***Supplementary data for***

The Axin2-Snail axis promotes bone invasion by activating cancer-associated fibroblasts in oral squamous cell carcinoma

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**Supplementary materials and methods**

Immunohistochemistry

In this study, mouse monoclonal anti-human alpha smooth muscle actin (α-SMA) antibody (Dako, Santa Clara, CA, USA), mouse monoclonal anti-human vimentin (Dako), rabbit monoclonal anti-human Axin2 (Abcam, Cambridge, UK), and rabbit polyclonal anti-human Snail[1] were used as primary antibody for immunochemical staining.

Formalin-fixed paraffin-embedded OSCC tissue samples were cut into 4 μm tissue sections for immunohistochemistry. After deparaffinization and hydration, antigen retrieval was performed using antigen retrieval buffer (Dako). For immunocytochemistry, the cells were seeded in 6-well plates and fixed with 95% ethanol for 30 min at room temperature. Primary antibody incubation was performed after blocking endogenous peroxidase activity with a mixture of methanol and hydrogen peroxide (ratio: 40:1), and REAL EnVision HRP Rabbit/Mouse Detection System (Dako) was used as secondary antibody. Visualization was performed by chromogen 3,3′-diaminobenzidine and hematoxylin was used in counterstaining. For the negative control, primary antibody was replaced by mouse or rabbit IgG (DakoCytomation Denmark A/S, Glostrup, Denmark) in the present study.

As described in a previous study [2], the total histoscore of the protein expression was analyzed according to staining intensity and the percentage of positive cells. The patients were subdivided into two groups, a low expression (total histoscore ≤100) and a high expression group (total histoscore>100), according to the total histoscore. For scoring of vessel density, we picked three areas with the highest CD31 positive vessel density on a slide, referred to as a “hot spot”. In each hot spot, we further counted CD31 positive vessels under three 200x magnifications. The mean value of the total CD31 positive vessel counts was considered the CD31 positive vessel density. The status of angiogenesis was subdivided into two groups, low- (vessel density ≤ mean vessel density of the cohort) and a high-angiogenic (vessel density > mean vessel density of the cohort) reaction.

OSCC cell culture and establishment of Axin2 knockdown CA9-22 and HSC-2 cells

CA9-22 and HSC-2 cells were purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and cultured in medium containing a 3:1 ratio of Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Grand Island, NY, USA) and Ham’s F12 Nutrient Mixture (F12, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 U/mL penicillin, 100 mg/mL streptomycin, 100 ng/mL cholera toxin, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, and 2×10−11M tri-iodothyronine.

Axin2 knockdown cells (CA9-22△Axin2 and HSC-2△Axin2) were constructed using pLKO-Tet-On vector (Addgene, Cambridge, MA, USA) expressing short hairpin RNA (shRNA) against Axin2 (Target sequence: 5'-ACCACCACTACATCCACCA-3')(pLKO-Tet-shAxin2). Both CA9-22 and HSC-2 cells transfected with pLKO-Tet-shAxin2 were grown in culture media supplemented with 10% Tet-approved FBS (HyClone Laboratories, Inc., Logan, UT, USA) and 1 μg/mL puromycin (CA9-22 Mock and HSC-2 Mock). Axin2 shRNA expression was induced by 5 μg/mL of doxycycline (Sigma-Aldrich, St. Louis, MO, USA), and knockdown of Axin2 expression in each stable cell line was confirmed by immunocytochemical staining.

CAF culture from OSCC tissues

Primary CAFs were prepared using OSCC surgical specimens. The tissue samples were cut into approximately 2 mm3 sized cubes and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 U/mL penicillin, 100 mg/mL streptomycin. After 2 weeks of culture, the outgrown cells were harvested and the identities of the cells were verified according to the expression of vimentin and α-smooth muscle actin (α-SMA). CAFs used in this study were at passage 4.

Wound healing and invasion assays

For the wound healing assay, groups of OSCC cells and CAFs were seeded in 24-well plates at densities of 3x105 and 1x105 cells, respectively. The wound closure was determined 20h after scratch wounding. The transwell chamber culture system (BD Biosciences, Bedford, MA, USA) was used to quantify the invasion ability of the subjected cells. OSCC cells and CAFs were seeded on the upper chamber of the transwell with culture medium containing 2% FBS at densities of 3x104 and 1x104, respectively. Culture medium containing 20% FBS was added in the lower chamber. After 36 h of incubation, penetrating cells were counted by light microscope.

Quantitative reverse transcription polymerase chain reaction

The level of mRNA expression for target genes was determined using 2×SYBR Premix Ex Taq II (Tli RnaseH Plus) (RR82LR; Takara, Ann Arbor, MI, USA) on an Applied Biosystems (Foster City, CA, USA) instrument by quantitative reverse transcription polymerase chain reaction. Total RNA extraction was performed in each group of cells using TRIzol Reagent (Invitrogen), and oligo (dT) primers were used to synthesize complementary DNA. All of the mRNA expression was normalized to that of actin, and primer sequences are listed in supplementary **Table 1**.

**Supplementary references**

[1] Yook JI, Li XY, Ota I, et al. A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. Nat Cell Biol 2006;8:1398-406.

[2] Zhang X, Zheng Z, Shin YK, et al. Angiogenic factor thymidine phosphorylase associates with angiogenesis and lymphangiogenesis in the intestinal-type gastric cancer. Pathology 2014;46:316-24.

**Supplementary figure legends**

**Figure S1.** Influence of Axin2 knockdown on the biological behavior of OSCC cell lines. (A) Expression of both Axin2 and Snail was strongly decreased after Axin2 knockdown in CA9-22 (i) and HSC-2 (iv) cell lines (original magnification, x400; scale bar, 25 μm). Both cell number and Ki67 mRNA expression were significantly decreased after Axin2 knockdown in CA9-22 (ii-iii) and HSC-2 cell lines (v-vi). Migration ability was significantly reduced after Axin2 knockdown in both CA9-22 (i-ii) and HSC-2 (iii-iv) cell lines (original magnification, x200; scale bar, 50μm). (C) Invasion ability was significantly decreased after Axin2 knockdown in both CA9-22 (i-ii) and HSC-2 (iii-iv) cell lines (original magnification, x100; scale bar, 100μm). (D) CCL2, CCL5, and IL8 mRNA expression was significantly decreased after Axin2 knockdown in both CA9-22 (i) and HSC-2 (ii) cells (\* *p*<0.05).