# The Axin2-snail Axis Promotes Bone Invasion by Activating Cancer-associated Fibroblasts in Oral Squamous Cell Carcinoma

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# Abstract

**Background**: In bone-invasive oral squamous cell carcinoma (OSCC), cancer-associated fibroblasts (CAFs) infiltrate into bony tissue ahead of OSCC cells. In the present study, we aimed to investigate the role of the Axin2-Snail axis in the biological behavior of CAFs and bone invasion in OSCC.

**Methods**: The clinicopathological significance of Axin2 and Snail expression was investigated by immunohistochemistry in an OSCC cohort containing 217 tissue samples of patients with long-term follow-up. The influence of the Axin2-Snail axis on the biological behavior of OSCC cells and CAFs was further investigated both *in vitro* and *in vivo*.

**Results**: Axin2 expression is significantly associated with Snail expression, status of desmoplasia, and bone invasion in patients with OSCC. In multivariate analysis, lymph node metastasis, desmoplasia, Axin2, and Snail expression were independent poor prognostic factors in our cohort. Supportively, OSCC cells demonstrated attenuated oncogenic activity as well as decreased expression of Snail and various cytokines after Axin2 knockdown *in vitro*. Among the related cytokines, C-C motif chemokine ligand 5 and interleukin 8 have a strong influence on the biological behavior of CAFs *in vitro*. Moreover, both the desmoplastic reaction and osteolytic lesions in the calvaria were predominantly decreased after Axin2 knockdown in OSCC cells *in vivo* using BALB/c athymic nude mice xenograft model.

**Conclusions**: Oncogenic activities of the Axin2-Snail axis are not limited to the cancer cells themselves, but rather extend to CAFs by regulation of cytokine-mediated cancer-stromal interaction, with further implications for bone invasion as well as poor prognosis of OSCC.


# Tables

Table 1. Clinicopathological characteristics of 217 OSCC patients

| Clinicopathological variables | No. of patients (%) |
|---|---|
| Total cases | 217 |
| Age, years | |
| Median age (range) | 61(27-85) |
| ≤61 | 113(52.1) |
| >61 | 104(47.9) |
| Gender | |
| Male | 141(65.0) |
| Female | 76(35.0) |
| Site | |
| Mandible | 112(51.6) |
| Maxilla | 67(30.9) |
| RMT | 38(17.5) |
| T stage | |
| T1 | 27(12.4) |
| T2 | 45(20.7) |
| T3 | 9(4.1) |
| T4 | 136(62.7) |
| N stage | |
| Nx | 48(22.1) |
| N0 | 95(43.8) |
| N1 | 23(10.6) |
| N2a | 9(4.1) |
| N2b | 25(11.5) |
| N2c | 1(0.5) |
| N3b | 16(7.4) |
| Histologic grade | |
| WD | 35(16.1) |
| MD | 149(68.7) |

| | |
|---|---|
| PD | 33(15.2) |
| Perineural invasion | |
| Negative | 202(93.1) |
| Postive | 15(6.9) |
| Vascular invasion | |
| Negative | 192(88.5) |
| Postive | 25(11.5) |
| Bone invasion | |
| Negative | 94(43.3) |
| Postive | 123(56.7) |

WD: well differenciated; MD: moderately differentiated; PD: poorly differentiated

Table 2. Clinicopathological significance of Axin2 and Snail expression in 217 OSCC patients

| Variables | | Axin2 | | *P* | Snail | | *P* |
|---|---|---|---|---|---|---|---|
| | Total | Low | High | | Low | High | |
| Age | | | | | | | |
| ≤59 | 113 | 65(57.5) | 48(42.5) | 0.211 | 60(53.1) | 53(46.9) | 0.46 |
| >59 | 104 | 51(49.0) | 53(51.0) | | 50(48.1) | 54(51.9) | |
| Gender | | | | | | | |
| Male | 141 | 69(48.9) | 72(51.1) | 0.069 | 74(52.5) | 67(47.5) | 0.472 |
| Female | 76 | 47(61.8) | 29(38.2) | | 36(47.4) | 40(52.6) | |
| Site | | | | | | | |
| Mandible | 112 | 66(58.9) | 46(41.1) | 0.173 | 60(53.6) | 52(46.4) | 0.681 |
| Maxilla | 67 | 34(50.7) | 33(49.3) | | 32(47.8) | 35(52.2) | |
| RMT | 38 | 16(42.1) | 22(57.9) | | 18(47.4) | 20(52.6) | |
| T stage | | | | | | | |
| T1-T2 | 72 | 51(70.8) | 21(29.2) | <0.001 | 44(61.1) | 28(38.9) | 0.031 |
| T3-T4 | 145 | 65(44.8) | 80(55.2) | | 66(45.5) | 79(54.5) | |
| N stage | | | | | | | |
| Nx | 48 | 31(64.6) | 17(35.4) | <0.001 | 29(60.4) | 19(39.6) | <0.001 |
| N0 | 95 | 62(65.3) | 33(34.7) | | 57(60.0) | 38(40.0) | |
| N1-3 | 74 | 23(31.1) | 51(68.9) | | 24(32.4) | 50(67.6) | |
| Histologic grade | | | | | | | |
| WD | 35 | 22(62.9) | 13(37.1) | 0.439 | 19(54.3) | 16(45.7) | 0.565 |
| MD | 149 | 78(52.3) | 71(47.7) | | 77(51.7) | 72(48.3) | |
| PD | 33 | 16(48.5) | 17(51.5) | | 14(42.4) | 19(57.6) | |
| Perineural invasion | | | | | | | |
| Negative | 202 | 110(54.5) | 92(45.5) | 0.279 | 105(52.0) | 97(48.0) | 0.163 |
| Postive | 15 | 6(40.0) | 9(60.0) | | 5(33.3) | 10(66.7) | |
| Vascular invasion | | | | | | | |
| Negative | 192 | 109(56.8) | 83(43.2) | 0.01 | 103(53.6) | 89(46.4) | 0.019 |
| Postive | 25 | 7(28.0) | 18(72.0) | | 7(28.0) | 18(72.0) | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Bone invasion | | | | | | | |
| Negative | 94 | 63(67.0) | 31(33.0) | <0.001 | 56(59.6) | 38(40.4) | 0.028 |
| Postive | 123 | 53(43.1) | 70(56.9) | | 54(43.9) | 69(56.1) | |

WD: well differenciated; MD: moderately differentiated; PD: poorly differentiated

Table 3. Prognostic impact of clinical variables and biomarkers in multivariate Cox regression analysis in 179 OSCC patients

| | Hazard ratio (95% CI) | *p* |
|---|---|---|
| Age | 0.825 (0.482-1.410) | 0.481 |
| Sex | 0.609 (0.339-1.093) | 0.096 |
| Lesion site | | |
| Mandible | 1 | 0.235 |
| Maxilla | 0.618(0.289-1.323) | 0.215 |
| RMT | 1.206(0.608-2.392) | 0.591 |
| T stage | | |
| T1 | | 0.99 |
| T2 | 0.989(0.319-3.066) | 0.985 |
| T3 | 1.059(0.119-9.453) | 0.959 |
| T4 | 1.157(0.386-3.467) | 0.795 |
| N stage | | |
| Nx | | 0.014 |
| N0 | 1.927(1.016-3.655) | 0.044 |
| N1-3 | 3.424(1.466-7.998) | 0.004 |
| Histologic grade | | |
| WD | 1 | 0.435 |
| MD | 1.209(0.592-2.467) | 0.602 |
| PD | 1.665(0.730-3.798) | 0.226 |
| Perineural invasion | 0.850(0.321-2.247) | 0.743 |
| Vascular invasion | 1.036(0.457-2.350) | 0.933 |
| Bone invasion | 1.357(0.572-3.219) | 0.489 |
| Desmoplasia | 2.491(1.240-5.004) | 0.01 |
| Angiogenesis | 1.449(0.860-2.441) | 0.164 |
| Axin2 | 2.488(1.358-4.559) | 0.003 |
| Snail | 1.984(1.097-3.588) | 0.024 |

WD: well differenciated; MD: moderately differentiated; PD: poorly differentiated; 95% CI: 95% confidence interval

# Background

Oral squamous cell carcinoma (OSCC) is the most common histological type of oral cancer. OSCC cells often penetrate underlying bone, and 12-56% of patients with OSCC present with bone invasion [1]. According to the American Joint Committee on Cancer (AJCC) classification, the presence of bone invasion can upstage this type of cancer regardless of tumor size, because bone invasion is a major poor prognostic indicator of OSCC [2-4]. However, the molecular mechanism underlying the invasion of adjacent bone by OSCC is not fully understood.

Desmoplasia refers to the growth of excessive stromal tissue around tumors, and fibroblasts located in this stromal tissue, termed cancer-associated fibroblasts (CAFs), are key players in the cancer stroma. The critical roles of CAFs have been investigated recently in various types of cancers [5-7]. CAFs can promote angiogenesis via production of pro-angiogenic factors such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA), and also contribute to immune surveillance of tumor cells by recruiting immunosuppressive cells such as myeloid-derived suppressor cells (MDSC) and M2 macrophages [8-10]. Moreover, CAF-derived hepatocyte growth factor (HGF), stromal cell-derived factor 1 (SDF1), and transforming growth factor beta (TGF-β) can promote proliferation, invasion, and metastasis of cancer cells via activation of various signaling pathways such as the MAPK and PI3K/AKT pathways, as well as the TGF-β/SMAD pathway [11-13]. In bone-invasive OSCC, an abundant population of stromal cells intervenes between OSCC cells and damaged bony tissue. In particular, alpha-smooth muscle actin (α-SMA)-positive CAFs, a major component of cancer-related stroma, are shown to infiltrate into bony tissue ahead of OSCC cells [14]. Biological characteristics of CAFs may be important in the pathogenesis of bone invasion in OSCC.

CAFs demonstrate heterogeneity in tumor tissues. In pancreatic adenocarcinomas, for example, CAFs display two distinct populations, myofibroblasts and inflammatory fibroblasts, while in breast cancer, four different populations of CAFs are observed, including those related to immune escape [15, 16]. Moreover, various cancers including OSCC have revealed the presence of a senescence-associated secretory phenotype of CAFs, which can secrete various cytokines and thereby influence the tumor microenvironment [17-19]. The stromal role may differ according to the particular population of CAFs.

The production as well as transdifferentiation of CAFs may be regulated by cancer-stromal crosstalk. Some tumor-derived factors, particularly proinflammatory cytokines, can trigger cancer-stromal crosstalk and further influence multifaceted signaling pathways responsible for cancer progression, thereby promoting formation of a favorable microenvironment for tumor cell survival, growth, and invasion [20, 21]. Recently, therefore, CAFs have come to be regarded as a potential target to increase the therapeutic efficacy of treatment of various cancers with desmoplastic features [22]. Accordingly, targeting the key genetic factors involved in the desmoplastic reaction in cancers may enable provide therapeutic advances for patients resistant to current anti-cancer therapy.

The epithelial to mesenchymal transition (EMT) is mediated by local activation of the canonical Wnt signaling pathway in various type of cancers, and promotes cell invasion and metastasis during cancer

progression by silencing epithelial traits and inducing mesenchymal phenotypes [23]. On the invasive front, cancer cells are exposed to the extracellular matrix and thereby given the opportunity to participate in cancer-stromal crosstalk. Snail, a zinc-finger transcription factor, is primarily known as a transcriptional repressor that mediates EMT via repression of E-cadherin transcription [24-26]. Recently, increasing evidence has demonstrated that Snail-mediated transactivation can remodel the tumor microenvironment during EMT progression. Transcription of multiple proinflammatory cytokines such as C-C motif chemokine ligand (CCL) 2 and CCL5 is known to be activated by Snail transactivation [27, 28]. Moreover, Snail can also directly activate interleukin (IL) 8 transcription by binding to the E3/E4 E-boxes [29]. In addition, Snail can activate TGF-β signaling pathway-related proteins such as connective tissue growth factor, secreted protein acidic and rich in cysteine, fibronectin1, and transgelin that are involved in fibrosis in various organs. Axis inhibition protein 2 (Axin2), a scaffolding protein of glycogen synthase kinase 3 (GSK-3), can promote cancer cell invasion and metastasis in various type of malignancies via inhibition of GSK-3-mediated Snail degradation [24, 30]. Axin2-mediated Snail stabilization may contribute to cancer-stromal crosstalk, and thereby may influence cancer prognosis.

In the present study, the association between Axin2 expression and various clinicopathological factors including status of desmoplasia as well as bone invasion was investigated in patients with OSCC. The influence of Axin2 expression on the biological behavior of CAFs was also investigated *in vitro* and *in vivo* with the aim of identifying the possible role of Axin2 expression in the pathogenesis of OSCC.

# Methods

Patients in the OSCC cohort

In this study, we retrospectively reviewed the archived files of the patients with OSCC at Dental Hospital, Yonsei University Medical Center, Seoul, Korea, from 1999 to 2017. Of the 432 patients reviewed, 217 patients with OSCC occurring in lesion sites that might involve bone of the maxilla and/or the mandible were included in the present study. Of those 217 patients in the OSCC cohort, 123 (56.7%) had been determined during follow-up to have bone invasion, while no bone invasion had been found in the other 94 (43.3%) patients (**Fig. 1**). The clinicopathological characteristics of the patients in OSCC cohort are shown in **Table 1**. This study was approved by the Institutional Review Board for Bioethics of Yonsei University College of Dentistry (IRB 2-2017-0006 and 2-2019-0050).

Immunochemical stainig

Protein expression for alpha smooth muscle actin (α-SMA), vimentin, CD31, Axin2, and Snail expression in cells and tissue samples were determined by immunochemical staining. Details about the procedures and scorinig methods of immunochemical staining are described in the Supplementary Materials and Methods.

Histomorphometry

As described in a previous study, the desmoplastic reaction status was evaluated according to the ratio of the area of tumor-associated stroma and whole tumor tissue [31]. The subjected areas were measured with ImageJ using hematoxylin and eosin-stained OSCC tumor tissue sections. The status of desmoplasia was further divided into two groups according to the calculated ratio: high- (ratio≥1) and low-desmoplastic (ratio<1) reaction.

Cell lines

Two kineds of CAFs (CAF1 and CAF2) and OSCC cell lines (CA9-22 and HSC-2) were used in this study. Details about the procedures of cell culture and establishment of Axin2 knockdown OSCC are described in the Supplementary Materials and Methods.

Recombinant proteins

All of the human recombinant proteins (CCL2, CCL5, and IL8) were purchased from R&D Systems (Minneapolis, MN, USA).

Proliferation assay

To investigate the proliferative ability of each group of cells, OSCC cells and CAFs, respectively, were seeded in 6-well plates at densities of $1x10^5$ and $5x10^4$. The number of cells was counted after trypan blue staining at each indicated time point.

Wound healing and invasion assays

The cell motility and invasion ability was determined for each group of cells using wound healing and matrigel invasion assays, respectively. Details about the procedures of wound healing and invasion assays are described in the Supplementary Materials and Methods.

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to determine mRNA expression of IL8, CCL2, CCL5, matrix metalloproteinase (MMP)-2, MMP-9, and Ki67 expression in the cell lines. Details of the RT-PCR procedure are described in the Supplementary Materials and Methods.

Nude mouse xenograft

The animal studies were performed according to experimental protocols approved by the animal ethics committee of Yonsei University College of Dentistry. Twenty female 5-week-old BALB/c athymic nude mice (6 weeks of age; Central Laboratory Animal Inc., Seoul, Korea) were housed in laminar-flow cabinets under specific-pathogen-free conditions. All of the mice were randomized into four groups (n=5 per group) and a total of $1x10^6$ HSC-$2^{Mock}$, HSC-$2^{\triangle Axin2}$, CA9-$22^{Mock}$, and CA9-$22^{\triangle Axin2}$ cells were subcutaneously injected into the calvaria of the mice. The length and width of the tumor nodules were measured every 3

days and the size of the tumors were calculated by the following formula: width$^2$xlengthx1/2. All of the mice were sacrificed after 9 weeks by $CO_2$ asphyxiation.

Statistical analysis

The association between protein expression and clinicopathological variables was analyzed using the chi-square test. The Mann-Whitney U-test was used for comparisons of cell groups in proliferation, migration, and invasion, as well as tumorigenesis. SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and a p-value less than 0.05 was considered statistically significant.

# Results

Clinicopathological significance of Axin2 and Snail expression in patients with OSCC

In the present study, Axin2 expression was found in the cytoplasm of cancer cells in 168 (77.4%) patients with OSCC, and immunoreactivity against Axin2 was high in 101 (high-Axin2, 46.5%) OSCC tissue samples and low in 116 (low-Axin2, 53.5%). Cancer cells demonstrated cytoplasmic and nuclear Snail expression in 186 (85.7%) patients with OSCC, and immunoreactivity against Snail was high in 107 (high-Snail, 49.3%) OSCC tissue samples and low in 110 (low-Snail, 50.7%). Significant association was found between Axin2 and Snail expression in patients with OSCC ($p$=0.006) (Fig. 2A). High-Axin2 or high-Snail expression both showed significant association with T stage ($p$<0.001 and $p$=0.031), lymph node metastasis (both $p$<0.001), vascular invasion ($p$=0.01 and $p$=0.019), and with bone invasion ($p$<0.001 and $p$=0.028) of OSCC in the present study (Table 2). Moreover, patients with high-Axin2 or high-Snail expression demonstrated increased vessel density ($p$<0.001 and $p$=0.002) and higher desmoplastic reaction (both $p$<0.001) than patients with low-Axin2 or low-Snail expression in our cohort (Fig. 2B and 2C). For identify the risk factors for prognosis of OSCC, multivariate analysis was performed in 179 patients who with follow up more than 5 years. Results showed that when using age, sex, lesion site, T stage, lymph node metastasis, histologic grade, vascular invasion, perineural invasion, bone invasion, status of desmoplasia, status of angiogenesis, Axin2, and Snail expression as cofactors, lymph node metastasis, status of desmoplasia, Axin2 expression, and Snail expression were independent risk factors for OSCC prognosis, with hazard ratios of 3.424 (95% confidence interval, 1.466–7.998; $p$=0.004), 2.491 (95% confidence interval, 1.240–5.004; $p$=0.01), 2.488 (95% confidence interval, 1.358–4.559; $p$=0.003), and 1.984 (95% confidence interval,1.097–3.588; $p$=0.024), respectively (Table 3).

Axin2 knockdown had a strong influence on the biological behavior of OSCC cells

Consistent with the results of a previous study [24], we found that Snail expression was predominantly decreased in both CA9-22$^{\triangle Axin2}$ and HSC-2$^{\triangle Axin2}$ cells compared to that in related control cells (Supplementary Fig. 1A, i and iv).

Proliferative ability was significantly reduced after Axin2 knockdown in both CA9-22 and HSC-2 cells. Compared with CA9-22 $^{Mock}$, decreases of 1.4-, 2.1-, and 2.2-fold in cell number were found in CA9-22$^{\triangle Axin2}$ cells after 24h, 48h, and 72h of culture (all $p$=0.008). Similarly, HSC-2$^{\triangle Axin2}$ cells also showed decreases of 1.4-, 1.6-, and 2.3-fold in number compared to the HSC-2$^{Mock}$ cells (all $p$=0.008) (Supplementary Fig. 1A, ii and v). Likewise, Ki67 expression was also significantly decreased in Axin2 knockdown cells compared to that in the controls in both HSC-2 and CA9-22 cells (both $p$=0.002) (Supplementary Fig. 1A, iii and vi). In addition, cell motility was decreased 1.5- and 1.8-fold, respectively, in Axin2 knockdown cells compared to the control CA9-22 and HSC-2 cells (both $p$=0.002) (Supplementary Fig. 1B, i-iv). Moreover, 2.3- and 1.6-fold decreases in numbers of invading cells were found in Axin2 knockdown cells compared to CA9-22 and HSC-2 control cells, respectively (both $p$=0.002). Axin2 may have oncogenic activity in OSCC cells (Supplementary Fig. 1C, i-iv). Interestingly, compared to the related control cells, expression of Snail-related cytokines IL8, CCL2, and CCL5 was 3.5-fold, 2.8-fold, and 3.3-fold decreased in CA9-22$^{\triangle Axin2}$ cells (all $p$=0.002), and 2.6-fold, 1.8-fold, and 1.5-fold decreased in HSC-2$^{\triangle Axin2}$ cells (all $p$=0.002) (Supplementary Fig. 1D, i-ii).

Cytokines related to Axin2-Snail axis reveal strong influences on the biological behavior of CAFs

To evaluate the effect of those cytokines on the biological behavior of CAFs, both CAF1 and CAF2 cells were treated with different does (0, 2, 5, and 10ng/ml) of human recombinant proteins (IL8, CCL2, and CCL5), after which the proliferation and invasion abilities of CAFs in each group were comparatively investigated. Strong influence of IL8 or CCL5 on biological behavior of CAFs was found from dose of 2ng/ml in the present study. We found that CAF1 showed increases of 2.5- and 2.0-fold in numbers of the cells after treatment with 2ng/ml IL8 or CCL5, compared to untreated controls (both $p$=0.002). Similar results were obtained using CAF2, with IL8 or CCL5 treatment leading to increases in cell numbers (both $p$=0.002). No significant differences were observed in the proliferative ability of CAFs after CCL2 treatment in this study (Fig. 3B, i-ii).

We also found that the invasion ability of CAF1 and CAF2 cells was 2.2- and 2.3-fold increased after IL8 (2ng/ml) treatment compared to that of untreated control cells (both $p$=0.002). Supportively, MMP-2 expression was 2.3-fold and 2.2-fold increased after IL8 (2ng/ml) treatment in both CAF1 and CAF2 cells compared to untreated controls (both $p$=0.002). No significant difference was found in MMP-9 expression in CAFs after IL8 treatment in our study (Fig. 3C, i-vi).

Tumor progression and bone invasion depends on Axin2 expression in tumor cells *in vivo*

As shown in Fig. 4, tumor volume was significantly decreased in mice injected with Axin2 knockdown cells compared to controls for both CA9-22 and HSC-2 cells (i-ii). In the micro-CT imaging analysis, extensive osteolytic lesions were observed in the calvaria from CA9-22$^{Mock}$ or HSC-2$^{Mock}$ cell-bearing mice when compared to those from the related Axin2 knockdown cell-bearing mice (iii-iv). Moreover, in the tissue sections, the area of tumor-associated stroma was predominantly increased at the tumor-bone

interface in CA9-22$^{Mock}$ or HSC-2$^{Mock}$ cell-bearing mice compared to that in the related Axin2 knockdown cell-bearing mice (v-vi).

# Discussion

Although earlier studies focused on the inhibitory role of Axin2 in the β-catenin degradation complex, recent studies have implied that Axin2 contributes to oncogenic progression. Increased expression of Axin2 was found after loss of adenomatous polyposis coli (APC), a key tumor suppressor gene, in colorectal cancer, and knockdown of Axin2 attenuated oncogenic and Wnt signaling activities of cancer cells [32, 33]. In addition, the GSK3 nuclear export function of Axin2-mediated abundance of nuclear Snail and β-catenin is also a sign of the oncogenic activity of Axin2 [24]. In our previous study, we found that Axin2 expression showed a positive correlation with Snail expression, and increased expression of both Axin2 and Snail was closely associated with malignant transformation of oral leukoplakia [34]. Consistent with these findings, abundance of Axin2 and Snail expression are significantly correlated in OSCC tissues. Moreover, we found that both Axin2 and Snail expression were significantly related to poor prognosis as well as poor prognostic indicators of OSCC, including bone invasion. Supportively, Axin2 knockdown cells showed decreased Snail expression and attenuated oncogenic activities compared to control cells. Axin2 may be implicated in OSCC pathogenesis as an oncogene.

Desmoplasia is known as a typical sign of aggressiveness in various type of cancers including OSCC [14, 35]. Some investigators have shown that abundant desmoplastic reaction is prominent in the cancer-bone interface of OSCC with bone invasion, but identifiable fibrous stroma has been less frequently found in ameloblastoma, a benign odontogenic epithelial tumor with features of bone invasion [14]. In the present study, we also found frequent desmoplastic reaction in OSCC tissues, especially in the area of bone invasion, and the status of desmoplasia was significantly associated with bone invasion in patients with OSCC. Interestingly, both Axin2 and Snail expression were positively correlated with desmoplastic reaction in our cohort. Moreover, in mouse xenograft analysis, the stromal component of tumor nodules as well as osteolytic bone resorption of mouse calvaria were predominantly decreased in the group of cells with Axin2 knockdown compared to the control cells. In addition, expression of the Snail-mediated proinflammatory cytokines such as IL8, CCL2, and CCL5 was significantly decreased after Axin2 knockdown in OSCC cells compared to that in control cells. Admittedly, all of these cytokines are related to characteristics of aggressiveness by means of promotion of migration, invasion, and metastatic abilities of cancer cells including those of OSCC, and are considered potential therapeutic targets of malignancies [36–40].

According to previous studies, all of these tumor-derived cytokines also influence different types of cells in the stromal component of tumors, such as CAFs, endothelial cells, and inflammatory cells, and thereby trigger multiple signaling pathways related to the malignant progression of tumors. Both CCL2 and CCL5 can mediate infiltration of tumor-associated macrophages and inhibit potential anti-tumor T-cell activities, thereby controlling the populations of leukocytes at tumor sites [39]. Moreover, distinct regulatory roles as well as underlying molecular mechanisms performed by CCL2 and IL8 in angiogenesis have been

indicated by some investigators. CCL2 can enhance angiogenic activity either by directly inducing endothelial cell retraction or by CCL2-induced release of angiogenic factors such as VEGFA [41, 42]. IL8 can promote the invasion ability of endothelial cells via increased expression of MMP-2 and MMP-9 in endothelial cells [43]. Moreover, IL8 can promote proliferation and tube formation of endothelial cells by activating extracellular signal-regulated protein kinase 1/2 (Erk 1/2) during interaction with CXCR2 in endothelial cells [44]. In the present study, we found that CD31-positive vessel density was significantly associated with Axin2 and Snail expression in patients with OSCC. In OSCC, the Axin2-Snail axis may also mediate angiogenic responses via control of related cytokines.

A previous study demonstrated that 12 different types of chemokine receptors were found in oral fibroblasts, including CXCR1, CXCR2, and CCR3 [45]. In the present study, we found that both CCL5 and IL8 have strong influence on biological behavior of CAFs. Those cytokines may influence CAFs via binding to related receptors in CAFs. All of those findings implied that Axin2-Snail axis may mediate a diffuse desmoplastic reaction in OSCC via control inflammation-stromal crosstalk.

In the present study, recombinant IL8 can increase the invasion ability of CAFs *in vitro.* In various type of cancer tissues, IL8 can participate in the degradation of the extracellular matrix by promoting the expression of MMP-2 and MMP-9 in cancer cells [46–49]. According to public data base analysis, MMP-9 acts in a context-dependent manner in different types of cancers [50]. In this study, no significant difference was found in MMP-9 expression in CAFs after IL8 treatment. Meanwhile, in the present study, we found that MMP-2 expression was significantly increased in CAFs after IL8 treatment. A previous study showed that MMP-2 was primarily expressed in fibroblasts of mouse lung tumors and concluded that CAFs were the main producer of MMP-2 [51]. MMPs can directly destroy bone matrix; thus, IL8-mediated MMP-2 overexpression may be one of the underlying molecular mechanisms of CAF infiltration into bony tissue ahead of OSCC cells in patients with high-Axin2 expression.

# Conclusions

Consistent with previous observations in other cancers [24], our results implied that the Axin2-Snail axis is a poor prognostic indicator of OSCC. We also found that the oncogenic activities of the Axin2-Snail axis are not limited to the cancer cells themselves, but rather extend to cancer-associated stromal cells such as endothelial cells and CAFs through regulation of cytokine-mediated cancer-stromal interaction, and thereby promote active desmoplastic reactions as well as bone invasion in OSCC. The Axin2-Snail axis may serve as a novel diagnostic and therapeutic target in bone-invasive OSCC.

# Abbreviations

OSCC
Oral squamous cell carcinoma; CAFs:Cancer-associated fibroblasts; AJCC:American Joint Committee on Cancer; FGF2:fibroblast growth factor 2; VEGFA:Vascular endothelial growth factor A; MDSC:Myeloid-derived suppressor cells; HGF:Hepatocyte growth factor; SDF1:Stromal cell-derived factor 1; TGF-

β:Transforming growth factor beta; α-SMA:Alpha-smooth muscle actin; EMT:Epithelial to mesenchymal transition; CCL:C-C motif chemokine ligand; Axin2:Axis inhibition protein 2; GSK-3:Glycogen synthase kinase 3; RT-PCR:Reverse transcription polymerase chain reaction; MMP:Matrix metalloproteinase; APC:Adenomatous polyposis coli; Erk 1/2:Extracellular signal-regulated protein kinase 1/2;

# Declarations

### Ethics approval and consent to participate

This study was approved by the Institutional Review Board for Bioethics of Yonsei University College of Dentistry (IRB 2-2017-0006 and 2-2019-0050). We used paraffin embedded tissue removed after surgery which is collected in pathology department. The block was the remaining component after pathologic reading and therefore patient consent for using the stored tissue was exempted by approval of IRB, in accordance with the guidelines of the local ethics committees.

### Consent to publish

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

XLZ, EC, and JQL designed and wrote the manuscript; XLA and YZA involved and performed most of the experiments. XLZ, YZA, and EC participated in data acquisition, data analysis, or data interpretation. All authors have read and approved the manuscript, and ensure that this is the case.


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# Figures

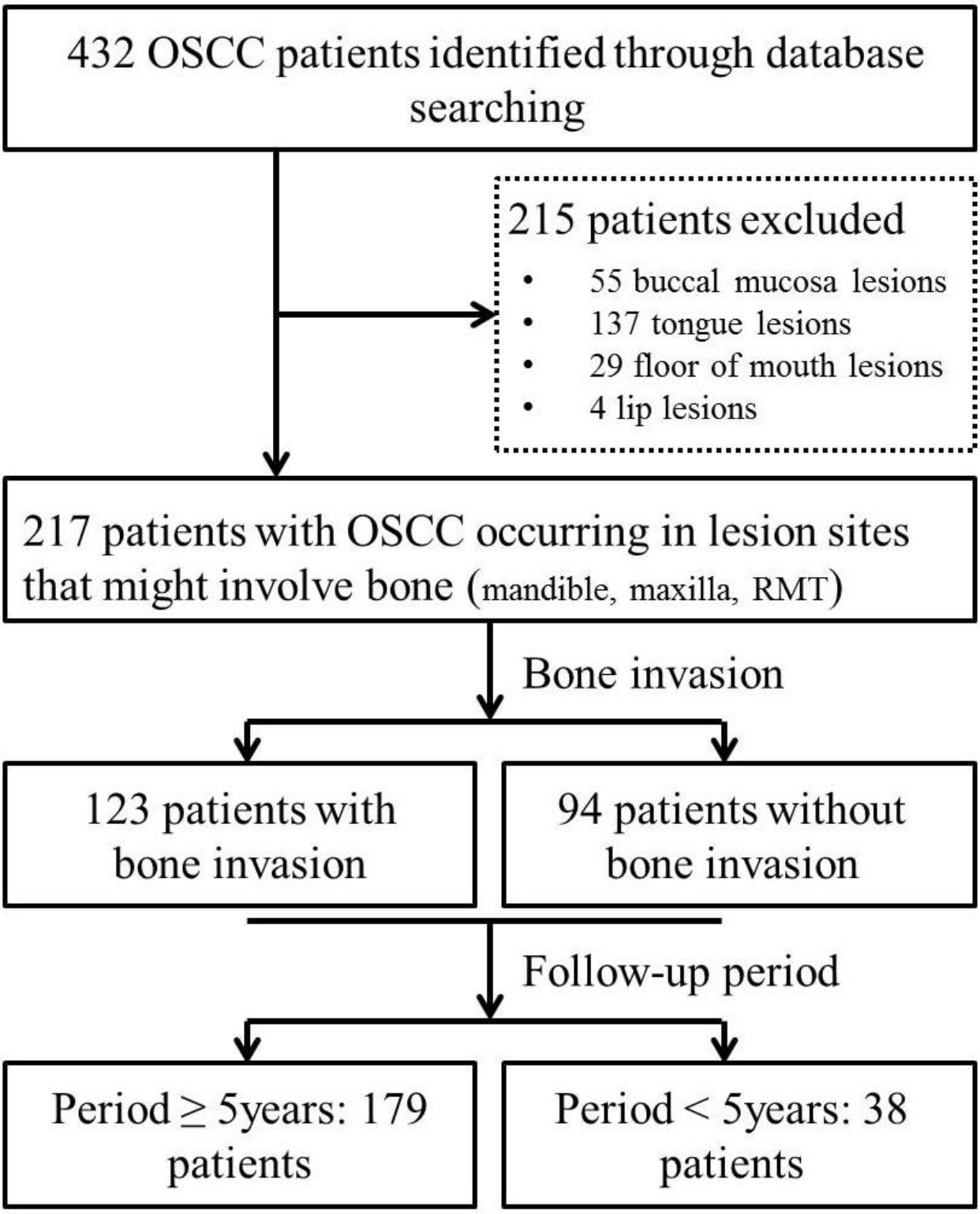
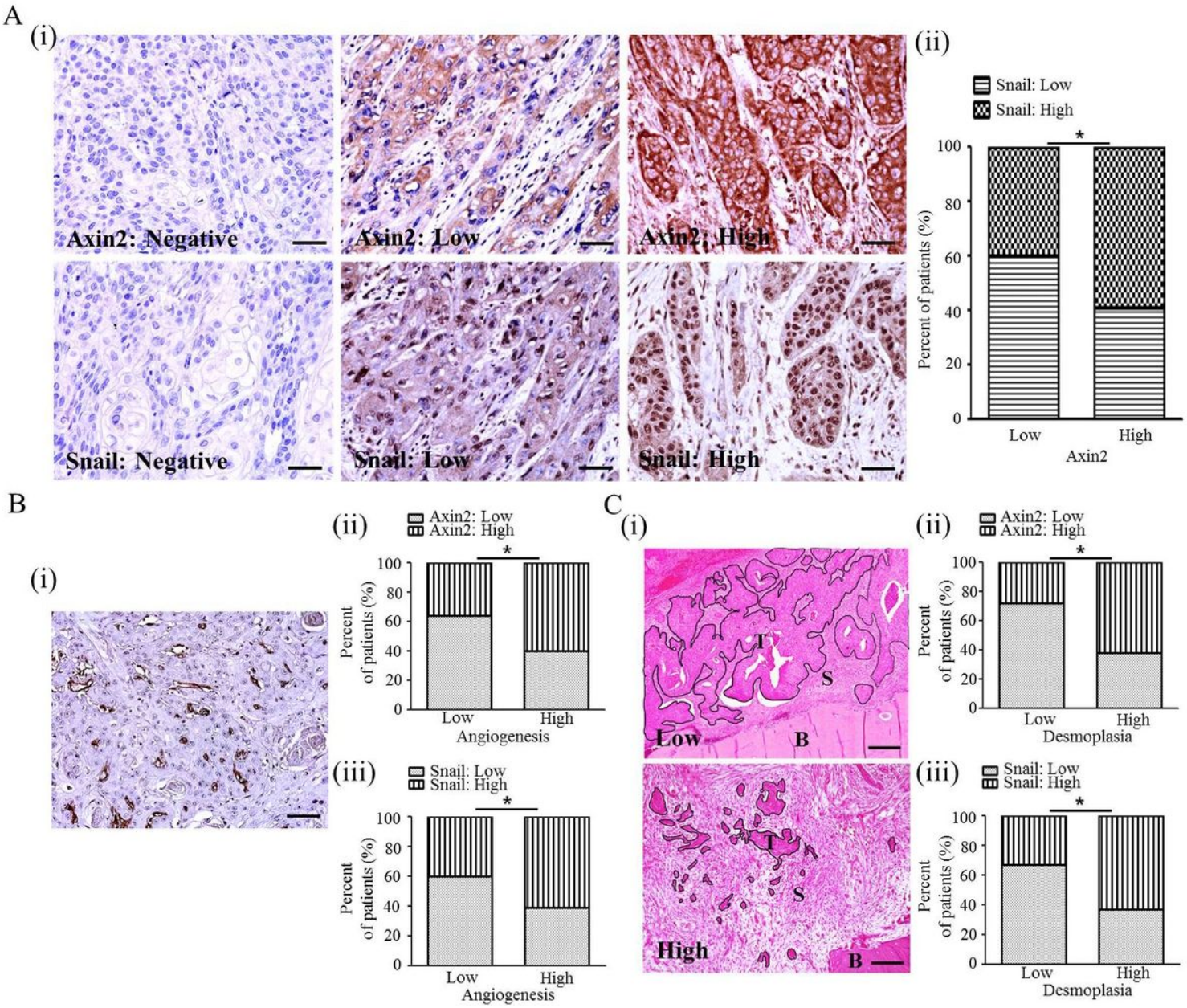


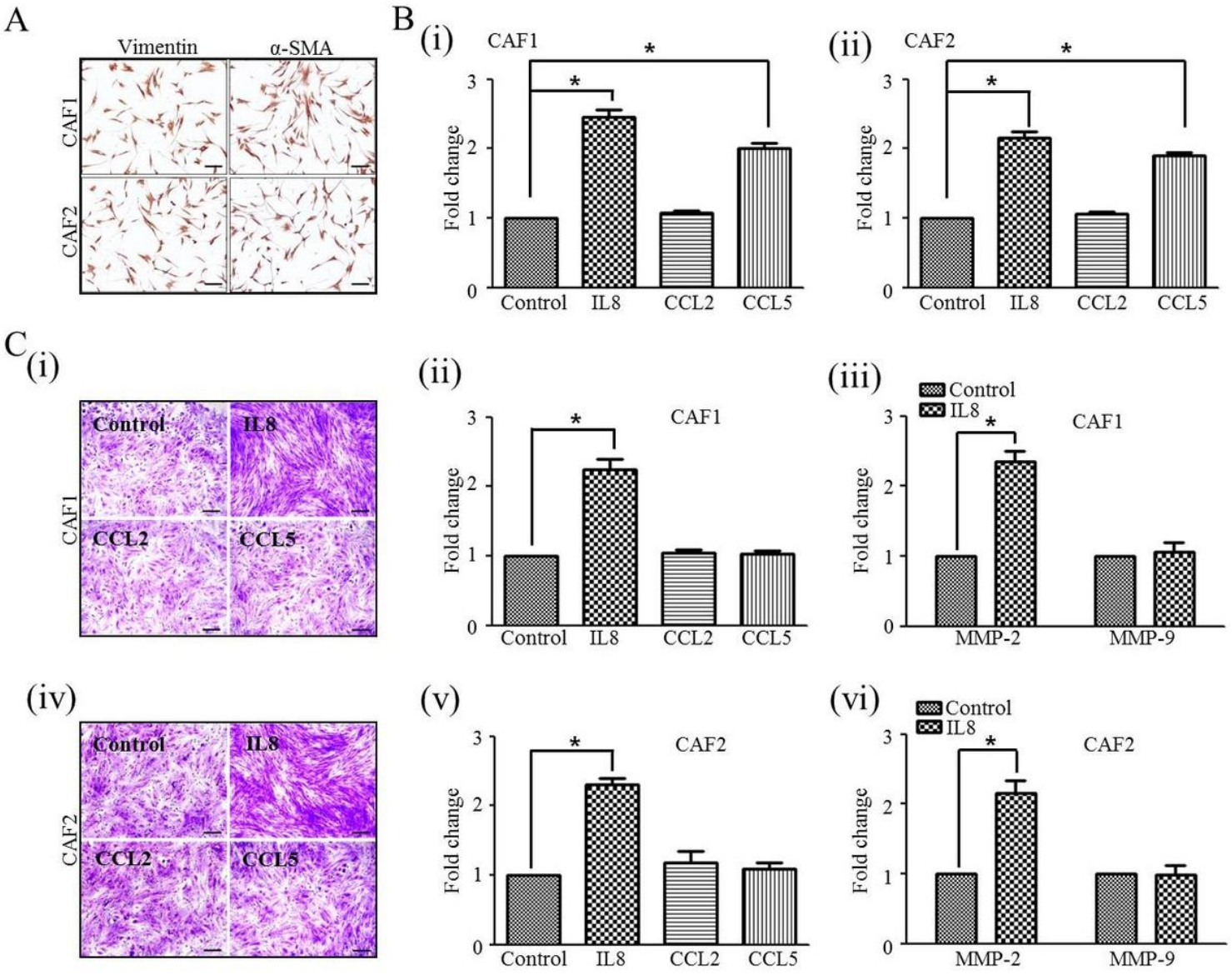

**Figure 1**

Flow diagram of sample selection and attrition for patients with OSCC.




## Figure 2

Clinicopathological significance of Axin2 and Snail expression in patients with OSCC: (A) Representative expression patterns for Axin2 and Snail in OSCC tissue samples (original magnification, x400; scale bar, 25 μm) (i). Axin2 and Snail expression are significantly correlated in OSCC tissues (ii). (C) Association between microvessel density and expression of Axin2 and Snail in OSCC tissues: Example of a hot spot in OSCC tissues (original magnification, x200; scale bar, 50 μm) (i). Microvessel density is significantly associated with Axin2 and Snail expression (ii-iii). (D) Association between desmoplastic reaction and expression of Axin2 and Snail in OSCC tissues: representative histological patterns of low and high desmoplastic reaction in OSCC tissues (i). Desmoplastic reaction is significantly associated with Axin2 and Snail expression (original magnification, x100; scale bar, 100 μm) (ii-iii) (* $p<0.05$) (T: tumor, S: stroma, B: bone).




## Figure 3

Cytokines related to Axin2-Snail axis demonstrate strong influences on the biological behavior of cancer-associated fibroblasts (CAFs) in vitro: (A) CAF identities were verified based on expression of vimentin and α-smooth muscle actin (α-SMA). (B) Proliferating ability was significantly increased after CCL5 or IL8 treatment in both CAF1 and CAF2 (i & ii). (C) Invasion ability as well as MMP-2 expression was significantly increased after IL8 treatment in both CAF1 (i-iii) and CAF2 (iv-vi) (original magnification, x100; scale bar, 100μm) (* $p<0.05$).

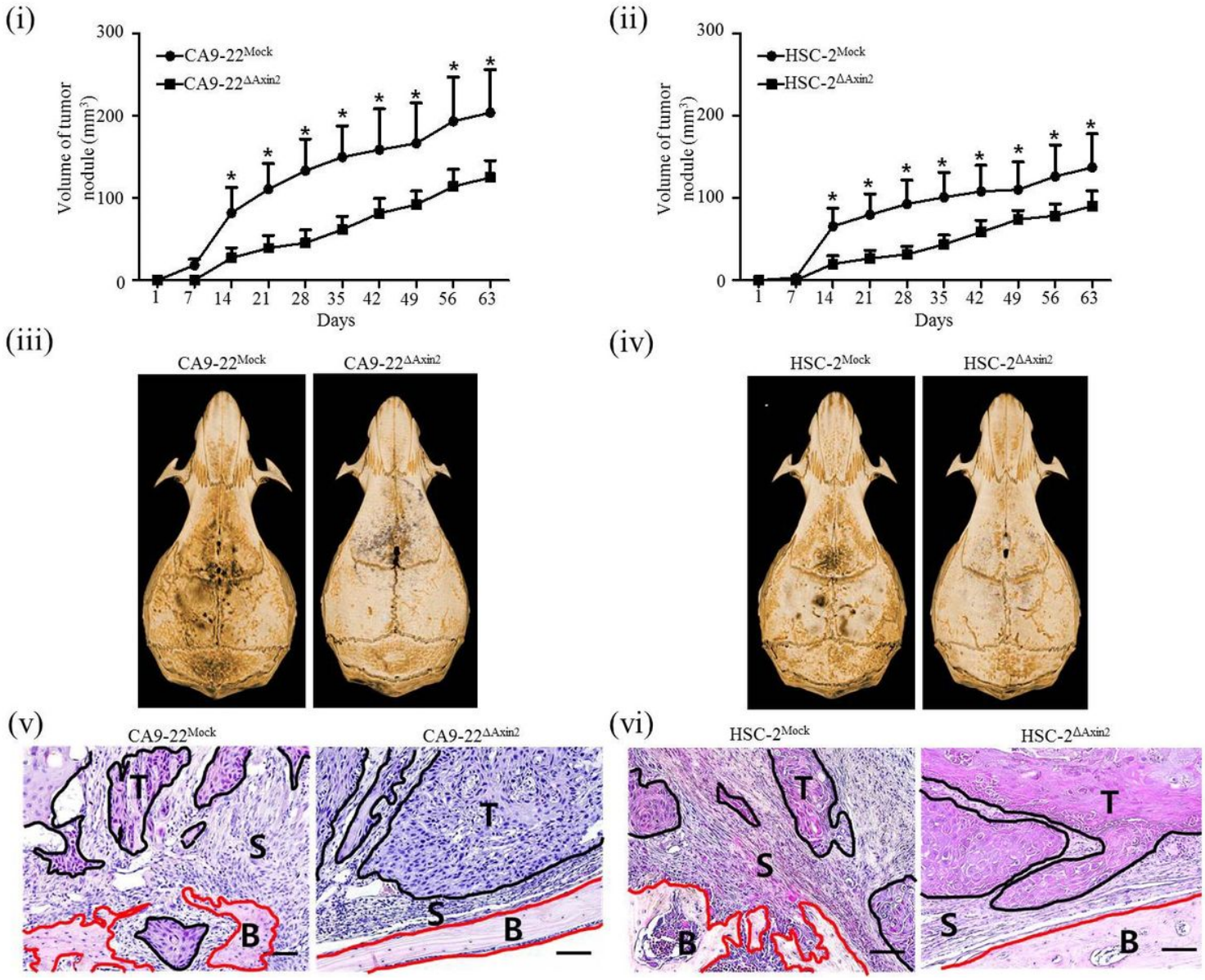



**Figure 4**

Tumor progression and bone invasion depends on Axin2 expression in tumor cells in vivo. (A) Tumor volume was predominantly decreased in mice injected with Axin2 knockdown cells compared to controls for both HSC-2 (i) and CA9-22 cells (ii). In micro-CT imaging analysis, extensive osteolytic lesions were observed in the calvaria from HSC-2Mock or CA9-22Mock cell-bearing mice when compared to those from the related Axin2 knockdown cell-bearing mice (iii-iv). The area of tumor-associated stroma was predominantly increased at the tumor-bone interface in HSC-2Mock or CA9-22Mock cell-bearing mice compared to that in the related Axin2 knockdown cell-bearing mice (v-vi) (T: tumor, S: stroma, B: Bone) (* $p<0.05$).

# Supplementary Files

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