Wnt/β-catenin-C-kit axis may play a role in adenoid cystic carcinoma prognostication

Fujii, Shinsuke
Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University

Hasegawa, Kana
Laboratory of Oral Pathology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University

Maehara, Takashi
Dento-craniofacial Development and Regeneration Research Center, Faculty of Dental Science, Kyushu University

Kari J. Kurppa
Institute of Biomedicine and MediCity Research Laboratories, University of Turku

他

出版情報：Pathology - Research and Practice. 254, pp.155148-, 2024-02. Elsevier
バージョン：

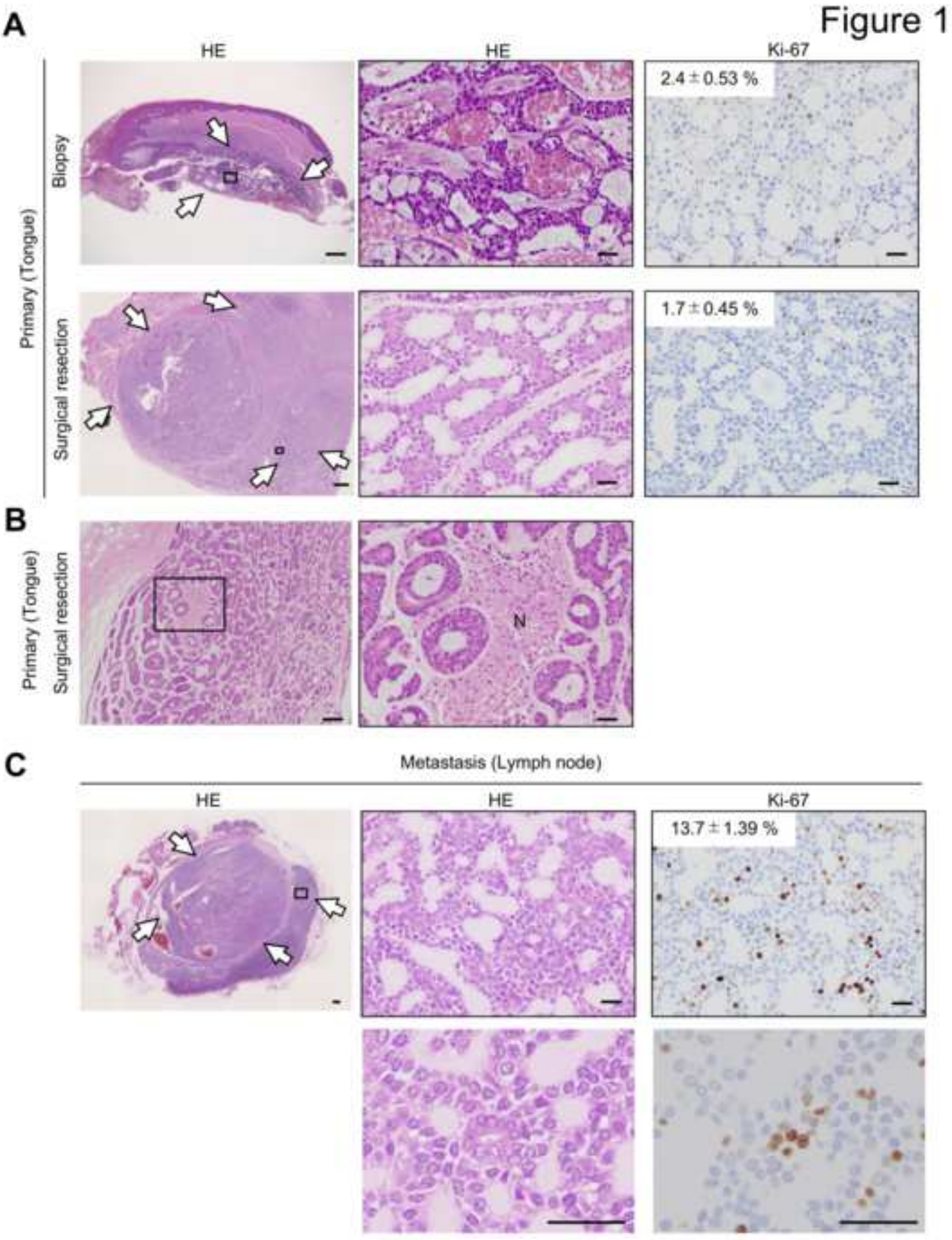


Figure 2

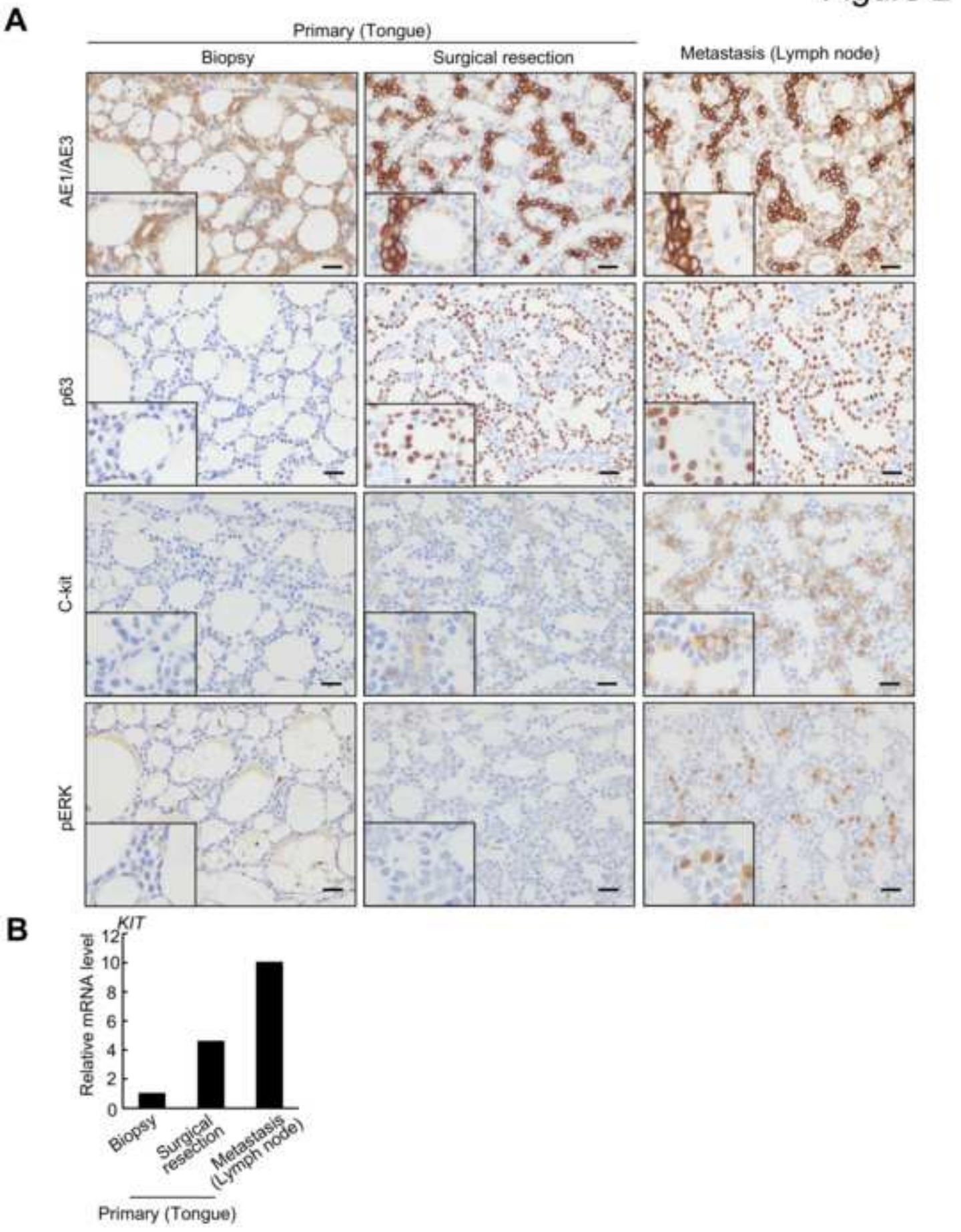


Figure 3

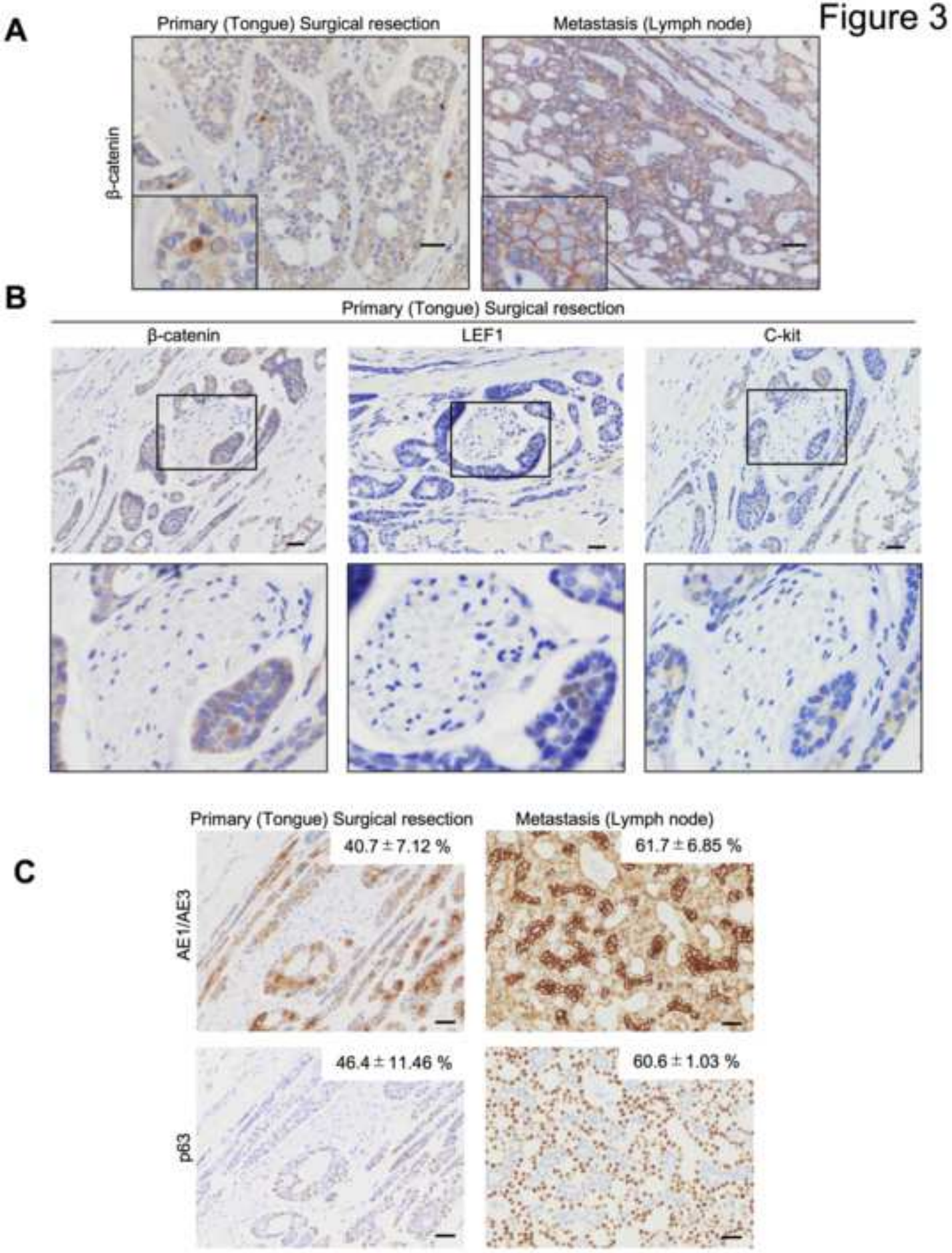
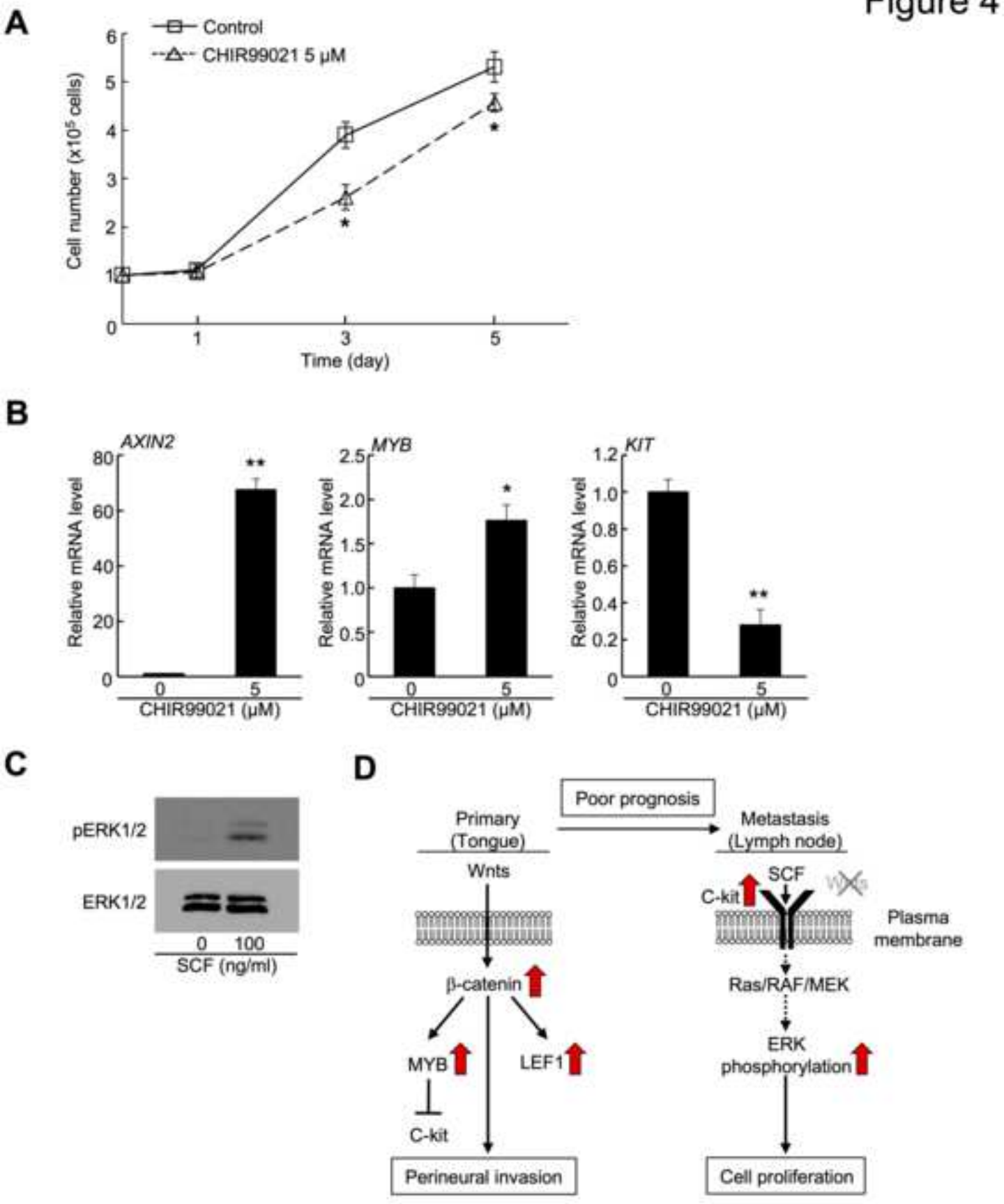


Figure 4



Wnt/β-catenin-C-kit axis may play a role in adenoid cystic carcinoma prognostication

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

Author Contributions

Shinsuke Fujii: Conceptualization, Methodology, Writing - Review & Editing, Funding acquisition.: **Kana Hasegawa:** Investigation, Writing - Original Draft, Funding acquisition.: **Kari J Kurppa, Kristiina Heikinheimo, Yudai Tajiri:** Supervision.: **Takashi Maehara, Kristy A. Warner, Satoshi Maruyama, Jacques E. Nör, Jun-ichi Tanuma, 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, R. Nagano, Y. Nakako, 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.

Wnt/β-catenin-C-kit axis may play a role in adenoid cystic carcinoma prognostication

Shinsuke Fujii[1,2,3,14,*], Kana Hasegawa[1,14], Takashi Maehara[2,4], Kari J Kurppa[3], Kristiina Heikinheimo[5], Kristy A. Warner[6], Satoshi Maruyama[7], Yudai Tajiri[1,8], Jacques E. Nör[6,9,10,11,12], Jun-ichi Tanuma[7,13], Shintaro Kawano[4] 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]Dento-craniofacial Development and Regeneration Research Center, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

[3]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.

[4]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.

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

[6]Department of Cariology, Restorative Sciences, and Endodontics, University of Michigan School of Dentistry, Ann Arbor, MI, USA.

[7]Oral Pathology Section, Department of Surgical Pathology, Niigata University Hospital, Niigata 951-8520, Japan.

[8]Department of Dentistry and Oral Surgery, National Hospital Organization, Fukuokahigashi Medical Center, 1-1-1 Chidori, Koga, Fukuoka 811-3195, Japan.

[9]Department of Otolaryngology-Head & Neck Surgery, University of Michigan School of Medicine, Ann Arbor, MI, USA.

[10]Department of Biomedical Engineering, University of Michigan College of Engineering, Ann Arbor, MI, USA.

[11]University of Michigan Rogel Cancer Center, Ann Arbor, MI, USA.

[12]Department of Otolaryngology-Head & Neck Surgery, University of Michigan School of Medicine, Ann Arbor, MI, USA.

[13]Division of Oral Pathology, Department of Tissue Regeneration and Reconstruction, Faculty of Dentistry & Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8514, Japan.

[14]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: ACC, Tongue, Late metastasis, Wnt/β-catenin, C-kit

Abstract

Adenoid cystic carcinoma (ACC) is one of the most common malignant salivary gland tumors. ACC is composed of myoepithelial and epithelial neoplastic cells which grow slowly and have a tendency for neural invasion. The long term prognosis is still relatively poor. Although several gene abnormalities, such as fusions involving *MYB* or *MYBL1* oncogenes and the transcription factor gene *NFIB*, and overexpression of *KIT* have been reported in ACC, their precise functions in the pathogenesis of ACC remain unclear. We recently demonstrated that the elevated expression of Semaphorin 3A (SEMA3A), specifically expressed in myoepithelial neoplastic cells, might function as a novel oncogene-related molecule to enhance cell proliferation through activated AKT signaling in 9/10 (90%) ACC cases. In the current study, the patient with ACC whose tumor was negative for SEMA3A in the previous study, revisited our hospital with late metastasis of ACC to the cervical lymph node eight years after surgical resection of the primary tumor. We characterized this recurrent ACC, and compared it with the primary ACC using immunohistochemical methods. In the recurrent ACC, the duct lining epithelial cells, not myoepithelial neoplastic cells, showed an elevated Ki-67 index and increased cell membrane expression of C-kit, along with the expression of phosphorylated ERK. Late metastasis ACC specimens were not positive for β-catenin and lymphocyte enhancer binding factor 1 (LEF1), which were detected in the nuclei of perineural infiltrating cells in primary ACC cells. In addition, experiments with the GSK-3 inhibitor revealed that β-catenin pathway suppressed not only *KIT* expression but also proliferation of ACC cells. Moreover, stem cell factor (SCF; also known as KIT ligand, KITL) induced ERK activation in ACC cells. These results suggest that inactivation of Wnt/β-catenin signaling may promote C-kit-ERK signaling and cell proliferation of in metastatic ACC.

1. Introduction

Adenoid cystic carcinoma (ACC) is a slow-growing and relentless salivary gland malignant tumor composed of epithelial and myoepithelial neoplastic cells[1]. Immunohistochemically, C-kit (also known as CD117) is typically restricted to duct lining epithelial cells, and p63 and smooth muscle actin (SMA) to peripheral myoepithelial cells[2]. Those neoplastic cells form various patterns, including tubular, cribriform and solid forms[1]. The annual incidence of ACC is approximately 2 cases per 100,000 population, but ACC exhibits malignant potential, including unpredictable growth, extensive perineural invasion, high rates of metastasis and low survival rates[3, 4]. Since ACC is thought to be an important cancer of submandibular salivary gland (SMG) clinically, given with its malignant potential, new anticancer therapies based on the molecular mechanisms underlying ACC tumorigenesis are needed.

Several gene abnormalities, such as fusions involving *MYB* or *MYBL1* oncogenes and the transcription factor gene *NFIB*, and overexpression of *KIT* and *EGFR*, and their involvement in the etiology and pathogenesis of ACC have been reported[1, 4-6]. We have recently demonstrated that an axonal guidance factor Semaphorin 3A (SEMA3A)[7], is specifically expressed in myoepithelial neoplastic cells and its elevated expression activates AKT signaling, resulting in enhanced cell proliferation of ACC cells[8]. SEMA3A expression was detected in 9/10 (90%) ACC cases. In contrast, the expression of SEMA3A was hardly detected in the specimen derived from one patient, suggesting that other unknown molecular mechanisms may be involved in ACC pathogenesis and malignancy. Eight years after the surgical resection, this patient revisited our hospital with late metastasis of ACC to the cervical lymph node.

It has been suggested that there could be mechanistic similarities between epithelial morphogenesis and carcinogenesis[9]. A recent report showed that Wnt/β-catenin signaling increases the expression of Myb to suppress C-kit expression, regulating the early stage of SMG development[10]. Currently, the effect of Wnt/β-catenin signaling on the expression of C-kit in ACC remains unknown.

In the present report, we characterized a case of ACC with late metastasis to the cervical lymph node using pathological specimens from the metastatic and primary tumors and demonstrated the role of Wnt/β-catenin-C-kit axis in ACC using an ACC cell line.

2. Material and methods

2.1. Immunohistochemistry

The protocol for this study was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#2022-50). All specimens were fixed in 10% (v/v) neutral buffered formalin solution and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 4-μm-thick sections, stained with HE, and examined by 3 experienced pathologists to confirm the diagnoses. Immunohistochemical staining was performed on 4-μm-thick paraffin sections. Antigen retrieval, elimination of the endogenous peroxide activity, and blocking were carried out as previously described[11, 12]. The sections were then incubated with each primary antibody (used at 1:100 for Ki-67, used at 1:5 for AE1/AE3, used at 1:100 for p63, used at 1:400 for C-kit, used at 1:100 for phospho-ERK1/2, used at 1:200 for β-catenin[13], used at 1:100 for LEF1[13], used at 1:100 for phospho-AKT[14], used at 1:300 for SEMA3A[8], used at 1:10 for EMA) at 4°C 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. The immunoreactivity was visualized with a DAB substrate solution (Nichirei). Subsequently, the sections were counterstained with hematoxylin.

2.2. Cell lines, reagents and antibodies

Human ACC cells, UM-HACC-2A[15], and human pleomorphic adenoma (PA) cells, SM-AP1[16], were used in this study. UM-HACC-2A cells were maintained in PriGrow III (Applied Biological Materials Inc. (abm), Richmond, BC, Canada) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 0.4 μg/ml

Hydrocortisone (FUJIFILM Wako, Osaka, Japan), 20 ng/ml EGF (abm), 5 μg/ml insulin (abm) and contained 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen)[15]. SM-AP1 cells were maintained in D-MEM (Invitrogen) with 10% FBS (Invitrogen) and contained 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen)[16]. When necessary, CHIR99021 (FUJIFILM Wako) was added[17].

Anti-Ki-67 (M7240) (for immunohistochemistry), anti-AE1/AE3 (IR053) (for immunohistochemistry), anti-C-kit (A4502) (for immunohistochemistry) and anti-EMA (E29) (for immunohistochemistry) antibodies were obtained from Dako (Carpentaria, CA, USA). Anti- SEMA3A (ab199475) (for immunohistochemistry) and anti-p63 (ab735) (for immunohistochemistry) antibodies were obtained from Abcam (Cambridge, UK). Anti-phospho-ERK1/2 (Thr202/Tyr204) (4370S) (for immunohistochemistry and western blotting), anti-ERK1/2 (4695S) (for western blotting), anti-LEF1 (2230) (for immunohistochemistry) and anti-phospho-AKT (4060S) (for immunohistochemistry) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-catenin (610153) (for immunohistochemistry) and anti-β-actin (A5441) (for western blotting) antibodies were from BD Biosciences (San Jose, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.3. RNA extraction and Quantitative RT-PCR

RNAs were extracted from an ACC pathological formalin fixed paraffin embedded (FFPE) tissue specimen, involving a biopsy and surgical resection of tongue and lymph node, as described previously[18]. Briefly, tumor lesions were identified by pathologists and were taken for RNA extraction by microdissection. RNAs were extracted using RNeasy FFPE Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocols. And

the first-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Then, the cDNA was used for PCR analysis as described previously[13, 19-21]. Forward and reverse primers were as follows: human Kit, 5'-TGCTCTGCTTCTGTACTGCC-3' and 5'-AGGGTGTGGGGATGGATTTG-3'; human Axin2, 5'-CTGGCTCCAGAAGATCACAAAG-3' and 5'-CATCCTCCCAGATCTCCTCAAA-3'; human MYB (for quantitative RT-PCR), 5'-GGACAGCAGACACAGAACCA-3' and 5'-GGAGTGGAGTGGTGTTCTCC-3'; human MYB (exon15), 5'-AATACCCAACTGTTCACGCA-3'; human MYBL1 (exon6), 5'-TCATATGCAAACCCAGAATCAG-3'; human NFIB (exon11), 5'-CCTCACTGGTACTGGGGTAT-3'; human NFIB (exon12), 5'-TGGACATTGGCCGGTAAGAT-3'; human NFIB (3'UTR (untranslated region)): 5'-TCTGCTTGAAGGAAAGGTTCTC-3'; human GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3'.

2.4. Statistical analysis

Significant differences were determined using one-way ANOVA with *post hoc* Tukey test. *P* value of < 0.05 or 0.01 was considered statistically significant.

3. Results

3.1. Clinical features and histological findings

A 71-year-old male visited at the Department of Oral and Maxillofacial Surgery at Kyushu University Hospital (Fukuoka, Japan) with a bite wound 2 year ago and mass showing gradual growth in the right anterior of the tongue. An oral examination revealed that about 20×28-mm tumor mass was hard-elastic and well-circumscribed without induration, and the overlying mucosal surface showed normal texture (Fig. S1A). Based on the examinations, including contrast-enhanced computed tomography (CT), positron emission tomography (PET)-CT and ultrasonography (data not shown), the lesion was clinically diagnosed as a malignant tumor. No distant metastasis, including cervical lymph node, was evident in clinical findings using PET-CT or abdominal ultrasonography (data not shown).

A biopsy was performed. HE-stained sections showed an invasive carcinoma tissue with cribriform pattern and low Ki-67 index (2.4% at the hot spot) (Fig. 1A; upper panels). Based on these histological and immunohistochemical findings, the lesion was diagnosed as an ACC. Two months after the biopsy, a surgical resection was carried out (Fig. S1B) and HE-stained sections showed an invasive growth of carcinoma cells with cribriform and/or strand patterns into the muscular layer as well as subepithelial layer and low Ki-67 index (1.7% at the hot spot) (Fig. 1A; lower panels). Furthermore, perineural invasion of the carcinoma cells was found in the lesion (Fig. 1B) but not in the right lingual nerve (data not shown). The patient underwent a regular follow-up without local recurrence or metastasis.

Eight years after the surgical resection, late metastasis to the left cervical lymph node was suspected in the regular follow-up and detected by ultrasonography and contrast

CT (data not shown). The supraomohyoid neck dissection was performed. HE-stained sections showed tumor lesion with strand and tubular structures in the lymph nodes (Fig. 1C). Duct-like structures with two-cell pattern were noted in the tumor nest and the cancer cells showed high Ki-67 index (13.7% at the hot spot), which was higher than those of the primary tumor biopsy and the surgical resection of the tongue ($P < 0.0001$) (Fig. 1C; upper panels). Notably, Ki-67 positive signals were mainly detected in eosinophilic duct lining cells (Fig. 1C; lower panels). Based on these histological and immunohistochemical findings, the lesion was diagnosed as a recurrence of ACC with late metastasis to the cervical lymph node. PET-CT also indicated a distant metastasis in upper lobe of left lung (data not shown) as well as in the cervical lymph node.

3.2. Characterization of the adenoid cystic carcinoma by an immunohistochemical analysis

We have recently demonstrated that the elevated expression of SEMA3A might function as a novel oncogene-related molecule to activate AKT signaling, resulting in enhanced cell proliferation in ACC[8]. In this report, the SEMA3A expression was detected in the peripheral myoepithelial cells of 9/10 (90%) ACC cases, while its expression was hardly detected in the specimen derived from the current patient. Indeed, the expression of SEMA3A and phosphorylated AKT was hardly detected in any of the specimens - primary tumor biopsy, surgical resection of tongue and lymph node metastasis (Fig. S2A). ACCs have been reported to harbor the key genomic alterations, such as a t(6;9) chromosomal translocation or more rarely a t(8;9) translocation, resulting in fusions involving the *MYB* or *MYBL1* oncogenes and the transcription factor gene *NFIB*[1]. However, the current pathological specimens did not show genomic rearrangements consisting of *MYB-NFIB*

or *MYBL1-NFIB* (*MYB* (exon15)-*NFIB* (exon11), *MYB* (exon15)-*NFIB* (exon12), *MYBL1*(exon6)-*NFIB* (3'UTR) or *MYBL1* (exon12)-*NFIB* (3'UTR)[20, 21] (Fig. S2B). These results indicated that an uncharacterized signaling pathway, except for SEMA3A-AKT signaling or *MYB/MYBL1* activations, may be involved in tumorigenesis as well as metastasis to the lymph nodes.

To clarify the characterization of the current ACC, further immunohistochemical analyses were carried out. The analyses showed that the pseudo-lumen lining epithelial cells were strongly positive for p63, faintly positive for AE1/AE3 and negative for EMA (Fig. 2A and Fig. S3A). Importantly, strong C-kit-positive signals were frequently detected in the cell membrane of AE1/AE3-positive duct lining cells of late metastasis lymph nodes, while its expression was faintly detected in the cytoplasm of tongue surgical resection (Fig. 2A). To compare the expression of *KIT*, RNA was extracted by macrodissection from the pathological specimens[18]. Quantitative RT-PCR analysis demonstrated that the expression of *KIT* in the surgical resection of tongue and lymph nodes was higher than in the primary tumor biopsy (Fig. 2B). Among the specimens, the expression of *KIT* was highest in late lymph node metastasis, which is consistent with the immunohistochemical analyses. Importantly, phosphorylated ERK, the expression of which appeared to be co-localized with Ki-67, was specifically detected in the AE1/AE3-positive duct lining cells of late lymph node metastasis (Fig. 2A and Fig. S3B). These results suggested that higher proliferation of the AE1/AE3-positive duct lining cells of lymph nodes might result from C-kit-ERK signaling.

3.3. The expression of β-catenin in adenoid cystic carcinoma cells

To further profile the ACC, additional immunohistochemical analyses were performed. Wnt/β-catenin signaling has been reported to be involved in the developmental processes, organ homeostasis and disease pathogenesis[22]. Previous reports demonstrated the activation of Wnt/β-catenin in ACC cells, evident as nuclear expression of β-catenin and/or mutations of β-catenin gene (*CTNNB1*)[23, 24], but the precise functions of Wnt/β-catenin pathway in the pathogenesis of ACC are poorly understood.

Immunohistochemically, β-catenin was focally localized in the nucleus of ACC cells in the surgical resection of tongue, but in the cell membrane of ACC cells in the late lymph node metastasis (Fig. 3A). Importantly, the expression of β-catenin and lymphoid enhancer binding factor 1 (LEF1, a target gene of the Wnt/β-catenin pathway[25, 26]) were detected in the perineurally invading cells (Fig. 3B) in the surgical resection of tongue. The ratio of AE1/AE3-positive duct lining cells and p63-positive myoepithelial cells did not show significant differences in perineural invasive lesions and metastatic lesions, respectively (Fig. 3C). These results suggest that Wnt/β-catenin signaling is focally activated in ACC cells of the surgical resection of tongue, while Wnt/β-catenin signaling is inactivated in ACC cells of the late lymph node metastasis. Since Wnt/β-catenin signaling has been reported to be involved in perineural invasion of tumor cells[27, 28], it is possible that activation of Wnt/β-catenin signaling may be involved in perineural invasion of ACC cells.

A recent report demonstrated that Wnt/β-catenin signaling induces the expression of the transcriptional regulator Myb[29] to suppress C-kit expression at the early stage of SMG development[10]. Intriguingly, our current immunohistochemical analysis demonstrated activation of Wnt/β-catenin signaling and low expression of C-kit in the surgical resection of tongue, and conversely, inactivation of Wnt/β-catenin signaling and

high expression of C-kit in the late lymph node metastasis, suggesting that Wnt/β-catenin may regulate C-kit expression in ACC.

3.4. Wnt/β-catenin signaling-dependent expression of KIT is involved in human adenoid cystic carcinoma cell proliferation

To examine the role of Wnt/β-catenin signaling in human ACC cells, UM-HACC-2A cells were treated with the GSK-3 inhibitor, CHIR99021, which is an activator of the β-catenin pathway[30,31]. The treatment suppressed the proliferation of UM-HACC-2A cells (Fig. 4A), suggesting that the β-catenin pathway might function as a negative regulator of ACC cell proliferation.

Consistent with a previous report[10], CHIR99021 dramatically reduced *KIT* expression, but promoted *Axin2* (a direct target gene of the Wnt/β-catenin pathway) and *MYB* expression (Fig. 4B). In contrast, CHIR99021 treatment promoted *Axin2* expression but did not affect the expression of *MYB* and *KIT* in human pleomorphic adenoma cells, SM-AP1 cells[16] (Fig. S4), suggesting that the β-catenin-dependent regulation of MYB/KIT expression is specific to UM-HACC-2A ACC cells. In addition, stem cell factor (SCF; also known as KIT ligand, KITL) induced ERK activation in UM-HACC-2A cells (Fig. 4C), demonstrating that KIT activation can lead to increased activity of ERK in ACC cells. Collectively, these results suggest that Wnt/β-catenin signaling regulates MYB/KIT expression and ACC cell proliferation.

4. Discussion

ACC occurs most frequently in major salivary glands, but more than one third of cases occur in minor salivary glands in the oral cavity, sinonasal tract or rarely other sites[1]. In addition, a recent report showed that most malignant salivary grand tumors of the tongue were located at the base of the tongue[32]. The primary ACC in this report arose from minor salivary glands of the anterior of the tongue (see Fig. S1A), which is an extremely rare entity. Further studies are needed to examine whether the malignancy might differ depending on the site of occurrence. Our present study demonstrated that ACC, which lately metastasized to the cervical lymph node, exhibited malignant potential with increased expression of C-kit, phosphorylation of ERK and an elevated Ki-67 index.

Our immunohistochemical analyses showed that the duct lining epithelial cells in the lymph node metastasis exhibit higher proliferative index, and this was associated with high C-kit expression and ERK activation, suggesting that C-kit may drive the proliferation of these tumor cells (See Fig. S3B). In contrast, in the primary tumor, we observed activation of the Wnt/β-catenin signaling, but low expression of C-kit. The treatment of ACC cells with the Wnt/β-catenin signaling agonist CHIR99021 activated Wnt/β-catenin signaling[10], suppressed the expression of *KIT* and the growth of ACC cells (see Fig. 4). These results suggested that Wnt/β-catenin signaling might control ACC cell proliferation *via* regulation of C-kit. On the other hand, since Wnt/β-catenin signaling is involved in perineural invasion of prostate cancer cells and colorectal cancer cells[27, 28], the activation of Wnt/β-catenin signaling in the primary tumor specimen might be involved in perineural invasion of ACC cells, and thus in the metastatic capacity of ACC. The cell composition of AE1/AE3-positive cells and p63-positive myoepithelial cells shows no significant difference in content in the perineural regions and metastatic lesions,

respectively (see Fig. 3C). On the other hand, the detailed mechanisms of whether Wnt/β-catenin signaling activation in which cell or both cells are required for the perineural invasion are unclear. Although the direct data connecting perineural invasion with lymph node metastasis is lacking at present, perineural invasion could potentially facilitate lymph node metastasis because of the close anatomical location of neural tissues and lymphatic vessels.

As our immunohistochemical analyses showed sparse nuclear expression of β-catenin in the primary tumor cells, it is possible that the sporadic ectopic expression of Wnt ligands might be activated Wnt/β-catenin signaling. Considering present results, the activation of Wnt/β-catenin signaling could play a pivotal role in the pathogenesis of ACC, although it might be difficult to examine the expression of all Wnt ligands, consisting of 19 members[33], in the pathological specimens. It is intriguing to speculate that the evaluation of the amounts of C-kit expression and its expression patterns, such as in cell membrane or in cytoplasm, would be a novel marker to predict the prognosis of ACC. Although the mechanism to regulate the expression pattern of C-kit needs to be clarified, the expression C-kit in cell membrane is thought to be advantages to function as a receptor tyrosine kinase (RTK)[34].

We recently reported that SEMA3A-positive myoepithelial neoplastic cells exhibit high proliferative capabilities through AKT activation[8]. In the current study, C-kit-positive duct lining epithelial cells demonstrated higher cellular growth through ERK activation. Further studies should assess the association of these signal transduction pathways to clinical long-term worse prognosis of ACC using large ACC patient cohort.

In summary, we characterized a case of recurrent ACC with late metastasis to the cervical lymph node 8 years after the surgical resection of tongue primary tumor. We

propose that activation of Wnt/β-catenin signaling and C-kit-ERK signaling might relate to the poor prognosis of ACC (Fig. 4D).

Figure legends

Figure l. Histological findings of the adenoid cystic carcinoma.

(A) HE staining of the biopsy (upper panels) and surgical resection (lower panels) of tongue. The boxes are enlarged images. The white arrows indicate the adenoid cystic carcinoma lesion. These sections were stained with anti-Ki-67 antibody and hematoxylin (right panels). Ki-67 index was shown in the upper left. (B) HE staining of the surgical resection of lymph nodes. The box is enlarged image. N; nerve bundle. (C) HE staining of the late metastasis of lymph nodes. The box is enlarged image. The white arrows indicate the adenoid cystic carcinoma lesion. The section was stained with anti-Ki-67 antibody and hematoxylin (right panels). Ki-67 index was shown in the upper left. Scale bars, 500 μm (A, C; left panels), 50 μm (A, C; middle and right panels, B).

Figure 2. Characterization of the adenoid cystic carcinoma by an immunohistochemical analysis.

(A) The adenoid cystic carcinoma lesion was stained with anti-AE1/AE3, anti-p63, anti-C-kit and anti-phospho-ERK antibodies as well as hematoxylin. Insets are further enlarged images. (B) *KIT* mRNA levels were measured in a biopsy, surgical resection of the tongue and the late metastasis lymph node lesion. *KIT* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in a biopsy. Scale bars, 50 μm.

Figure 3. The expression of β-catenin in adenoid cystic carcinoma cells.

(A) The adenoid cystic carcinoma lesions of tongue and lymph nodes were stained with anti-β-catenin antibody as well as hematoxylin. Insets are further enlarged images. (B)

The adenoid cystic carcinoma lesion in the surgical resection of tongue was stained with anti-β-catenin, anti-LEF1 and anti-C-kit antibodies as well as hematoxylin. The boxes show enlarged images. (C) The adenoid cystic carcinoma lesions of tongue and lymph nodes were stained with anti-AE1/AE3 or anti-p63 antibody as well as hematoxylin. Results are expressed as the percentage of AE1/AE3 or p63 positively stained cells compared with total cells (n = 4,906). Scale bars, 50 μm.

Figure 4. Wnt/β-catenin signaling-dependent expression of KIT is involved in human adenoid cystic carcinoma cell proliferation.

(A) UM-HACC-2A cells were cultured with or without 5 μM CHIR99021 in the presence of 10% FBS for indicated numbers of days, and cell numbers were counted. (B) UM-HACC-2A cells were cultured with or without 5 μM CHIR99021 for 24h. *AXIN2*, *MYB* and *KIT* mRNA levels were measured by quantitative RT-PCR. Relative *AXIN2*, *MYB* and *KIT* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control cells. (C) UM-HACC-2A cells were cultured with or without 100 ng/ml SCF for 24h. Cell lysates were probed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. (D) Schematic model of stepwise activation of Wnt/β-catenin signaling and C-kit-ERK signaling in poor prognosis of ACC. Results are shown as means ± s.d. of three independent experiments. *P<0.05, **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

Shinsuke Fujii: Conceptualization, Methodology, Writing - Review & Editing, Funding acquisition.: **Kana Hasegawa:** Investigation, Writing - Original Draft, Funding acquisition.: **Kari J Kurppa, Kristiina Heikinheimo, Yudai Tajiri:** Supervision.: **Takashi Maehara, Kristy A. Warner, Satoshi Maruyama, Jacques E. Nör, Jun-ichi Tanuma, 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, R. Nagano, Y. Nakako, 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. Slootweg. Tumours of salivary glands, Adenoid cystic carcinoma, WHO classification of Head and Neck Tumours, (fourth ed.), IARC press, Lyon (2017), pp. 164-165

[2] Andreadis D, Epivatianos A, Poulopoulos A, Nomikos A, Papazoglou G, Antoniades D, Barbatis C, Detection of C-KIT (CD117) molecule in benign and malignant salivary gland tumours, Oral Oncol 42 (2006) 57-65, https://doi.org/10.1016/j.oraloncology.2005.06.014

[3] Ellington CL, Goodman M, Kono SA, Grist W, Wadsworth T, Chen AY, Owonikoko T, Ramalingam S, Shin DM, Khuri FR, Beitler JJ, Saba NF, Adenoid cystic carcinoma of the head and neck: Incidence and survival trends based on 1973-2007 Surveillance, Epidemiology, and End Results data, Cancer 118 (2012) 4444-51, https://doi.org/10.1002/cncr.27408

[4] Ho AS, Kannan K, Roy DM, Morris LG, Ganly I, Katabi N, Ramaswami D, Walsh LA, Eng S, Huse JT, Zhang J, Dolgalev I, Huberman K, Heguy A, Viale A, Drobnjak M, Leversha MA, Rice CE, Singh B, Iyer NG, Leemans CR, Bloemena E, Ferris RL, Seethala RR, Gross BE, Liang Y, Sinha R, Peng L, Raphael BJ, Turcan S, Gong Y, Schultz N, Kim S, Chiosea S, Shah JP, Sander C, Lee W, Chan TA, The mutational landscape of adenoid cystic carcinoma, Nat Genet 45 (2013) 791-8, https://doi.org/10.1038/ng.2643

[5] Holst VA, Marshall CE, Moskaluk CA, Frierson HF, Jr., KIT protein expression and analysis of c-kit gene mutation in adenoid cystic carcinoma, Mod Pathol 12 (1999) 956-60

[6] Agulnik M, Cohen EW, Cohen RB, Chen EX, Vokes EE, Hotte SJ, Winquist E, Laurie S, Hayes DN, Dancey JE, Brown S, Pond GR, Lorimer I, Daneshmand M, Ho J, Tsao MS, Siu LL, Phase II study of lapatinib in recurrent or metastatic epidermal growth factor receptor and/or erbB2 expressing adenoid cystic carcinoma and non adenoid cystic carcinoma malignant tumors of the salivary glands, J Clin Oncol 25 (2007) 3978-84, https://doi.org/10.1200/JCO.2007.11.8612

[7] Wright DE, White FA, Gerfen RW, Silos-Santiago I, Snider WD, The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons, J Comp Neurol 361 (1995) 321-33, https://doi.org/10.1002/cne.903610209

[8] 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

[9] Nieto MA, Epithelial plasticity: a common theme in embryonic and cancer cells, Science 342 (2013) 1234850, https://doi.org/10.1126/science.1234850

[10] Matsumoto S, Kurimoto T, Taketo MM, Fujii S, Kikuchi A, The WNT/MYB pathway suppresses KIT expression to control the timing of salivary proacinar differentiation and duct formation, Development 143 (2016) 2311-24, https://doi.org/10.1242/dev.134486

[11] 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

[12] 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

[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, 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

[15] Warner KA, Oklejas AE, Pearson AT, Zhang Z, Wu W, Divi V, Rodriguez-Ramirez C, Castilho RM, Polverini PJ, Nör JE, UM-HACC-2A: MYB-NFIB fusion-positive human adenoid cystic carcinoma cell line, Oral Oncol 87 (2018) 21-8, https://doi.org/10.1016/j.oraloncology.2018.10.012

[16] Maruyama S, Cheng J, Shingaki S, Tamura T, Asakawa S, Minoshima S, Shimizu Y, Shimizu N, Saku T, Establishment and characterization of pleomorphic adenoma cell systems: an in-vitro demonstration of carcinomas arising secondarily from adenomas in the salivary gland, BMC Cancer 9 (2009) 247, https://doi.org/10.1186/1471-2407-9-247

[17] 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

[18] 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

[19] 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

[20] Mitani Y, Liu B, Rao PH, Borra VJ, Zafereo M, Weber RS, Kies M, Lozano G, Futreal PA, Caulin C, El-Naggar AK, Novel MYBL1 Gene Rearrangements with Recurrent MYBL1-NFIB Fusions in Salivary Adenoid Cystic Carcinomas Lacking t(6;9) Translocations, Clin Cancer Res 22 (2016) 725-33, https://doi.org/10.1158/1078-0432.CCR-15-2867-T

[21] McIntyre JB, Ko JJ, Siever J, Chan AMY, Simpson RHW, Hao D, Lau HY, MYB-NFIB gene fusions identified in archival adenoid cystic carcinoma tissue employing NanoString analysis: an exploratory study, Diagn Pathol 14 (2019) 78, https://doi.org/10.1186/s13000-019-0855-8

[22] 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.

[23] Daa T, Kashima K, Kaku N, Suzuki M, Yokoyama S, Mutations in components of the Wnt signaling pathway in adenoid cystic carcinoma, Mod Pathol 17 (2004) 1475-82, https://doi.org/10.1038/modpathol.3800209

[24] Sato M, Yamamoto H, Hatanaka Y, Nishijima T, Jiromaru R, Yasumatsu R, Taguchi K, Masuda M, Nakagawa T, Oda Y, Wnt/β-catenin signal alteration and its diagnostic utility in basal cell adenoma and histologically similar tumors of the salivary gland, Pathol Res Pract 214 (2018) 586-92, https://doi.org/10.1016/j.prp.2017.12.016

[25] Filali M, Cheng N, Abbott D, Leontiev V, Engelhardt JF, Wnt-3A/β-catenin signaling induces transcription from the LEF-1 promoter, J Biol Chem 277 (2002) 33398-410, https://doi.org/10.1074/jbc.M107977200

[26] Hovanes K, Li TW, Munguia JE, Truong T, Milovanovic T, Lawrence Marsh J, Holcombe RF, Waterman ML, β-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer, Nat Genet 28 (2001) 53-7, https://doi.org/10.1038/ng0501-53

[27] Hassounah NB, Nagle R, Saboda K, Roe DJ, Dalkin BL, McDermott KM, Primary

cilia are lost in preinvasive and invasive prostate cancer, PLoS One 8 (2013) e68521, https://doi.org/10.1371/journal.pone.0068521

[28] Terrin L, Agostini M, Ruvoletto M, Martini A, Pucciarelli S, Bedin C, Nitti D, Pontisso P, SerpinB3 upregulates the Cyclooxygenase-2/β-Catenin positive loop in colorectal cancer, Oncotarget 8 (2017) 15732-43, https://doi.org/10.18632/oncotarget.14997

[29] Vandenbark GR, Chen Y, Friday E, Pavlik K, Anthony B, deCastro C, Kaufman RE, Complex regulation of human c-kit transcription by promoter repressors, activators, and specific myb elements, Cell Growth Differ 7 (1996) 1383-92

[30] 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

[31] 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

[32] Goldblatt LI, Ellis GL, Salivary gland tumors of the tongue. Analysis of 55 new cases and review of the literature, Cancer 60 (1987) 74-81, https://doi.org/10.1002/1097-0142(19870701)60:1<74::aid cncr2820600113>3.0.co;2-b

[33] 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

[34] Pathania S, Pentikäinen OT, Singh PK, A holistic view on c-Kit in cancer: Structure, signaling, pathophysiology and its inhibitors, Biochim Biophys Acta Rev Cancer 1876 (2021) 188631, https://doi.org/10.1016/j.bbcan.2021.188631