigenesis. Although it has been reported that overexpression of CCN1 is associated with the cell proliferation and migratory ability in oral squamous cell carcinoma (OSCC), the role of CCN1 on the biological behavior of OSCC has been rarely investigated. Herein, we investigated the effect of CCN1 downregulation on proliferation and migration of OSCC cells in vitro. We also examined the relation between CCN1 expression and clinicopathological parameters in patients with OSCC.

Methods: We generated stable CCN1-knockdowned clones (KOSCC-25B_shCCN1) from the KOSCC-25B OSCC cell lines by lentiviral delivery. Cell proliferation and *in vitro* migration assays were used to investigate the effect of CCN1 downregulation on cell proliferation and migration in KOSCC-25B_shCCN1. Immunohistochemistry was performed to evaluate the correlation between CCN1 expression and clinicopathological parameters in OSCC tissue samples.

Results: CCN1-knockdowned KOSCC-25B_shCCN1 cells showed enhanced proliferative and migratory ability compared with the control vector-infected cells. Also, KOSCC-25B_shCCN1 cells showed increased phosphorylated ERK expression, compared with the control vector-infected cells. Of 52 OSCC cases, 35 (67.3%) showed high CCN1 expression profile, while 17 (32.7%) did low CCN1 expression profile. Higher level of CCN1 expression significantly correlated with smaller tumor size (P=0.020), lower clinical stage (P=0.030) and negative lymph node metastasis (P=0.002). However, there were no significant correlations of CCN1 expression level with age, gender, smoking, histologic differentiation of cancer cells, and recurrence. Also, there were no correlations between CCN1 expression and patient overall survival (P=0.689).

Conclusion: This study demonstrated that the downregulation of CCN1 enhanced cell proliferation and migration of OSCC cells, and that high CCN1 expression in patients with OSCC significantly correlated with smaller tumor size, lower clinical stage, negative lymph node metastasis. These results suggested that CCN1 might be a negative regulator on the tumor progression of OSCC.

Keywords: Oral squamous cell carcinoma, CCN1 (Cyr61), cell proliferation, migration, knockdown

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목 차

I. Introduction

Oral squamous cell carcinoma (OSCC), the most common malignant neoplasm in oral cavity, is a significant global public health threat. [1] The prognostic evaluation and decisions on treatment strategy are mainly based on the TNM classification. [2] Despite progress in treatment modalities over the past few decades, oral cancer still has a poor survival rate, with a high incidence of metastasis. [3, 4] Therefore, molecular targeting research could bring about a revolution in the treatment and prevention of the disease. [5, 6]

Connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family of proteins consists of six members (CCN1-CCN6) also known as cysteine-rich 61 (CCN1); connective tissue growth factor (CCN2); nephroblastoma overexpressed gene (CCN3); and Wnt-1–induced secreted proteins 1, 2, and 3 (CCN4-CCN6). Studies from the past decade showed that CCN proteins are involved in numerous cellular functions, including proliferation, differentiation, and neoplastic transformation. [7-9]

CCN1, a prototypical member of the CCN family, is a proangiogenic early response gene and has been shown to regulate angiogenesis, cell proliferation, adhesion, migration, and differentiation. [10] An increasing body of evidence indicates that abnormal expression of the CCN1 is related to carcinogenesis. [11] Overexpression of CCN1 protein is associated with growth and progression of gastric cancer, [12, 13] breast cancer, [14-18] ovarian cancer, [19] and glioma. [20, 21] Paradoxically, CCN1 has also been shown to behave as a tumor suppressor in prostate cancer, [22] uterine leiomyoma, [23] lung cancer, [24, 25] and endometrial cancer. [26]

Although it has been reported that CCN1 is associated with the cell proliferation and migratory ability in OSCC, [27, 28] the precise role of CCN1 on the biological behavior and clinical significance has not been completely investigated. In the present study, therefore, we investigated the effect of CCN1 downregulation on proliferation and migration of OSCC cells in vitro. We also examined the relation between CCN1 expression and clinicopathological parameters in patients with OSCC.

II. Materials and Methods

Cell Culture and Reagents

SCC-4, SCC-9, HSC-2, HSC-3, Ca9-22, HO-1-U1, and KOSCC-25B (human OSCC cell lines) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin). Antibodies against CCN1, ERK, phosphorylated ERK (Tyr 204), AKT, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphorylated Akt (Ser 473) was obtained from Cell Signaling Technology (Danvers, MA, USA). α-Tubulin was purchased from Sigma (St. Louis, MO, USA).

Generation of DNA Constructs and Stably Transformed Tumor Cells

Lentiviral shRNA-expression vectors for targeting CCN1 mRNA were constructed by inserting synthetic double-strand oligonucleotides (for CCN1 5'-GCA AAC AGA AAT CAG GTG TTT-3') into *EcoR I* restriction enzyme sites of the shLenti3.4GFP lentiviral vector (Fig. 1). The nucleotide sequence of the construct was verified by sequencing. The shLenti3.4GFP lentiviral vector was designed to produce siRNAs promoted from the U6 promoter and to express eGFP protein from the hCMV promoter. As a control, a scrambled shRNA sequence (5'-AAT CGC ATA GCG TAT GCC GTT-3') was inserted into the shLenti3.4GFP vector.

KOSCC-25B cells were infected by control vector or shLenti3.4GFP/CCN1 with 6μg polybrene (Sigma). After an overnight incubation, infected cells were selected with 3μg/μl puromycin for a minimum of 7 days. Transformed cells were named to KOSCC-

25B_vec and KOSCC-25B_shCCN1, respectively. All cells were grown to passage 20.

RT-PCR Analysis

mRNA was purified from the cells using the Trizol reagent (Invitrogen) according to the manufacturer's recommended protocol. Two micrograms of RNA was converted into cDNA using random primers and reverse transcriptase. cDNA was PCR amplified with 30 cycles of 94℃ for 30sec, 58℃ for 50sec, and 72℃ for 50sec. The primer pairs for CCN1 and GAPDH were as follows: CCN1 forward, 5' -CGA GGT GGA GTT GAC GAG AAA C -3'; CCN1 reverse, 5' - AGG ACT GGA TCA TCA TGA CGT TCT - 3'; GAPDH forward, 5'-GAA GGT GAA GGT CGG AGT C-3'; and GAPDH reverse, 5'-CAA AGT TGT CAT GGA TGA CC-3'.

Immunoblotting

Briefly, 70-80% confluent cells were homogenized with 1 ml of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice. To the homogenates was added 125 μl of 10% NP-40 solution, and the mixture was then centrifuged for 30 sec at 12,000×g. Supernatant protein was normalized. Forty micrograms of protein was size-fractionated through a 10% SDS-PAGE gel, transferred to PDVF membrane, and immunoblotted with specific antibodies.

Cell proliferation assay

To determine the proliferation rate, cells were seeded on 12-well plates (BD Falcon, Franklin Lakes, NJ, USA) at 3×10^4 cells per well in DMEM with or without 10% FBS.

After 24h, 48h, and 72h, the cells were typsinized and stained with 0.4% Trypan Blue (Gibco). The total cells number and the proportion of dead cells were counted by hemocytometer. Cell death was determined by the presence of cytoplasmic Trypan Blue. This assay was performed in triplicate in each experiment, and each experiment was repeated three times.

In vitro migration assay

A total of 1 X 10^5 cells were seeded in the upper compartment (8μm pore size) in DMEM medium with or without 10% FBS. Medium was added to the lower compartment. After 24h and 48h incubation, the cells on the upper surface of the filter were wiped off with a cotton swab, and the remaining cells were stained with the Diff-Quick stain set (Sysmex, Kobe, Japan). Using a microscope at X100 magnification, migration was quantified by counting the number of cells that migrated through the pores to the lower side of the filter. This assay was performed in triplicate in each experiment, and each experiment was repeated three times.

Patients and Tissue Samples

Samples from 52 patients (39 men and 13 women) with OSCC were examined by immunohistochemistry. All tumors were surgically removed at the department of oral and maxillofacial surgery, Seoul National University Dental Hospital, South Korea, between 1997 and 2002. The age of the patients ranged from 39 to 84 years, with a mean of 59.6 years. Clinical data obtained from patient charts included age, gender, smoking habits, TNM stage, and recurrence. Tumors were staged according to the current TNM

classification as recommended by the AJCC. [29] Tumors were re-reviewed by two pathologists to determine the histological grade (well differentiated, moderately differentiated, or poorly differentiated). [30] Survival was calculated from the date of diagnosis until the date of death or last follow-up.

Immunohistochemistry

Tissues were fixed in 10% buffered-formalin and embedded in paraffin. Immuno-histochemistry was performed on 4 μm paraffin sections mounted on silicon-coated glass slides, using streptavidin-biotin peroxidase technique as previous described. [31, 32]

The staining intensities to CCN1 of the cancer nests were estimated according to the criteria of Haque *et al*'s study. [33] The tumor cells were scored and divided into the following four groups: (-), No stain, (+), light brown, (++), moderately dark brown, and (+++), dark brown. (-) and (+) were defined as low CCN1 expression, and (++) and (+++) as high CCN1 expression.

Statistical Analysis

In both cell proliferation assay in vitro migration assay, statistical significance was assessed by comparing mean (±SD) values with Student's *t*-test for independent groups. Relationships between CCN1 and the various clinicopathological factors were examined using the χ^2 test. Survival curves were calculated using the Kaplan–Meier method and analyzed using the log rank test. $P < 0.05$ was considered statistically significant.

III. Results

Screening of Oral Squamous Cell Carcinoma Cell Lines

We screened 7 OSCC cell lines to obtain a suitable CCN1-knowndowned model with RT-PCR and western blot analysis. All of those expressed CCN1mRNA and protein (Fig. 2). Because KOSCC-25B showed high CCN1 mRNA and protein levels in RT-PCR and western blot analysis, the KOSCC-25B cell line were chosen for the present study.

Confirmation of CCN1 Knockdown by shRNA

We assayed for siRNA-mediated CCN1 knockdown by RT-PCR analysis, and we saw CCN1 downregulation in the KOSCC-25B_shCCN1 cells compared with the KOSCC-25B_vec cells (Fig. 3A). By western blot analysis, CCN1 was also downregulated in the KOSCC-25B_shCCN1 cells (Fig. 3B). Therefore, CCN1 was knocked down at both the mRNA and protein levels in KOSCC-25B_shCCN1 cells.

Effect on Cell Proliferation of CCN1 Downregulation

From the cell proliferation assay, we found that the CCN1-knockdowned KOSCC-25B_shCCN1 cells grew significantly faster than the KOSCC-25B_vec control cells ($P<0.05$; Fig. 4). In the serum-free culture condition, the proliferation rates of KOSCC-25B_shCCN1 were 149.0%, 141.6%, and 133.3% for 24h, 48h, and 72h, respectively, compared with the KOSCC-25B_vec cells ($P<0.05$). In the presence of 10% FBS, the proliferation rates of the KOSCC-25B_shCCN1 cells were 162.2%, 310.1%, and 214.3% for 24h, 48h, and 72h, respectively ($P<0.05$). These data indicate that the downregulation

of CCN1 enhances cell proliferation of KOSCC-25B OSCC cells.

Effect on Cell Migration by CCN1 Downregulation

CCN1-knockdowned cells showed increased migratory ability (Fig. 5). The migratory percentages of the KOSCC-25B_shCCN1 cells without FBS were 180.0% and 270.0% for 24h and 48h, respectively, compared with the KOSCC-25B_vec control cells ($P<0.05$). In the presence of 10% FBS, the migratory percentages of the KOSCC-25B_shCCN1 cells were 37.0% and 50.0% for 24h and 48h, respectively ($P<0.05$). These data indicate that downregulation of CCN1 induces cell migration of KOSCC-25B OSCC cells.

Effect on Signaling Molecules by CCN1 Downregulation

CCN1-knockdowned KOSCC-25B_shCCN1 cells showed increased phosphorylated ERK expression compared with the KOSCC-25B_vec control cells (Fig. 6). However, The expression of ERK, AKT, NF-κB (p65) signaling molecules did not change irrespective of CCN1 knockdown (Fig. 6).

Correlation between CCN1 Expression and Clinicopathological Parameters in OSCC Tissues

Immunoreactivity to CCN1 was localized in cytoplasm of OSCC cells, whereas only faint immunoreactivity was observed in normal epithelium (Fig. 7, Fig. 8). Of 52 OSCC cases, 35 (67.3%) showed high CCN1 expression profile, while 17 (32.7%) did low CCN1 expression profile.

Table 1 shows the association of several clinicopathological factors with CCN1

expression. Higher level of CCN1 expression significantly correlated with smaller tumor size (P=0.020), lower clinical stage (P=0.030), negative lymph node metastasis (P=0.002). However, there were no significant correlations of CCN1 expression level with age, gender, smoking, histologic differentiation of cancer cells, and recurrence.

Survival Analysis

Of 52 OSCC patients, 22cases were deceased and 5-year overall survival was 57.7% (n=30). There were no correlations between CCN1 expression and patient overall survival (P=0.689; Fig. 8).

IV. Discussion

CCN1 is involved in a wide range of biological activities including angiogenesis and tumorigenesis [7, 8]. However, previous studies showed that CCN1 has disparate functions in tumorigenesis of different cell types, and this phenomenon may result from its multimodular architecture and ability to interact with other signaling molecules. [10] Elevated levels of CCN1 are associated with a more advanced stage of breast cancer, and its overexpression in normal human breast cells promotes their cell proliferation and tumor formation in immunodeficient mice [18, 34]. Cells from glioblastoma multiforme expressed higher levels of CCN1 and these brain tumors with high levels of CCN1 had a worse prognosis than those with lower levels of CCN1. [21] Paradoxically, CCN1 acts as a tumor suppressor in non-small cell lung cancers and is associated with enhanced expression of p53 and p21 and decreased levels of cdk2 kinase activity [24, 25]. Induction of CCN1 has recently been shown to be important for neuronal cell death through c-Jun N-terminal kinase activation [35]. Taken together, the biological properties of CCN1 are dependent upon their interacting molecules, be they either positive or negative effectors [7, 8].

In vitro studies concerning the functional role of CCN1 in OSCC are rare. Kang *et al* [27] found that CCN1 was overexpressed in an invasive oral SCC subline, compared with its parental cell line. Knockdown of CCN1 with RNAi technique led to significant suppression of in vitro cell growth, cell migration, and invasion. [27] In a recent study, Kok *et al* [28] reported that forced expression of CCN1 stimulated the motility and growth of OSCC cells in vitro and enhanced xenograft growth in SCID mice. In the

present study, we knocked down CCN1 by shRNA in KOSCC-25B OSCC cells and generated CCN1-knockdowned clone, KOSCC-25B_shCCN1. We then investigated the effect of CCN1 downregulation on cell proliferation and migratory ability. Against our expectation, downregulation of CCN1 increased cell proliferation and migration of the OSCC clone. These results were contrary to the previous reports of Kang *et al* [27] and Kok *et al* [28]. These discrepancies raised the possibility that the CCN1 could function as positive or negative regulator of OSCC cells in cell line-dependant manner. Additional data on more OSCC cell lines should be needed to elucidate the issue.

Recently, studies on the signaling pathway of CCN1 have been reported. Tanaka *et al* [36] found that OSCC cell lines overexpressing CCN1 exhibited constitutive activation of Rho A and upregulated invasiveness without the disruption of homophilic cell attachment and that humoral CCN1 enhanced further production of endogenous CCN1 by OSCC cells, which stimulated collective cell migration and the development of an invasive tumor nest. Chuang *et al* [37] reported that CCN1 enhances the migration of OSCC cells by increasing MMP-3 expression through the $\alpha_v\beta_3$ or $\alpha_6\beta_1$ integrin receptor, FAK, MEK, ERK, and NF-kB signal transduction pathway. In the present study, CCN1-knockdowned KOSCC-25B_shCCN1 OSCC cells showed enhanced phosphorylated ERK expression compared with the KOSCC-25B_vec control cells. However, The expression of ERK, AKT, NF-κB (p65) signaling molecules did not change irrespective of CCN1 knockdown. Considering our result on the signaling molecules, phosphorylation of ERK is, at least partly, associated with CCN1-mediated proliferation and migration of OSCC cells.

Data on the correlations between CCN1 expression and clinicopathological parameters

are extremely rare; only a study has been reported to our knowledge. Kok *et al* [28] reported that the level of CCN1 expression positively correlates with tumor size and stage of OSCC. However, the relation between lymph node status and CCN1 expression was not statistically significant in their clinical study. Nevertheless, they demonstrated that the expression level of CCN1 is an independent prognostic indicator for OSCC; patients with high-CCN1 tumors had significantly shorter overall survival than those with low CCN1 expression. On the other hand, our study showed that higher level of CCN1 expression significantly correlated with smaller tumor size (P=0.020), lower clinical stage (P=0.030), negative lymph node metastasis (P=0.002). Also, there were no correlations between CCN1 expression and patient overall survival (P=0.689). Our results are extremely opposite to that of Kok *et al* as shown in our *in vitro* data. These reverse results might result from the regional or environmental difference because Areca nut is one of important etiologic factors for carcinogenesis in Taiwan, not in Korea. Additional studies from other countries should be needed for determining more accurate clinical significance of CCN1 in patients with OSCC.

V. Conclusion

This study demonstrated that the downregulation of CCN1 enhances cell proliferation and migration of OSCC cells, and that high CCN1 expression in patients with OSCC significantly correlates with smaller tumor size, lower clinical stage, negative lymph node metastasis. These results suggested that CCN1 might be a negative regulator on the tumor progression of OSCC.

VI. References

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Figure 1 shLenti3.4GFP lentiviral vector construct

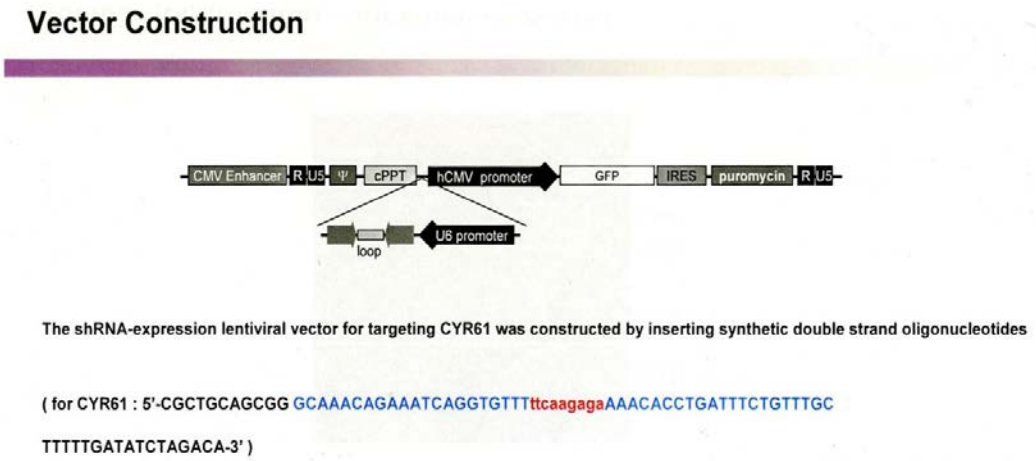
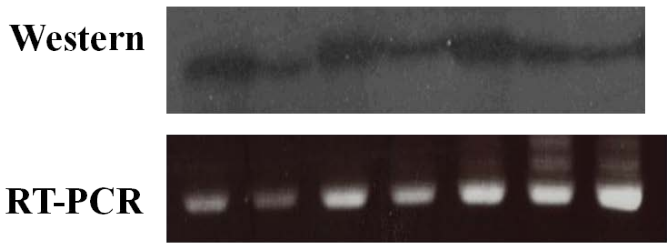


Figure 2 Screening of Oral Squamous Cell Carcinoma Cell Lines



Cell line	
1.	SCC-4
2.	SCC-9
3.	Ca9-22
4.	HO-1-U-1
5.	KOSCC-25B
6.	HSC-2
7.	HSC-3

Figure 3 Confirmation of CCN1 Knockdown by shRNA: (A) RT-PCR and (B) Western blot analysis

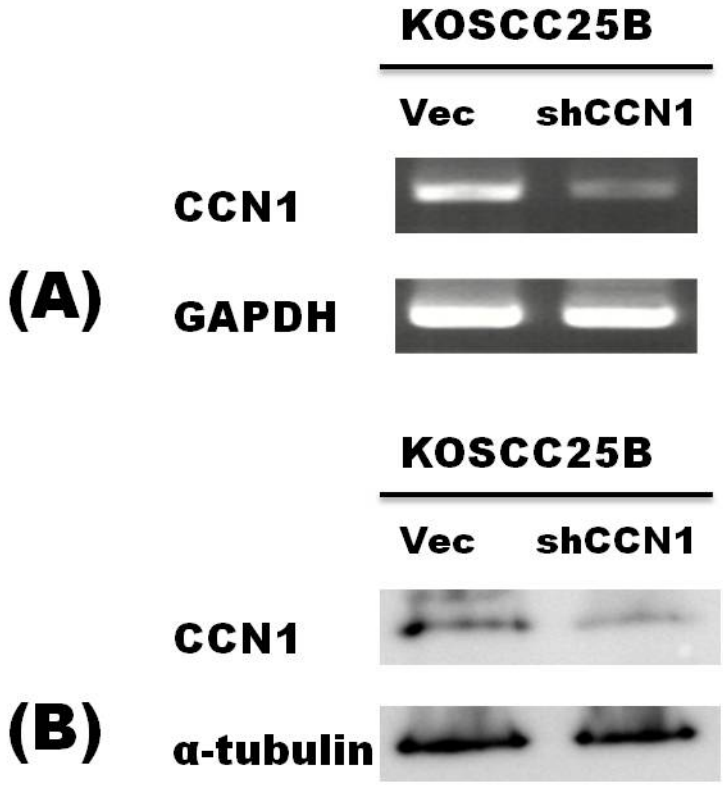


Figure 4 Cell proliferation assay in KOSCC-25B cells

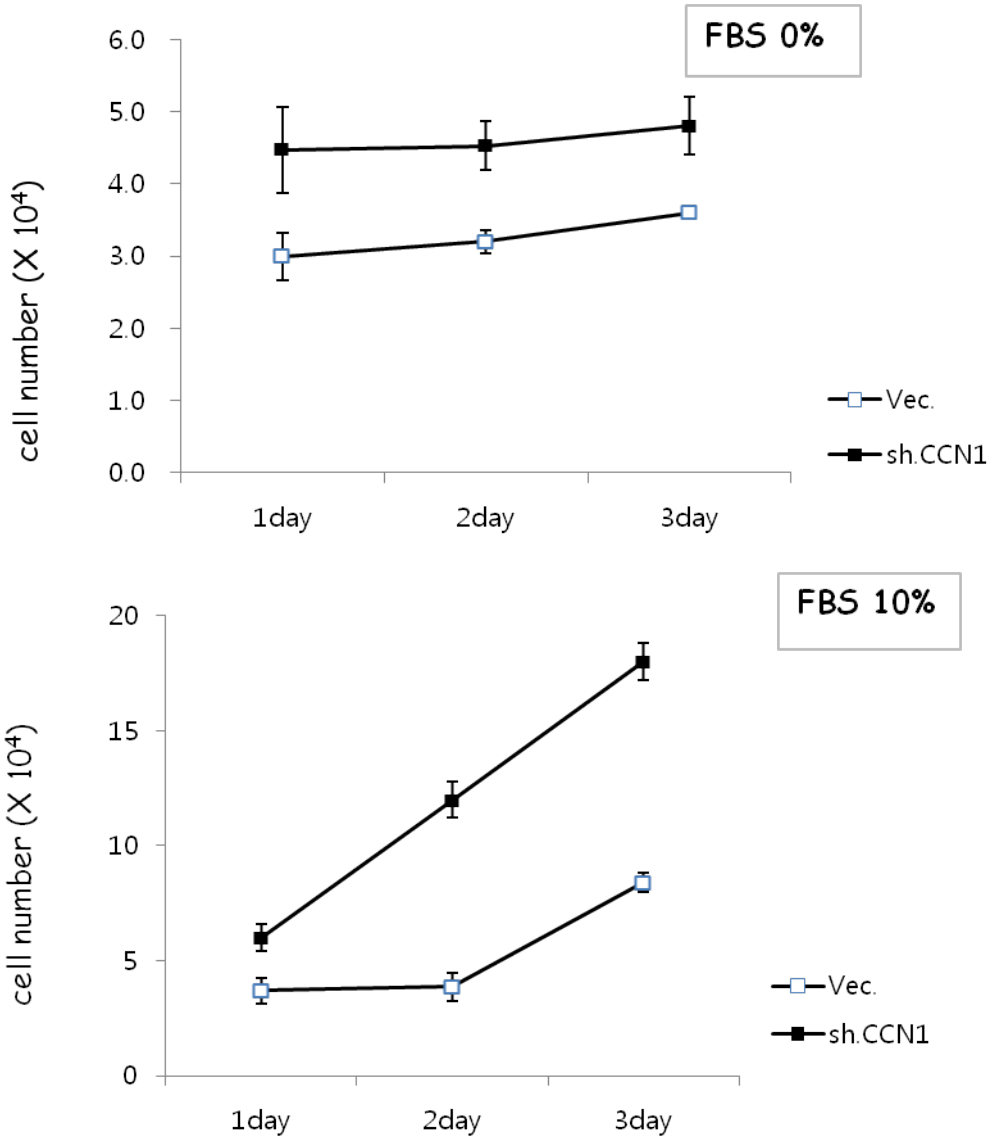


Figure 5 In vitro cell migration assay in KOSCC-25B cells

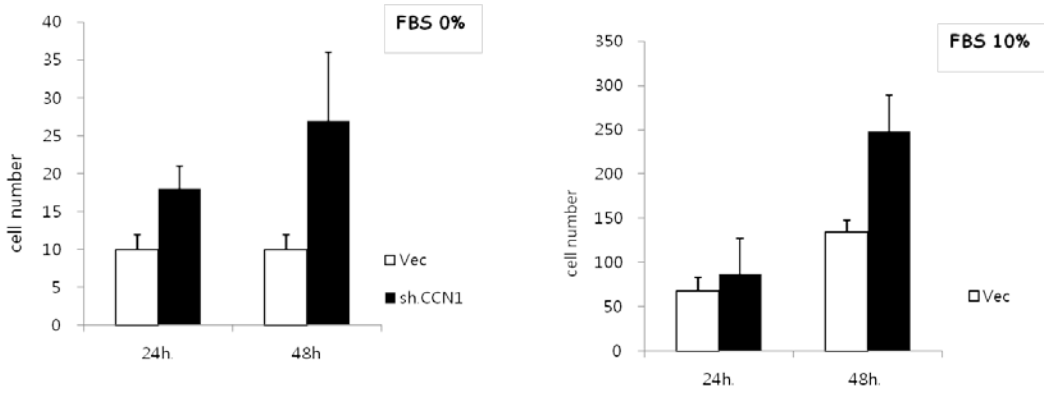


Figure 6 Effect on signaling molecules by CCN1 downregulation in KOSCC-25B cells

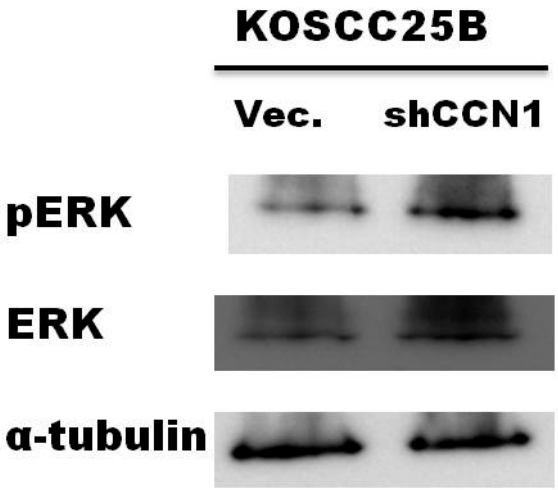


Figure 7 CCN1 immunohistochemical staining in normal mucosa (X100)

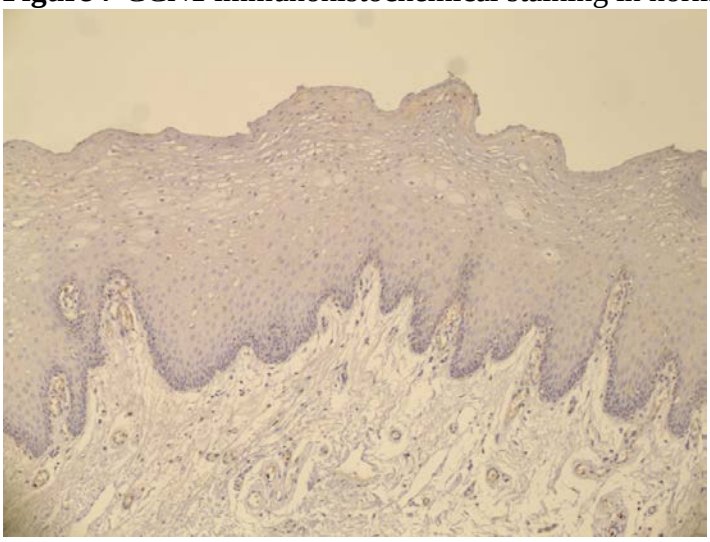


Figure 8 CCN1 immunohistochemical staining in OSCC (X200).
(A) grade (-); (B) grade (+); (C) grade (++); (D) grade (+++)

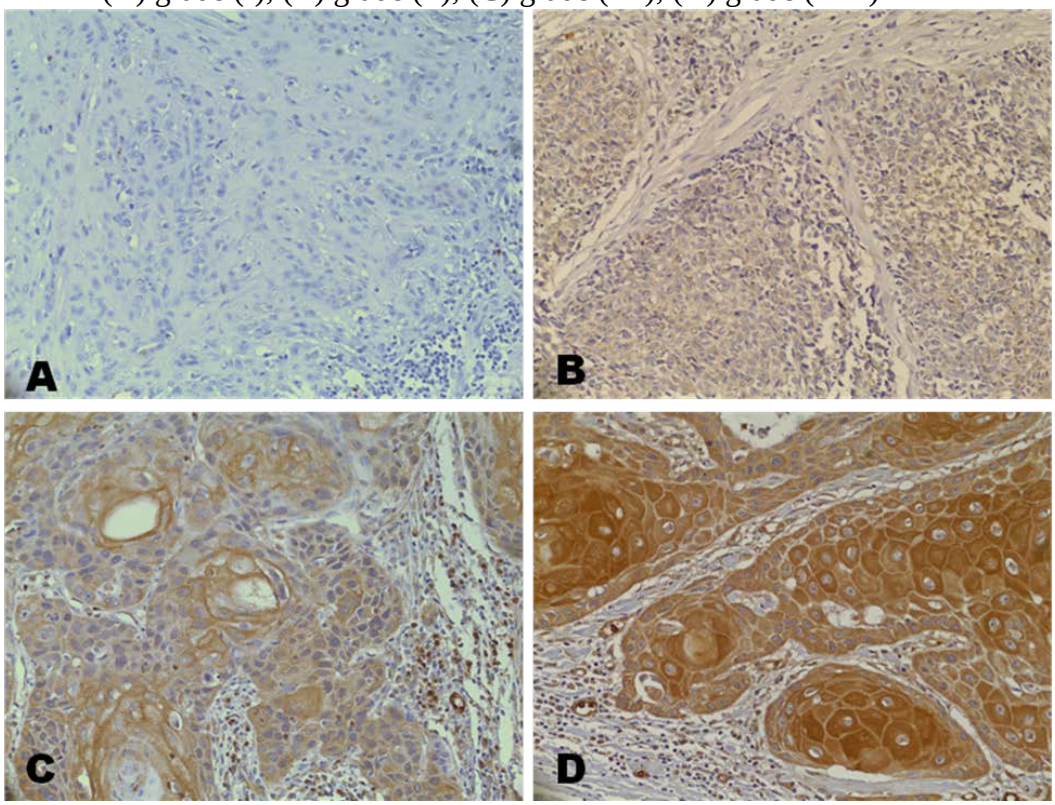


Figure 9 Survival analyses of 52 patients with OSCC.

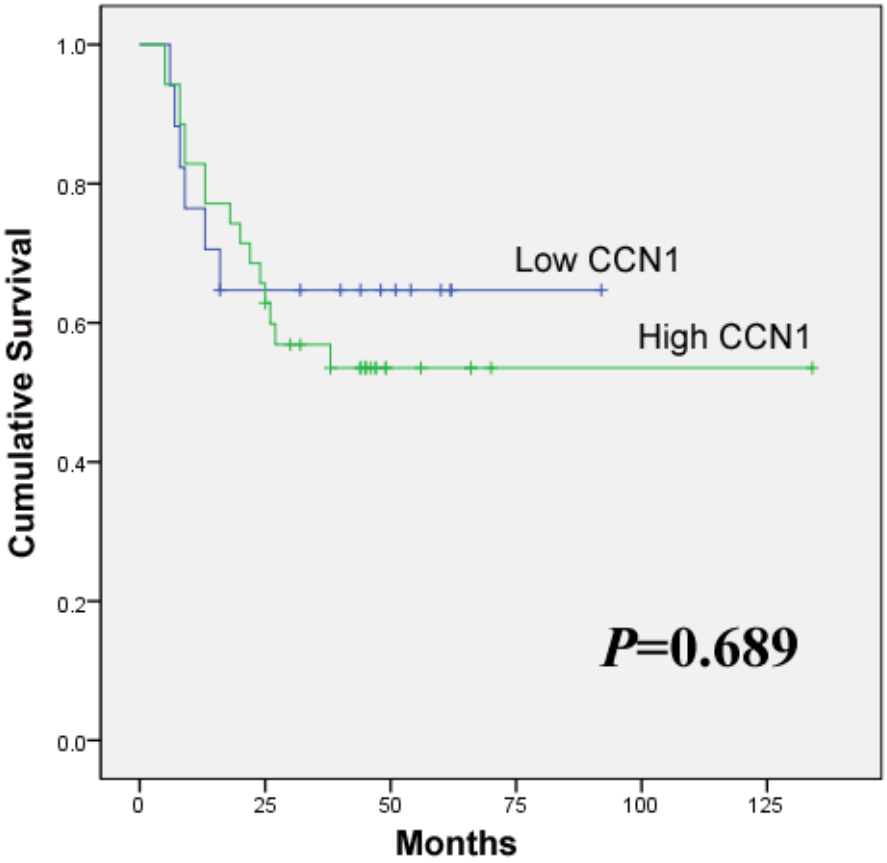


Table 1 Relationships between clinicopathological factors and CCN1 expression of 52 OSCC patients

Variable	*n*	*Low CCN1*	*High CCN1*	*P-value*
Age				
≤ 60	28	10	18	0.616
> 60	24	7	17	
Gender				
Male	38	13	25	0.701
Female	14	4	10	
Smoking				
Yes	23	8	15	0.775
No	29	9	20	
Tumor size				
T1 and T2	33	7	26	0.020
T3 and T4	19	10	9	
Lymph node metastasis				
Positive	17	9	8	0.030
Negative	35	8	27	
Stage				
Ⅰ and Ⅱ	28	4	24	0.002
Ⅲ and Ⅳ	24	13	11	
Differentiation				
Well	37	12	25	0.950
Moderate-poor	15	5	10	
Recurrence				
Yes	16	6	10	0.622
No	36	11	25	

국문 초록

구강편평상피암종의 진행 과정에서 Cyr61 (CCN1)의 역할

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1. 연구 목적

CCN1 (Cyr61)은 신생혈관 형성, 세포 증식, 부착, 이동 등의 관여하는 것으로 알려져 있다. 이러한 CCN1 단백의 발현 이상은 종양 발생 및 진행과 연관있다는 보고들이 증가하고 있다. 최근에 구강암에서 CCN1 의 과발현이 세포 증식 및 이동과 관련있다는 보고가 있지만, 구강암에서 CCN1 의 역할은 거의 규명된 바 없다. 본 연구에서는 CCN1 의 발현 저하가 구강암 세포의 증식과 이동에 미치는 영향을 분석하고, 또한 구강암 조직에서 CCN1 의 발현 양상과 임상병리학적 지표와의 상관관계를 조사하고자 한다.

2. 연구 방법

Lentiviral vector 를 사용하여 구강암 세포주 Ca9-22 와 KOSCC25B 에서 CCN1 의 발현을 저하시킨 Ca9-22_shCCN1 와 KOSCC25B_shCCN1 세포주를 획득하였다. Ca9-22_shCCN1 와 KOSCC25B_shCCN1 에서 CCN1 의 발현 저하가 구강암 세포의 증식과 이동에 미치는 영향을 분석하기 위해 cell proliferation assay 와 in vitro migration assay 를 시행하였다. 구강암 조직에서

CCN1 의 발현 양상과 임상병리학적 지표와의 상관관계를 조사하고자 면역조직화학적 염색을 시행하였다.

3. 연구 결과 및 결론

① Ca9-22_shCCN1 는 세포의 증식 및 이동 능력이 control vector 로 감염시킨 대조군에 비해 현저하게 감소하였다. 반면에, KOSCC25B _shCCN1 는 세포의 증식 및 이동 능력이 control vector 로 감염시킨 대조군에 비해 현저하게 증가하였다.

② Ca9-22_shCCN1 에서는 인산화 ERK 의 발현이 감소하였고, KOSCC25B _shCCN1 에서는 인산화 ERK 의 발현이 증가하였다.

③ 59 증례의 구강암 조직 중에서 38 증례 (64.4%)에서 CCN1 의 발현이 관찰되었지만, CCN1 의 발현과 임상병리학적 지표와의 상관관계는 통계학적 유의성이 관찰되지 않았다.

④ 이상의 결과로 볼 때, CCN1 은 구강암 발생 유도 와 발생 억제 양방향으로 기능을 보유한 것으로 생각되며, 방향을 결정하는 세포내 환경을 규명하는 추가적인 연구가 필요하다.

주요어: 구강암, CCN1 (Cyr61), 세포 증식, 이동 능력, 발현저하
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