Wnt/β-catenin-YAP axis may be involved in primary intraosseous carcinoma, NOS, derived from odontogenic keratocyst

Yusuke Nakako[1,2,8], Kana Hasegawa[1,8], Shinsuke Fujii[1,3,4,*], Yukiko Kami[5], 口腔外科の先生[2], Masafumi Moriyama[6], Kari J Kurppa[4], Kristiina Heikinheimo[7], Kazunori Yoshiura[5], Shintaro Kawano[2] and Tamotsu Kiyoshima[1]

[1]Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[2]Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[3]Dento-craniofacial Development and Regeneration Research Center, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[4]Institute of Biomedicine and MediCity Research Laboratories, University of Turku, and Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, FI-20520, Finland.

[5]Department of Oral and Maxillofacial Radiology, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

[6]Section of Oral and Maxillofacial Surgery, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[7]Department of Oral and Maxillofacial Surgery, Institute of Dentistry, University of Turku and Turku University Hospital, FI-20520, Finland.

[8]These authors contributed equally to this work.

*Corresponding author: Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-l Maidashi, Higashi-ku, Fukuoka 811-8582, Japan.

Phone: +81-92-642-6326

Fax: +81-92-642-6329

E-mail: sfujii@dent.kyushu-u.ac.jp

Key words: Primary intraosseous carcinoma, NOS (PIOC), Mandible, Wnt/β-catenin, YAP, Odontogenic keratocyst

Abstract

Odontogenic tumors (OGTs), which originate from cells of odontogenic apparatus and their remnants, are rare entities. Primary intraosseous carcinoma, NOS (PIOC), is one of the OGTs, but it is even rarer and has a worse prognosis. The characteristics of PIOC, especially in immunohistochemical features and its pathogenesis, remain unclear. We characterized a case of PIOC arising from the left mandible, in which histopathological findings showed a transition from the odontogenic keratocyst to the carcinoma. The tumor lesion of this PIOC exhibited malignant potentials with an invasive growth of carcinoma cells into the bone tissue, an elevated Ki-67 index, and lower signal for CK13 and higher signal for CK17 compared with the non-tumor region, histopathologically and immunohistopathologically. Further immunohistochemical analyses demonstrated increased expression of ADP-ribosylation factor (ARF)-like 4c (ARL4C) (accompanying expression of β-catenin in the nucleus) and yes-associated protein (YAP) in the tumor lesion. On the other hand, YAP was expressed and the expression of ARL4C was hardly detected in the non-tumor region. In addition, quantitative RT-PCR analysis using RNAs and dot blot analysis using genomic DNA showed the activation of Wnt/β-catenin signaling and epigenetic alterations, such as an increase of 5mC levels and a decrease of 5hmC levels, in the tumor lesion. A DNA microarray and gene ontology analysis demonstrated that numerous gene expression variations and signal activation, including Notch signaling, were altered in non-tumor and tumor lesions within this PIOC. Experiments with the GSK-3 inhibitor revealed that β-catenin pathway increased not only mRNA levels of *ankyrin repeat domain1* (*ANKRD1*) but also protein levels of YAP and TAZ in oral squamous cell carcinoma cell lines. These results suggested that further activation of YAP signaling by Wnt/β-catenin signaling may be associated with the

development of PIOC derived from odontogenic keratocyst in which YAP signaling is activated.

1. Introduction

Odontogenic tumors (OGTs), which originate from cells of odontogenic apparatus and their remnants, are rare, constituting < 1% of all oral tumors [1]. Most OGTs are benign, and malignant OGTs are low incidence rate with 0.3-5.7% of all OGTs [2]. Both benign and malignant OGTs are subclassified into epithelial tumors, mixed epithelial and mesenchymal tumors and mesenchymal tumors. In addition, malignant OGTs are classified into odontogenic carcinoma, odontogenic carcinosarcoma and odontogenic sarcoma. Odontogenic carcinoma consists of ameloblastic carcinoma, primary intraosseous carcinoma, NOS (PIOC), sclerosing odontogenic carcinoma, clear cell odontogenic carcinoma and ghost cell odontogenic carcinoma [1].

PIOC, which frequently occurs in the posterior body and ramus of the mandible, cannot be categorized as any other type of carcinoma. Carcinoma arising in the oral mucosa and infiltrating the mandible, an antral primary and metastatic carcinoma must be excluded, and ulceration to the oral cavity is normally considered to preclude definitive diagnosis [1]. PIOC is a rare entity and cases arising in cysts are more common in the mandible [3,4]. In PIOC occurrence, radicular/residual cysts are the most common precursors, followed by dentigerous cysts and odontogenic keratocysts [1]. Radiographically, cases arising in cysts may produce an apparently multilocular or scalloped radiolucency. When the tumor is detected early, the radiological features appear benign one and the carcinoma is an incidental histological finding on enucleation [5,6]. When cases arise in odontogenic cysts, there may be a histological transition between the benign precursor and the carcinoma. Although an insufficient number of cases has been reported to determine outcome, prognosis is generally poor [1]. Several reports have demonstrated that the 5-year survival rate of reported case with PIOC is 52% [5,6].

Therefore, new anticancer therapies based on the molecular mechanisms underlying PIOC tumorigenesis are needed.

We recently reported several activated intracellular signaling molecules, such as ADP-ribosylation factor (ARF)-like 4c (ARL4C) [7-9] and yes-associated protein (YAP) [10,11], may be not only specific markers of oral epithelial tumors but also exerting an oncogenic role in their tumorigenesis. However, the characteristics of PIOC, including its immunohistochemical features, such as ARL4C and YAP expression, are unclear,

In the present report, we conducted a study using a rare case of PIOC pathological specimen, which appeared to be derived from odontogenic keratocyst, and cell lines to elucidate the role of Wnt/β-catenin-YAP axis in PIOC tumorigenesis.

2. Material and methods

2.1. Patients and immunohistochemistry

A 79-year-old female, the case of this person is mentioned below, and total of 5 patients with odontogenic keratocyst diagnosed for treatment at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, Japan from May 2021 to May 2023 were examined. Details for each patient were as follows; #1, 65-year-old, male, mandible; #2, 21-year-old, female, mandible; #3, 40-year-old, female, mandible; #4, 26-year-old, female, maxilla; #5, 75-year-old, female, mandible. The protocol for this study was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#2022-219).

All specimens for histological examination were fixed in 10% (v/v) neutral buffered formalin solution and embedded in paraffin blocks. The paraffin-embedded specimens were sliced into 4-μm-thick sections, stained with HE, and examined by three experienced pathologists to confirm the diagnoses. Immunohistochemical staining was performed on 4-μm-thick paraffin-embedded sections. Antigen retrieval, elimination of the endogenous peroxide activity, and blocking were carried out as previously described [11]. The sections were then incubated with each primary antibody (used at 1:300 for Ki-67, used at 1:100 for YAP, used at 1:100 for ARL4C, used at 1:2000 for CK13, used at 1:100 for CK17, used at 1:400 for β-catenin) at 4 ℃ overnight. The details of the antibodies used are mentioned below. The sections were incubated with secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 1 h at room temperature (RT). The immunoreactivity was visualized with a DAB substrate solution (Nichirei). Subsequently, the sections were counterstained with hematoxylin.

2.2. Cell lines and reagents

Human oral squamous cell carcinoma (OSCC) cell lines HSC-2 and HSC-3 (Japanese Cancer Research Resources Bank) were used in this study. HSC-2 and HSC-3 cells were maintained in α-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen) and contained 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen). All of these cell lines were incubated at 37 ℃ in a 5% CO_2 atmosphere. When necessary, CHIR99021 (FUJIFILM Wako, Osaka, Japan) was added [12].

Anti-Ki-67 (M7240) (for immunohistochemistry), anti-β-catenin (M3539) (for immunohistochemistry) and anti-CK17 (M7046) (for immunohistochemistry) antibodies were obtained from Dako (Carpentaria, CA, USA). Anti-CK13 (ab16112) (for immunohistochemistry) and anti-double strand (ds) DNA (ab27156) (for dot blot) antibody was obtained from Abcam was obtained from Abcam (Cambridge, UK). Anti ARL4C (HPA028927) (for immunohistochemistry) antibody was obtained from Atlas Antibodies (Voltavägen, SWE). Anti-YAP (sc-101199) (for immunohistochemistry) antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-5mC (39649) (for dot blot) and anti-5hmC (39769) (for dot blot) antibodies were obtained from Active Motif (Carlsbad, CA, USA). Anti-β-catenin (610154) (for western blotting) antibody was obtained from BD biosciences (San José, CA, USA). Anti-YAP/TAZ (D24E4) (for western blotting) antibody was obtained from Cell signaling technology (Beverly, MA, USA). Anti-β-actin (A5441) (for western blotting) antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. *RNA extraction, DNA extraction and Quantitative RT-PCR*

The total RNAs were isolated using the SV Total RNA Isolation System (Promega,

Madison, WI, USA), and cDNAs were generated from isolated total RNAs using synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Then, the cDNA was used for quantitative PCR analysis [13]. Forward and reverse primers were as follows: human CK13, 5'-CCAACACTGCCATGATTCAG-3' and 5'-TCTGGCACTCCATCTCACTG-3'; human CK17, 5'-GCTGCTACAGCTTTGGCTCT-3' and 5'-TCACCTCCAGCTCAGTGT-3'; human Axin2, 5'-CTGGCTCCAGAAGATCACAAAG-3' and 5'-CATCCTCCCAGATCTCCTCAAA-3'; human Wnt1, 5'-CGGCGTTTATCTTCGCTATC-3' and 5'-GCCTCGTTGTTGTGAAGGTT-3'; human Wnt2b, 5'-AAGATGGTGCCAACTTCACC-3' and 5'-GCCACAGCACATGATTTCAC-3'; human Wnt3, 5'-ACTTTTGTGAGCCCAACCCA-3' and 5'-TTCTCCGTCCTCGTGTTGTG-3'; human Wnt4, 5'-GCTGTGACAGGACAGTGCAT-3' and 5'-GCCTCATTGTTGTGGAGGTT-3'; human Wnt7a, 5'-CCCACCTTCCTGAAGATCAA-3' and 5'-ACAGCACATGAGGTCACAGC-3'; human Wnt8a, 5'-CCATTGTCTATCCCCATTC-3' and 5'-GTGGGTGGAGAGCTGAAGAG-3'; human Wnt8b, 5'-TCGGAGAGGCGATTTCCAAG-3' and 5'-GTTGTGCAGGTTCATGGCTG-3'; human Wnt10a, 5'-AAGCTGCACCGCTTACAACT-3' and 5'-ATTCTCGCGTGGATGTCTCT-3'; human Wnt10b, 5'-GAAAACCTGAAGCGGAAATG-3' and 5'-GGGTCTCGCTCACAGAAGTC-3'; human Wnt16, 5'-GCTCCTGTGCTGTGAAAACA-3' and 5'-TGCATTCTCTGCCTTGTGTC-3'; human ARL4C, 5'-CTAACATCTCGGCCTTCCAG-3' and 5'-TCTGCTTGAGGGACTTCCTG-3'; human ANKRD1, 5'-

ACGCCAAAGACAGAGAAGGA-3’ and 5’-TTCTGCCAGTGTAGCACCAG-3’; human SNAI2, 5’-CTTTTTCTTGCCCTCACTGC-3’ and 5’-ACAGCAGCCAGATTCCTCAT-3’; human DLX1, 5’-CAAGGCGGTGTTTATGGAGT-3’ and 5’-TGCTGACCGAGTTGACGTAG-3’; human DLX2, 5’-GCACATGGGTTCCTACCAGT-3’ and 5’-TCCTTCTCAGGCTCGTTGTT-3’; human IFT74, 5’-GGAAATAGCCAGCATGGAAA-3’ and 5’-GTGCTCCAAGAGTGCAACAA-3’; human SIX1, 5’-CTCCTCCTCCAACAAGCAGA-3’ and 5’-CTGTTAAGCCCGGGAGAGAA-3’; human GAPDH, 5’-GCACCGTCAAGGCTGAGAAC-3’ and 5’-TGGTGAAGACGCCAGTGGA-3’.

2.4. Plasmid construction and infection using lentivirus harboring a cDNA

The YAP^{5SA} plasmid, CSII-CMV-MCS-IRES2-Bsd/FLAG-YAP^{5SA}, was used [10,14]. The vectors were transfected along with the packaging vectors, pCAG-HIV-gp (RDB04394) and pCMV-VSV-G-RSV-Rev (RDB04393), into X293T cells using the Lipofectamine LTX reagent (Invitrogen) to generate lentiviruses [15]. To generate OSCC cells that stably express YAP^{5SA} parental cells (5×10^4 cells/well in a 12-well plate) were treated with lentivirus and 10 μg/ml polybrene. The cells were then centrifuged at 1080 × g for 1 h, and incubated for another 24 h. The cells that demonstrated stable expression of YAP^{5SA} were selected and maintained in culture medium containing 5 μg/ml Blasticidin S (FUJIFILM Wako)

2.5. Dot blot analysis

Dot blot analysis was performed as previously described [7,11] with modification.

Genomic DNA was diluted to 50 ng/μl in 20 μl total volume. 2.5 μl of 1 M NaOH was added to each sample and then the samples were incubated at 95 ℃ for 5 min. The samples were put on ice and neutralized with 3.3 μl of 5 M ammonium acetate. 2.1 μl and 1.05 μl of each mixture were spotted onto a nitrocellulose membrane and allowed to air dry for 10 min. The membrane was baked for 2 h at 80 ℃ and then blocked with 5% milk for 2 h at RT. Anti-5hmC antibody (1:10000), anti-5mC antibody (1:1000) or anti-ds DNA antibody (1:1000) was incubated at 4 ℃ overnight, and subsequent incubation with goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP for 2 h. ECL Western Blotting Detection Reagents (GE Healthcare, Chicago IL, USA) were used for detection.

2.6. DNA microarray analysis

DNA microarray analysis was performed using RNAs extracted from the tumor lesion and the non-tumor region of PIOC. The raw data reported in this study were deposited in NCBI GEO under the accession number (GSE24459003). Gene Ontology (GO) analysis was performed using the DAVID database (https://david.abcc.ncifcrf.gov/) as previously described [9,16].

2.7. Immunofluorescence staining

HSC-3 and HSC-2 cells were fixed in 4% (w/v) paraformaldehyde buffered by phosphate buffered saline (PBS) for 30 min at RT. After that, cells were permeabilized in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA (FUJIFILM Wako) for 10 min at RT, and then blocked with 1% BSA to prevent non-specific binding for 30 min at RT. The cells were incubated with primary antibody (used at 1:300 for β-catenin) for 3 h at RT, and then with secondary antibody (Jackson) and Hoechst 33342 (Dojindo, Kumamoto,

Japan) for 2 h at RT. The samples were viewed with an All-in-one Fluorescence Microscope BZ 9000 (Keyence, Osaka, Japan) [17,18].

2.8. Immunoprecipitation

Immunoprecipitation was performed as described previously with modification [19]. HSC-3 cells expressing YAP^{5SA} (10 cm diameter dish) were lysed in 400 μl of NP40 buffer (20 mM Tris-HCl pH 8.0, 10% glycerol, 137 mM NaCl, and 1% NP40) with protease inhibitors (2% Protease inhibitor cocktail (Sigma-Aldrich), 1 μM Clasto-Lactacystin β-Lactone (Sigma-Aldrich), 25 mM β-glycerophosphate (Sigma-Aldrich), 1 mM Na_3VO_4 (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (FUJIFILM Wako)) for 10 min on ice. After centrifugation, the supernatant was collected and incubated with antibody for 1 h at 4 °C and further incubated with 40 μl of 50% slurry of protein G Sepharose beads (GE healthcare) for 1 h at 4 °C. Beads were washed three times with 1 ml of NP40 buffer and were dissolved in Laemmli's sample buffer.

2.8. Statistical analysis

Statistical analyses were performed using JMP Pro 16 software. Significant differences were determined using one-way ANOVA with post hoc Tukey's test. P value of < 0.01 was considered to indicate statistical significance.

2.9. Additional assays

Western blotting was performed as described previously [20] and data were representative of at least three independent experiments.

3. Results

3.1. Clinical features and histological findings

A 79-year-old female visited at the Department of Oral and Maxillofacial Surgery at Kyushu University Hospital (Fukuoka, Japan) with exophytic swelling and pain in the left mandible, and fistula on the skin (Fig. 1A; left panel). She had no clinical evidence of cervical lymphadenopathy and no medical history of any significant diseases. An oral examination revealed the bulge of the left mandibular molar area without ulceration. A panoramic radiograph showed multilocular translucent areas (Fig. S1; upper panel). Contrast-enhanced computed tomography (CT) indicated well defined multilocular translucent areas and accumulation of keratin-like material in the cystic lesion (Fig. 1A; right panel and Fig. S1; lower panel). Based on these findings, the lesion was clinically diagnosed as a benign lesion, such as odontogenic keratocyst or ameloblastoma, with no apparent findings with malignant tumors.

A biopsy was performed under local anesthesia. HE-stained sections showed cyst wall-structures lined by stratified squamous epithelium. Palisading of basaloid cells was seen in the lining epithelium and many keratin materials were observed in the cavity (Fig. 1B; left panels), of which histological findings were similar to those of odontogenic keratocyst. In addition, hyperparakeratinized lining epithelia, such as atypical verrucous hyperplasia with advanced stage, and budding-like elongation of rete ridges with atypical epithelial cells into subepithelial layer were included in the lesion (Fig. 1B; right panels). Therefore, the lesion was diagnosed as squamous cell carcinoma, histopathologically. Immunohistochemically, non-tumor region showed moderate Ki-67 index (24.2% at the hot spot) (Fig. 1C; upper left panel). In contrast, its index was high (69.3% at the hot spot) in the tumor lesion (Fig. 1C; upper right panel), which showed a significant difference

(P<0.0001). Non-tumor region and tumor lesion in the Figs. 1B and 1C are consecutive sections. Based on these histological and immunohistochemical findings, the lesion was suspected as PIOC, which would be derived from odontogenic keratocyst.

To profile the tumor lesion, further immunohistochemical analyses were performed. The hippo pathway regulates cell proliferation through the nuclear localization of major downstream effectors, YAP and transcriptional co-activator with PDZ-binding motif (TAZ), resulting in the induction of target gene transcription [21,22]. We have recently demonstrated that YAP signaling regulates OSCC cell proliferation [10]. Indeed, YAP was detected in the nucleus of the atypical epithelial cells in the tumor lesion, suggesting its malignant potential (Fig. 1C; middle right panel). It was surprised that YAP expression was detected in the nucleus of the parabasal and/or basal layer in the non-tumor region (Fig. 1C; middle left panel). Previous report demonstrated that YAP was especially detected in the nucleus of basaloid cells in the pathological specimens with odontogenic keratocyst [23], which was confirmed by our immunohistochemical analyses (Fig. S2; middle panels). These data suggested that the immunohistochemical characteristics of epithelial cells in the non-tumor region might be similar to those of odontogenic keratocyst (Fig. 1C; middle left panel).

It has been reported that ARL4C, the expression of which is upregulated in several tumors and associated with tumorigenesis [8,14], promotes cell proliferation including OSCC [9]. Consistent with these reports, the expression of ARL4C was highly detected in cytoplasm at the invasion front of the tumor lesion but its expression was only seen in the basal cells of the non-tumor region (Fig. 1C; lower panels). The expression of ARL4C in the present non-tumor region appeared to be higher than those of the specimens with odontogenic keratocyst (Fig. S2; right panels), suggesting that the characteristics of

the present non-tumor region might be different from those of odontogenic keratocyst. However, its pathological meanings remained unclear. Based on these immunohistochemical analyses, the tumor lesion may exhibit malignant potential. Therefore, after 1 month later of the biopsy, a surgical resection was carried out and HE-stained section, of which section was indicated as area 3 in the picture, showed an invasive growth of carcinoma cells into the bone tissue (Fig. 1D). Results from the histological and immunohistochemical analyses, the lesion was diagnosed as PIOC, where at least two kinds of oncogene signaling, such as YAP signaling and ARL4C signaling, were activated.

3.2. Characterization of the PIOC

From the surgical resection sample, we collected the tissues from non-tumor region, which is indicated as area 1 in the picture (Fig. 1D; left panels), and from tumor lesion, which is indicated as area 2 in the picture (Fig. 1D; left panels), which were clinically diagnosed and collected by an oral surgeon. We divided each sample into two specimens with or without formalin fixation.

Immunohistochemical analyses showed that the cyst lining epithelial cells were positive for CK13 and negative for CK17 in the non-tumor region (Fig. 2A; upper and middle left panels). In contrast, decreased signal for CK13 and increased signal for CK17 were noted in the tumor lesion (Fig. 2A; upper and middle right panels). RNAs were extracted from the above samples without formalin fixation. Consistent with the immunohistochemical findings, *CK13* mRNA expression was decreased and *CK17* mRNA expression was increased in the tumor lesion and vice versa in non-tumor region (Fig. 2B). As OSCC is characterized by an increase in CK17 and a decrease in CK13 [24-

26], tumor lesion might exhibit malignant potential, and conversely, non-tumor region did not.

Wnt/β-catenin signaling has been reported to be involved in the disease pathogenesis as well as in the developmental processes [27,28]. As no mutations in components of Wnt/β-catenin-dependent signaling have been identified in OSCC pathological specimens and cell line [29-31], the activation of Wnt/β-catenin-dependent pathway would be unlikely to be involved in OSCC tumorigenesis. It is noteworthy that β-catenin was sparsely detected in the nucleus of the tumor lesion (Fig. 2A; lower right panel). In addition, the expression of *Axin2* (a direct target gene of the Wnt/β-catenin pathway [32]) was increased in the lesion (Fig. 2C). Wnt ligand consists of 19 members [28]. Among them, the expression of Wnt ligands, which activates Wnt/β-catenin-dependent signaling [33], such as *Wnt1*, *Wnt2b*, *Wnt3*, *Wnt4*, *Wnt7a*, *Wnt8a*, *Wnt8b*, *Wnt10b* and *Wnt16*, was higher in the tumor lesion than in the non-tumor region (Fig. 2D). Furthermore, DNA methylation status is associated with tumorigenesis [34]. 5-methylcytosine (5mC), which is a marker for global DNA methylation, was increased and 5-hydroxymethylcytosine (5hmC), which is converted from 5mC to promote transcriptional activity [35], was reduced in the tumor lesion (Fig. 2E). These results suggested that Wnt/β-catenin signaling would be focally activated, which depends on sporadic expression of several Wnt ligands, and epigenetic alteration might occur in the PIOC.

To further clarify the characteristics of the present PIOC, a DNA microarray analysis was performed using RNAs, which were used in quantitative RT-PCR (Fig. 2B and C). Fig. 3A shows scatterplots representing differences in gene expression amounts comparing the tumor lesion and the non-tumor region. The expression of *CK17* was

higher and the expression of *CK13* was lower in the tumor lesion (Fig. 3A). These expression patterns were consistent with previous our report [9] and the data with immunohistochemical findings and quantitative RT-PCR analysis (Figs. 2A and 2B), suggesting that the current DNA microarray data would reflect genetic background in the present PIOC. Next, we carried out an enrichment analysis of upregulated genes in the tumor lesion using the DAVID database. As we recently identified the target molecules and signaling using the DNA microarray analysis based on the criteria of which expression is 1.5 fold change [12,13], we followed these criteria in this study. 2,193 genes were upregulated in the tumor lesions (Fig. 3A). These genes were subjected to a gene ontology (GO) analysis and the result showed that various cellular functions would be altered in the PIOC (Fig. 3B). Among them, Notch signaling pathway was enriched in the tumor lesion (Fig. 3B). Notch signaling pathway is involved in tumorigenesis including OSCC [36,37]. The expression of Notch signaling pathway target genes, such as *SNAI2*, *DLX1*, *DLX2*, *IFT74* and *SIX1*, was indeed upregulated in the tumor lesion by quantitative RT-PCR analysis (Fig. S3). These results suggest that Notch signaling pathway might be activated in the present PIOC.

3.3. Wnt/β-catenin signaling activates YAP signaling in OSCC cells.

In this PIOC, squamous cell carcinoma cells were invaded into the subepithelial layer and the bone tissue (see Fig. 1B; right panels and Fig. 1D). We have recently demonstrated that YAP signaling is activated in OSCC cells [10]. It has been reported that Wnt/β-catenin signaling regulates YAP activity at the protein or transcriptional level [38,39]. Since the effect of Wnt/β-catenin signaling on YAP signaling remains unclear in OSCC, we used OSCC cell lines to investigate its effect. Human OSCC cells, HSC-2 and HSC-3 cells

were treated with the GSK-3 inhibitor, CHIR99021, which is an activator of the β-catenin pathway [19,40]. The protein levels and nuclear translocation of β-catenin were increased by CHIR99021 treatment (Fig. 4A and Fig. S4A). Consistent with previous reports [8,14], treatment with CHIR99021 induced the expression of *ARL4C* (Fig. 4B; left graph and Fig. S4B; left graph). In addition, the treatment increased the mRNA levels of *ankyrin repeat domain1* (*ANKRD1*, a direct target gene of the YAP signaling) (Fig. 4B; right graph and Fig. S4B; right graph), and the protein levels of YAP and TAZ (Fig. 4C and Fig. S4C). Furthermore, consistent with a previous report [38], YAP^{5SA}, in which five possible phosphorylation serine residues are changed to alanine [10], formed a complex with endogenous β-catenin in OSCC cells (Fig. 4D). These results suggested that the β-catenin signaling may promote YAP signaling in OSCC cells.

4. Discussion

PIOC is a diagnosis of exclusion. Therefore, it is required histological, radiographical and clinical information to exclude metastasis, malignant odontogenic tumors of specific types, carcinomas of the maxillary antrum and nasal mucosa, and intraosseous salivary gland neoplasms [1]. Because the histopathological findings demonstrated a transition from the odontogenic keratocyst to the carcinoma, this case was diagnosed as PIOC together with clinical findings. In the present case, retrospective radiographical features showed no findings suspicious for malignancy. Considering the current result, even in cystic cases in which malignancy is not suspected clinically, it is necessary to perform routine biopsy and pathological examination.

The immunohistochemical findings, quantitative RT-PCR data and a DNA microarray analysis revealed that several oncogene-related pathways, such as Wnt/β-catenin signaling (accompanying increased expression of ARL4C), YAP signaling and Notch signaling, were activated in the present PIOC. It is generally accepted that tumor cell genomes are hypomethylated relative to non-tumor counterparts [41]. Additionally, several reports demonstrated that alterations in DNA methylation occur in cancer, including hypermethylation of tumor suppressor genes and hypomethylation of oncogenes in non-small cell lung cancer [42-47]. Consistent with these reports, epigenetic alterations, such as an increase of 5mC levels and a decrease of 5hmC levels (see Fig. 2E), were occurred in this PIOC. At present, there is no direct data to conclude which one is necessary in PIOC tumorigenesis. Since it has been reported that there is no activation of Wnt/β-catenin signaling in OSCC tumorigenesis [29-31], we hypothesized that its activation as in this case may be related to the rarity of PIOC. Further studies should

assess the association of these signal transduction pathways and/or epigenetic changes to PIOC tumorigenesis using large PIOC patient cohort.

Reportedly, Wnt/β-catenin signaling promotes YAP activity at the protein or transcriptional level [38,39]. Our present data demonstrated that Wnt/β-catenin signaling could induce YAP activity at protein level, which might be regulated through the protein association of β-catenin and YAP (see Fig. 4C and D), because CHIR99021 treatment did not affect mRNA level of *YAP* in OSCC cells (data not shown).

Odontogenic keratocyst is a developmental cyst derived from odontogenic epithelium, but is previously classified as neoplastic lesions based on their potential aggressive and infiltrative behavior [48]. This neoplastic-like potentials of odontogenic keratocyst may be supported by the frequent YAP expression and its nuclear localization with high positive rate of Ki-67 staining (see Fig. S2). It is intriguing to speculate that YAP signaling is activated in odontogenic keratocyst, and the activation of Wnt/β-catenin signaling may further activate YAP signaling, which may be involved in the development of this PIOC. In contrast, a recent report showed that Wnt/β-catenin signaling suppress YAP signaling, which would be mildly activated, in odontogenic epithelial cells [12]. In this study, we used OSCC cells to investigate the relationship between Wnt/β-catenin signaling and YAP signaling. Since Wnt/β-catenin signaling is inactivated while YAP signaling is hyperactivated in OSCC cells [10,29-31], it is reasonable to use OSCC cells than odontogenic epithelial cells to clarify the etiology of PIOC, at least in a SCC type, derived from odontogenic keratocyst, where YAP signaling is highly activated, immunohistochemically (see Fig. 1C; middle left panel). Further studies are needed to generate an odontogenic keratocyst-derived cell line and verify our hypothesis.

In summary, we characterized a case of PIOC arising from odontogenic keratocyst by immunohistochemical analyses and proposed that further activation of YAP signaling by Wnt/β-catenin signaling may relate to the development of PIOC.

Figure legends

Figure l. Clinical features and histological findings of the primary intraosseous carcinoma, NOS.

(A) A mass in the left mandible was shown in a clinical photograph (left panel). Image of CT (right panel). The box is enlarged image. The white arrows indicate the mass of the left mandible. Asterisks indicate the accumulation of keratin-like material in the cystic lesion. Dotted lines indicate the border between keratin-like materials and cyst fluid. (B) HE staining of the biopsy of the left mandibular molar area with non-tumor region and tumor lesion. The solid box and the dashed box indicate enlarged images. (C) The sections of (B) were stained with anti-Ki-67, YAP and ARL4C antibodies and hematoxylin. Ki-67 index was shown in the upper left. (D) A macroscopic photograph of the surgical resection sample, which were 87 × 59 × 73 mm (left panels). HE staining of the surgical resection sample of area 3 (right panels). The samples of area 1 and area 2 were used in Fig 2A. The solid box and the dashed box indicate enlarged images. Scale bars, 10 mm (D; left panels), 200 μm (B; upper panels and D; upper middle panel), 100 μm (B; middle panels and D; lower middle panel), 20 μm (B; lower panels, C, and D; right panel).

Figure 2. Characterization of the primary intraosseous carcinoma, NOS.

(A) The surgically resected specimens with non-tumor region and tumor lesion (indicated in Fig. 1D) were stained with anti-CK13, CK17 and β-catenin antibodies as well as hematoxylin. (B) *CK13* and *CK17* mRNA levels were measured in non-tumor region and tumor lesion of PIOC. *CK13* and *CK17* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in non-tumor region. (C) *Axin2* mRNA levels were measured in non-tumor region and tumor lesion of PIOC. *Axin2* mRNA levels

were normalized by *GAPDH* and expressed as fold-changes compared with levels in non-tumor region. (D) *Wnt1*, *Wnt2b*, *Wnt3*, *Wnt4*, *Wnt7a*, *Wnt8a*, *Wnt8b*, *Wnt10a, Wnt10b* and *Wnt16* mRNA levels were measured in non-tumor region and tumor lesion. *Wnt1*, *Wnt2b*, *Wnt3*, *Wnt4*, *Wnt7a*, *Wnt8a*, *Wnt8b*, *Wnt10a, Wnt10b* and *Wnt16* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in non-tumor region of PIOC. (E) Analysis of 5mC and 5hmC levels in genomic DNA isolated from non-tumor region and tumor lesion of PIOC was performed by dot blot assay using an anti-5mC and anti-5hmC antibodies. Anti-ds DNA antibody was probed as a control. Scale bars, 20 μm. Results are shown as means ± s.d. of three independent experiments. $^{**}P<0.01$.

Figure 3. The characteristics of the primary intraosseous carcinoma, NOS by a DNA microarray analysis.

(A) Scatterplots representing differences in gene expression amounts comparing the tumor lesion and the non-tumor region of PIOC. (B) Gene enrichment analysis of the upregulated genes in the tumor lesion for gene ontology biological processes was carried out.

Figure 4. Wnt/β-catenin signaling activates YAP signaling in oral squamous cell carcinoma cells.

(A-C) HSC-3 cells were cultured with or without 5 μM CHIR99021 for 24h. (A) Cell lysates were probed with anti-β-catenin and anti-β-actin antibodies (left panels). After the culture, the cells were stained with anti-β-catenin (Green) and Hoechst 33342, and then cells in which β-catenin localizes to the nucleus and Hoechst 33342-stained cells were

counted, respectively (right panels and graph). (B) *ARL4C* and *ANKRD1* mRNA levels were measured by quantitative RT-PCR. Relative *ARL4C* and *ANKRD1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. (C) Cell lysates were probed with anti-YAP/TAZ and anti-β-actin antibodies. (D) Lysates of HSC-3 cells expressing YAP^{5SA} were immunoprecipitated with control IgG or anti-FLAG antibody and the immunoprecipitates were probed with the β-catenin antibody. Scale bars, 20 μm. Results are shown as means ± s.d. of three independent experiments. $^{**}P<0.01$.

Funding: This work was supported by JSPS KAKENHI Grants to S.F. (2023-2025) (JP22KK0262), K.H. (2021-2023) (JP21K09843), and T.K. (2023-2026) (JP23H03102), and Takeda Science Foundation, The Shinnihon Foundation of Advanced Medical Treatment Research, The Mochida Memorial Foundation for Medical and Pharmaceutical Research, TERUMO LIFE SCIENCE FOUNDATION and The Ube Industries Foundationto S.F., Takeda Science Foundation and Fukuoka Public Health Promotion Organization Cancer Research Fund and Kaibara Morikazu Medical Science Promotion Foundation to K.H., and Maritza and Reino Salonen Foundation to K.H..

Conflict of Interest Disclosures: None of the authors have any relevant financial relationship(s) with a commercial interest.

Author contributions

Yusuke Nakako: Investigation, Writing - Original Draft.: **Kana Hasegawa:** Investigation, Writing - Original Draft, Funding acquisition.: **Shinsuke Fujii:** Conceptualization, Methodology, Writing - Review & Editing, Funding acquisition.: **Yukiko Kami, Kari J Kurppa, Kristiina Heikinheimo, Kazunori Yoshiura:** Supervision.: 口腔外科の先生**, Masafumi Moriyama, Shintaro Kawano:** Resources.: **Tamotsu Kiyoshima:** Project administration, Writing - Review & Editing, Funding acquisition. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgments

The authors thank, Drs. S. Nakamura, H. Wada, TTK. Truong and T. Fujimoto and Mr H. Otoba, for valuable technical support in this research. We appreciate the technical

assistance from The Joint Use Laboratories, Faculty of Dental Science, Kyushu University.

References

[1] A.K. El-Nagger, J.K.C. Chan, J.R. Grandis, T. Takata, P.J. WHO classification of odontogenic and maxillofacial bone tumours. WHO classification of Head and Neck Tumours, 4th ed., IARC press, Lyon, 2017, pp. 204-209.

[2] Marin C, Dave M, Hunter KD, Malignant Odontogenic Tumours: A Systematic Review of Cases Reported in Literature, Front Oral Health, 2 (2021), 775707, https://doi.org/10.3389/froh.2021.775707.

[3] Bodner L, Manor E, Shear M, van der Waal I, Primary intraosseous squamous cell carcinoma arising in an odontogenic cyst: a clinicopathologic analysis of 116 reported cases, J Oral Pathol Med, 40 (2011), 733-8, https://doi.org/10.1111/j.1600-0714.2011.01058.x

[4] de Morais EF, Carlan LM, de Farias Morais HG, Pinheiro JC, Martins HDD, Barboza CAG, de Almeida Freitas R, Primary Intraosseous Squamous Cell Carcinoma Involving the Jaw Bones, A Systematic Review and Update. Head Neck Pathol, 15 (2021), 608-16, https://doi.org/10.1007/s12105-020-01234-z.

[5] Keszler A, Piloni MJ, Malignant transformation in odontogenic keratocysts. Case report, Med Oral, 7 (2002), 331-5.

[6] Makowski GJ, McGuff S, Van Sickels JE, Squamous cell carcinoma in a maxillary odontogenic keratocyst, J Oral Maxillofac Surg, 59 (2001), 76-80, https://doi.org/10.1053/joms.2001.19297.

[7] Fujii S, Shinjo K, Matsumoto S, Harada T, Nojima S, Sato S, Usami Y, Toyosawa S, Morii E, Kondo Y, Kikuchi A, Epigenetic upregulation of ARL4C, due to DNA hypomethylation in the 3'-untranslated region, promotes tumorigenesis of lung squamous cell carcinoma, Oncotarget, 7 (2016), 81571-87, https://doi.org/10.18632/oncotarget.13147.

[8] Fujii S, Ishibashi T, Kokura M, Fujimoto T, Matsumoto S, Shidara S, Kurppa KJ, Pape J, Caton J, Morgan PR, Heikinheimo K, Kikuchi A, Jimi E, Kiyoshima T, RAF1-MEK/ERK pathway-dependent ARL4C expression promotes ameloblastoma cell proliferation and osteoclast formation, J Pathol, 256 (2022), 119-33, https://doi.org/10.1002/path.5814.

[9] Alkhatib DZR, Thi Kim Truong T, Fujii S, Hasegawa K, Nagano R, Tajiri Y, Kiyoshima T, Stepwise activation of p63 and the MEK/ERK pathway induces the expression of ARL4C to promote oral squamous cell carcinoma cell proliferation, Pathol Res Pract, 246 (2023), 154493, https://doi.org/10.1016/j.prp.2023.154493.

[10] Hasegawa K, Fujii S, Matsumoto S, Tajiri Y, Kikuchi A, Kiyoshima T, YAP signaling induces PIEZO1 to promote oral squamous cell carcinoma cell proliferation, J Pathol,

253 (2021), 80-93, https//doi.org/10.1002/path.5553.
[11] Hasegawa K, Fujii S, Kurppa KJ, Maehara T, Oobu K, Nakamura S, Kiyoshima T, Clear cell squamous cell carcinoma of the tongue exhibits characteristics as an undifferentiated squamous cell carcinoma, Pathol Res Pract, 235 (2022), 153909, https://doi.org/10.1016/j.prp.2022.153909.
[12] Nagano R, Fujii S, Hasegawa K, Maeda H, Kiyoshima T, Wnt signaling promotes tooth germ development through YAP1-TGF-β signaling, Biochem Biophys Res Commun, 630 (2022), 64-70, https://doi.org/10.1016/j.bbrc.2022.09.012
[13] Fujii S, Nagata K, Matsumoto S, Kohashi KI, Kikuchi A, Oda Y, Kiyoshima T, Wada N, Wnt/β-catenin signaling, which is activated in odontomas, reduces Sema3A expression to regulate odontogenic epithelial cell proliferation and tooth germ development, Sci Rep, 9 (2019), 4257, https://doi.org/10.1038/s41598-019-39686-1.
[14] Fujii S, Matsumoto S, Nojima S, Morii E, Kikuchi A, Arl4c expression in colorectal and lung cancers promotes tumorigenesis and may represent a novel therapeutic target, Oncogene, 34 (2015), 4834-44, https://doi.org/10.1038/onc.2014.402.
[15] Fujii S, Tajiri Y, Hasegawa K, Matsumoto S, Yoshimoto RU, Wada H, Kishida S, Kido MA, Yoshikawa H, Ozeki S, Kiyoshima T, The TRPV4-AKT axis promotes oral squamous cell carcinoma cell proliferation via CaMKII activation. Lab Invest, 100 (2020), 311-23, https://doi.org/10.1038/s41374-019-0357-z.
[16] Truong TTK, Fujii S, Nagano R, Hasegawa K, Kokura M, Chiba Y, Yoshizaki K, Fukumoto S, Kiyoshima T, Arl4c is involved in tooth germ development through osteoblastic/ameloblastic differentiation, Biochem Biophys Res Commun, 679 (2023), 167-74, https://doi.org/10.1016/j.bbrc.2023.09.014.
[17] Mikami Y, Fujii S, Kohashi KI, Yamada Y, Moriyama M, Kawano S, Nakamura S, Oda Y, Kiyoshima T, Low-grade myofibroblastic sarcoma arising in the tip of the tongue with intravascular invasion: A case report, Oncol Lett, 16 (2018,), 3889-94, https://doi.org/10.3892/ol.2018.9115.
[18] Fujii S, Fujimoto T, Hasegawa K, Nagano R, Ishibashi T, Kurppa KJ, Mikami Y, Kokura M, Tajiri Y, Kibe T, Wada H, Wada N, Kishida S, Higuchi Y, Kiyoshima T, The Semaphorin 3A-AKT axis-mediated cell proliferation in salivary gland morphogenesis and adenoid cystic carcinoma pathogenesis, Pathol Res Pract, 236 (2022), 153991, https://doi.org/10.1016/j.prp.2022.153991.
[19] Matsumoto S, Fujii S, Sato A, Ibuka S, Kagawa Y, Ishii M, Kikuchi A, A combination of Wnt and growth factor signaling induces Arl4c expression to form epithelial tubular structures, EMBO J, 33 (2014), 702-18,

https://doi.org/10.1002/embj.201386942.

[20] Kimura H, Fumoto K, Shojima K, Nojima S, Osugi Y, Tomihara H, Eguchi H, Shintani Y, Endo H, Inoue M, Doki Y, Okumura M, Morii E, Kikuchi A, CKAP4 is a Dickkopf1 receptor and is involved in tumor progression, J Clin Invest, 126 (2016), 2689-705, https://doi.org/10.1172/JCI84658.

[21] Saucedo LJ, Edgar BA, Filling out the Hippo pathway, Nat Rev Mol Cell Biol, 8 (2007), 613-21, https://doi.org/10.1038/nrm2221.

[22] Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S, Role of YAP/TAZ in mechanotransduction, Nature, 474 (2011), 179-83. https://doi.org/10.1038/nature10137.

[23] Man QW, Ma YQ, Liu JY, Zhao Y, Liu B, Zhao YF, Expression of YAP/TAZ in Keratocystic Odontogenic Tumors and Its Possible Association with Proliferative Behavior, Biomed Res Int, (2017), 4624890, https://doi.org/10.1155/2017/4624890.

[24] Mikami T, Cheng J, Maruyama S, Kobayashi T, Funayama A, Yamazaki M, Adeola HA, Wu L, Shingaki S, Saito C, Saku T, Emergence of keratin 17 vs. loss of keratin 13: their reciprocal immunohistochemical profiles in oral carcinoma in situ, Oral Oncol, 47 (2011), 497-503, https://doi.org/10.1016/j.oraloncology.2011.03.015.

[25] Khanom R, Nguyen CT, Kayamori K, Zhao X, Morita K, Miki Y, Katsube K, Yamaguchi A, Sakamoto K, Keratin 17 Is Induced in Oral Cancer and Facilitates Tumor Growth, PLoS One, 11 (2016), e0161163, https://doi.org/10.1371/journal.pone.0161163.

[26] Mikami Y, Fujii S, Nagata K, Wada H, Hasegawa K, Abe M, Yoshimoto RU, Kawano S, Nakamura S, Kiyoshima T, GLI-mediated Keratin 17 expression promotes tumor cell growth through the anti-apoptotic function in oral squamous cell carcinomas. J Cancer Res Clin Oncol, 143 (2017), 1381-93, https://doi.org/10.1007/s00432-017-2398-2.

[27] Nusse R, Clevers H, Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities, Cell, 169 (2017), 985-99, https://doi.org/10.1016/j.cell.2017.05.016.

[28] Fujii S, Kiyoshima T, The role of Wnt, ARL4C, and Sema3A in developmental process and disease pathogenesis, Pathol Int, 73 (2023), 217-33, https://doi.org/10.1111/pin.13325.

[29] Iwai S, Katagiri W, Kong C, Amekawa S, Nakazawa M, Yura Y, Mutations of the APC, beta-catenin, and axin 1 genes and cytoplasmic accumulation of beta-catenin in oral squamous cell carcinoma, J Cancer Res Clin Oncol, 131 (2005), 773-82, https://doi.org/10.1007/s00432-005-0027-y.

[30] Tsuchiya R, Yamamoto G, Nagoshi Y, Aida T, Irie T, Tachikawa T, Expression of adenomatous polyposis coli (APC) in tumorigenesis of human oral squamous cell carcinoma, Oral Oncol, 40 (2004), 932-40, https://doi.org/10.1016/j.oraloncology.2004.04.011.

[31] Yeh KT, Chang JG, Lin TH, Wang YF, Chang JY, Shih MC, Lin CC, Correlation between protein expression and epigenetic and mutation changes of Wnt pathway-related genes in oral cancer, Int J Oncol, 23 (2003), 1001-7.

[32] Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F, Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway, Mol Cell Biol, 22 (2002), 1172-83, https://doi.org/10.1128/MCB.22.4.1172-1183.2002.

[33] Miller JR, The Wnts, Genome Biol, 3 (2002), Reviews3001, https://doi.org/10.1186/gb-2001-3-1-reviews3001.

[34] Song CX, He C, Balance of DNA methylation and demethylation in cancer development, Genome Biol, 13 (2012), 173, https://doi.org/10.1186/gb-2012-13-10-2012.

[35] Meng H, Cao Y, Qin J, Song X, Zhang Q, Shi Y, Cao L, DNA methylation, its mediators and genome integrity, Int J Biol Sci, 11 (2015), 604-17, https://doi.org/10.7150/ijbs.11218.10.7150/ijbs.11218.

[36] Sethi N, Kang Y, Notch signalling in cancer progression and bone metastasis, Br J Cancer, 105 (2011), 1805-10, https://doi.org/10.1038/bjc.2011.497.

[37] Sakamoto K, Notch signaling in oral squamous neoplasia, Pathol Int, 66 (2016), 609-17, https://doi.org/10.1111/pin.12461.

[38] Liu T, Zhou L, Yang K, Iwasawa K, Kadekaro AL, Takebe T, Andl T, Zhang Y, The β-catenin/YAP signaling axis is a key regulator of melanoma-associated fibroblasts, Signal Transduct Target Ther, 4 (2019), 63, https://doi.org/10.1038/s41392-019-0100-7.

[39] Konsavage WM, Jr., Kyler SL, Rennoll SA, Jin G, Yochum GS, Wnt/β-catenin signaling regulates Yes-associated protein (YAP) gene expression in colorectal carcinoma cells, J Biol Chem, 287 (2012), 11730-9, https://doi.org/10.1074/jbc.M111.327767.

[40] Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond ME, Harrison SD, Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo, Diabetes, 52 (2003), 588-95, https://doi.org/10.2337/diabetes.52.3.588.

[41] Brown R, Strathdee G, Epigenomics and epigenetic therapy of cancer, Trends Mol Med, 8 (2002), S43-8, https://doi.org/10.1016/s1471-4914(02)02314-6.

[42] Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG, Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer, Hum Mol Genet, 10 (2001), 687-92, https://doi.org/10.1093/hmg/10.7.687.

[43] Kim H, Kwon YM, Kim JS, Han J, Shim YM, Park J, Kim DH, Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer, Cancer, 107 (2006), 1042-9, https://doi.org/10.1002/cncr.22087.

[44] Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM, MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B, Proc Natl Acad Sci U S A, 104 (2007), 15805-10, https://doi.org/10.1073/pnas.0707628104.

[45] Lin RK, Hsu HS, Chang JW, Chen CY, Chen JT, Wang YC, Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer, Lung Cancer, 55 (2007), 205-13, https://doi.org/10.1016/j.lungcan.2006.10.022.

[46] Lin RK, Wu CY, Chang JW, Juan LJ, Hsu HS, Chen CY, Lu YY, Tang YA, Yang YC, Yang PC, Wang YC, Dysregulation of p53/Sp1 control leads to DNA methyltransferase-1 overexpression in lung cancer, Cancer Res, 70 (2010), 5807-17, https://doi.org/10.1158/0008-5472.CAN-09-4161.

[47] Lin SH, Wang J, Saintigny P, Wu CC, Giri U, Zhang J, Menju T, Diao L, Byers L, Weinstein JN, Coombes KR, Girard L, Komaki R, Wistuba, II, Date H, Minna JD, Heymach JV, Genes suppressed by DNA methylation in non-small cell lung cancer reveal the epigenetics of epithelial-mesenchymal transition, BMC Genomics, 15 (2014), 1079, https://doi.org/10.1186/1471-2164-15-1079.

[48] Leon Barnes, John W. Eveson, Peter Reichart, David Sidransky. Odontogenic tumours. WHO classification of Head and Neck Tumours., IARC press, Lyon, 2005, pp. 306-307.

Supplementary Information

Wnt/β-catenin-YAP axis may be involved in primary intraosseous carcinoma, NOS, derived from odontogenic keratocyst

Yusuke Nakako[1,2,8], Kana Hasegawa[1,8], Shinsuke Fujii[1,3,4,*], Yukiko Kami[5], 口腔外科の先生[2], Masafumi Moriyama[6], Kari J Kurppa[4], Kristiina Heikinheimo[7], Kazunori Yoshiura[5], Shintaro Kawano[2] and Tamotsu Kiyoshima[1]

[1]Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[2]Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[3]Dento-craniofacial Development and Regeneration Research Center, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[4]Institute of Biomedicine and MediCity Research Laboratories, University of Turku, and Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, FI-20520, Finland.

[5]Department of Oral and Maxillofacial Radiology, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

[6]Section of Oral and Maxillofacial Surgery, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[7]Department of Oral and Maxillofacial Surgery, Institute of Dentistry, University of Turku and Turku University Hospital, FI-20520, Finland.

[8]These authors contributed equally to this work.

*Corresponding author: Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-l Maidashi, Higashi-ku, Fukuoka 811-8582, Japan.

Phone: +81-92-642-6326

Fax: +81-92-642-6329

E-mail: sfujii@dent.kyushu-u.ac.jp

Key words: Primary intraosseous carcinoma, NOS (PIOC), Mandible, Wnt/β-catenin, YAP, odontogenic keratocyst

Supplementary Figure 1. Radiograph images and histological findings of the primary intraosseous carcinoma, NOS.

A panoramic radiograph image (upper panel). Image of contrast-enhanced CT (lower panel). The box is enlarged image. The white arrows indicate the mass of the left mandible. Asterisk indicates the accumulation of keratin-like material in the cystic lesion. Dotted line indicates the border between keratin-like materials and cyst fluid.

Supplementary Figure 2. Characterization of the odontogenic keratocyst by an immunohistochemical analysis.

Odontogenic keratocyst tissues (n = 5) were stained with anti-Ki-67, YAP and ARL4C antibodies and hematoxylin. Ki-67 index was shown in the upper left. Scale bars, 20 μm.

Supplementary Figure 3. The expression of Notch signaling pathway target genes in the primary intraosseous carcinoma, NOS.

SNAI2, DLX1, DLX2, IFT74 and *SIX1* mRNA levels were measured in non-tumor region and tumor lesion of PIOC. *SNAI2, DLX1, DLX2, IFT74* and *SIX1* mRNA levels (white column) were normalized by *GAPDH* (black column) and expressed as fold-changes compared with levels in non-tumor region. Results are shown as means ± s.d. of three independent experiments. *P<0.05, **P<0.01.

Supplementary Figure 4.

(A-C) HSC-2 cells were cultured with or without 5 μM CHIR99021 for 24 h. (A) Cell lysates were probed with anti-β-catenin and anti-β-actin antibodies (left panels). After the culture, the cells were stained with anti-β-catenin (Green) and Hoechst 33342, and then cells in which β-catenin localizes to the nucleus and Hoechst 33342-stained cells were

counted, respectively (right panels and graph). (B) *ARL4C* and *ANKRD1* mRNA levels were measured by quantitative RT-PCR. Relative *ARL4C* and *ANKRD1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. (C) Cell lysates were probed with anti-YAP/TAZ and anti-β-actin antibodies. Scale bars, 20 μm. Results are shown as means ± s.d. of three independent experiments. $^{**}P<0.01$.