Clinicopathological significance of genetic alterations in tumor-free surgical margins of oral squamous cell carcinoma

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Clinicopathological significance of genetic alterations in tumor-free surgical margins of oral squamous cell carcinoma

Directed by Professor In-Ho Cha

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ABSTRACT

Clinicopathological significance of genetic alterations in tumor-free surgical margins of oral squamous cell carcinoma

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Considering the epithelial tissue is composed of epithelial cells and stromal tissues, genetic alteration can occur in both epithelial and stromal components. However, the genetic alterations on stromal cells in resection margins of oral squamous cell carcinoma (OSCC) are largely unknown. This study evaluated the alteration of some genetic factors in epithelial cells and the stroma component of resection margins and further investigated the clinicopathological relevance in OSCC patients.

The 202 OSCC patients were included in this study. ARID1A (AT-Rich Interaction Domain 1A), ARID1B (AT-Rich Interaction Domain 1B), α-SMA (alpha-smooth muscle

actin), and IL-6 (Interleukin 6) expression were determined by immunohistochemistry in resection margins of OSCC. The influence of ARID1A and ARID1B expression on the biological behavior of spontaneously immortalized human oral keratinocytes (IHOK) was investigated *in vitro*.

Decreased ARID1A and ARID1B expression in resection margin were found in 47 (23.3%) and 50 (24.8%) OSCC patients, respectively. Kaplan-Meier analysis showed that patients with high-ARID1A/B expression in resection margins showed favorable prognoses in our cohort. Moreover, increased invasion ability and both TNF-α (Tumor Necrosis Factor-alpha) and IL-8 (Interleukin 8) mRNA expression were found in IHOK cells after downregulation of ARID1B. Similarly, increased TNF-α and IL-6 mRNA expression were found in IHOK cells after downregulation of ARID1A. Increased abundance of α-SMA/IL-6 positive fibroblasts was found in 72 (39.1%) and 58 (31.5%) OSCC patients, respectively. Kaplan-Meier analysis showed that patients with a high distribution of α-SMA/IL-6 positive fibroblasts in resection margins showed poor prognosis in our cohort.

In conclusion, this study supports that genetic alterations may occur in both epithelial cells and stroma components in the resection margins of OSCC. Combined factors that can represent both epithelial and stroma changes may need risk assessment for resection margins in OSCC. ARID1A/B, α-SMA/IL-6 positive fibroblasts may contribute to carcinogenesis of resection margins in OSCC, and it can serve as a novel molecular marker for risk assessment of resection margins in OSCC.

Keywords: Oral squamous cell carcinoma, Resection margin, Recurrence, ARID1A, ARID1B, α-SMA/IL-6 positive fibroblasts

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INTRODUCTION

The most widely used treatment option for oral squamous cell carcinoma (OSCC) is surgical resection, and the characteristics of the surgical resection margin in OSCC are thought to be powerful predictors of patient survival and recurrence (Binahmed et al., 2007; Chen et al., 2012; Kurita et al., 2010; Nason et al., 2009; Ow and Myers, 2011). Compared with other cancers, OSCC patients' oral cavity anatomy makes it difficult to archive adequate resection margins. During OSCC surgery, surgeons should provide adequate resection margins and preserve the surrounding oral cavity organs' appropriate anatomical structure and functionality. Consequently, following surgical resection, almost all OSCC

patients have unsatisfactory surgical margins, which will play a significant role in the recurrence of OSCC.

Within the current scope, the guidelines issued by the Royal College of Pathologists (RCPath) for histopathological assessment of resection margins are universally recognized worldwide. According to the definition, margins can be classified as follows: clear = the minimum distance between the tumor and the resection margin is >5mm; close = the minimum distance between the tumor and the resection margin is between 1mm to 5mm; positive = the distance between the tumor and the resection margin is <1mm(Priya et al., 2012). There is still significant controversy regarding the optimal cutoff point between different distances of resection margins in OSCC(Brinkman et al., 2022). For example, the RCPath defines 5 mm as the boundary between close and clear margins, but other authors question the significance of close margins defined under this criterion(Bajwa et al., 2020; Brandwein-Gensler et al., 2005; Solomon et al., 2021; Tasche et al., 2017; Zanoni et al., 2017). The significance of the distance between resection margins and tumors has been effectively determined by the College of American Pathologists (CAP) using values ranging from 3mm to 7mm(Brinkman et al., 2022). The David team found that the boundary between high-risk and low-risk margins is 3 mm(Brinkman et al., 2020). Furthermore, a margin cutoff point between 2 and 3 mm has been reported by different researchers for distinguishing low-risk from high-risk patients(Binahmed et al., 2007; Chiou et al., 2010; Dik et al., 2014; Zanoni et al., 2017). Therefore, using current criteria, predicting the recurrence and prognosis of OSCC patients is challenging.

Recently, researchers discovered a variety of genetic changes in the resection margins of OSCC, and molecular risk factors associated with these margins have drawn considerable interest in OSCC research. This phenomenon, known as "field cancerization," was first identified by Slaughter et al. in 1953 when they discovered histologically changed cells in the tissues around tumors(Slaughter et al., 1953). These days, researchers have discovered that "field cancerization" is caused by a variety of genetic alterations, including aberrant methylations, gene mutations, and amplifications. Even histologically normal cells found next to cancerous tissues have also been shown to have genetic changes(Jelovac et al., 2016; Sorroche et al., 2021; van Houten et al., 2004). Some researchers have drawn attention to the aberrant expression of p53, p16, and epidermal growth factor receptor (EGFR) in histologically normal resection margins of OSCC. Recently, we found that Snail expression in resection margins and axis inhibition protein 2 may have an impact on OSCC recurrence(Pei et al., 2022).

Since epithelial tissue consists of stromal tissues and epithelial cells, genetic modification can affect both the stromal and epithelial components. The genetic alterations on stromal cells in the OSCC resection margins are still mainly unknown. In addition to investigating the clinicopathological significance in OSCC patients, this study investigated alterations in some genetic factors in the stroma and epithelial cells of resection margins.

MATERIALS & METHODS

Patients in the OSCC cohort

A total of 278 patients diagnosed with oral squamous cell carcinoma (OSCC) underwent surgical procedures at the Department of Oral and Maxillofacial Surgery, Dental Hospital, Yonsei University Health System between 2009 and 2018. The study retrospectively reviewed these cases. Out of these, 33 patients were excluded due to inadequate tissue available for analysis, and 43 patients were excluded because of positive resection margins. Tissue samples from surgical resection margins were obtained from 202 patients with OSCC at the Department of Oral Pathology. The Institutional Review Board approved this study for Bioethics of Yonsei University College of Dentistry. Clinicopathologic characteristics are presented in **Table 1**.

Table 1. Clinicopathologic characteristics of 202 OSCC patients

Variables	No. patients (%)
Total cases	202
Age	
Median age (range)	61 (23-85)
≤61	107(53.0)
>61	95(47.0)
Gender	
Male	114(56.4)
Female	88 (43.6)
Lesion site	
Tongue	65 (32.2)
FOM	12 (5.9)

RMT	10 (5.0)
Lower gingiva	50 (24.8)
Upper gingiva	34 (16.8)
Buccal cheek	29 (14.4)
Lip	2 (1.0)
T stage	
T1	56 (27.7)
T2	69 (34.2)
T3	18 (8.9)
T4	59 (29.2)
LN status	
N0	149 (73.8)
N1	15 (7.4)
N2	25 (12.4)
N3	13 (6.4)
Extranodal extension	
Absent	182 (90.1)
Present	20 (9.9)
Histological grade	
WD	31 (15.3)
MD	139 (68.8)
PD	32 (15.8)
Peri-neural invasion	
Absent	183 (90.6)
Present	19 (9.4)
Vascular invasion	
Absent	181 (89.6)
Present	21 (10.4)
Dysplasia in RM	

Absent	145 (71.8)
Present	57 (28.2)
Width of RM	
<1mm	16 (7.9)
≥1mm to <3mm	94 (46.5)
≥3mm to <5mm	69 (34.2)
≥5mm	23 (11.4)

FOM, Floor of mouth; RMT, Retromolar trigone; LN, lymph node; WD, Well differentiated; MD, Moderately differentiated; PD, Poorly differentiated; RM, Resection margin.

Immunohistochemical staining

Immunohistochemistry was performed on 4μm sections of paraffin-embedded tissue samples obtained from resection margins of 202 OSCC patients. ARID1A (Abcam, Cambridge, UK), ARID1B (Abcam), α-SMA (Abcam), and IL-6 (Abcam) antibodies were used as primary antibody in this study. Immunohistochemistry was conducted on 4μm sections of paraffin-embedded tissue samples obtained from resection margins of 202 OSCC patients. We use primary antibodies against ARID1A (Abcam, Cambridge, UK), IL-6 (Abcam), α-SMA (Abcam), and ARID1B (Abcam). A 1:40 ratio of hydrogen peroxide to methanol was used to inhibit endogenous peroxidase activity. Antigen retrieval was achieved by pressure cooking the deparaffinized tissue sections in citrate buffer (pH 6.0, Sigma-Aldrich, Darmstadt, Germany). The secondary antibody was the Real Envision HRP Rabbit/Mouse Detection System (Dako, Glostrup, Denmark). Using 3,3'-diaminobenzidine, immunoreactivity against these antibodies was observed.

The protein expression levels were evaluated using the weighted histoscore method based on the staining intensity and the percentage of positive cells(Zhang et al., 2014). There were four categories for staining intensity: 0 (negative), 1 (light brown), 2 (brown), and 3 (dark brown). The formula used to calculate the final histoscore is: (0 × percentage of negative cells) + (1 × percentage of light brown staining cells) + (2 × percentage of brown staining cells) + (3 × percentage of dark brown staining cells). Patients were split into two groups based on their histoscores: low expression (histoscore 0-100) and high expression (histoscore 101-300).

Cell lines and cell culture

A spontaneously immortalized human keratinocyte line derived from oral mucosa (IHOK) and cultured in a medium composed of Dulbecco's modified Eagles medium (DMEM; Gibco BRL, USA) and Ham's nutrient mixture F-12 (Ham's F12; Gibco BRL, USA) at a ratio of 3:1. The medium used in this study was supplemented with the following components: 10% Tet-approved FBS (HyClone Laboratories, Inc., Logan, UT, USA), 1% penicillin/streptomycin (Sigma-Aldrich), 0.01 μg/ml cholera toxin (Sigma-Aldrich), 0.04 μg/ml hydrocortisone (Sigma-Aldrich), 0.5 μg/ml insulin, 0.5 μg/ml apo-transferrin (Sigma-Aldrich), and 0.2 μg/ml 3'-5-triiodo-1-thyronine (Sigma-Aldrich).

Small interfering RNA (siRNA) transfection

Three independent pools of small interfering RNA (siRNA) oligonucleotides targeting ARID1A/B were utilized to silence ARID1A/B expression. Various numbers of cells were suspended in antibiotic-free culture media and seeded into plates. The cell density was maintained at 30-50% confluence at the time of transfection. Stealth siRNAs (Bioneer, Oakland, CA, USA) targeting ARID1A/B were combined with Opti-MEM medium (Invitrogen, Carlsbad, CA, USA). Subsequently, this mixture was mixed with an equal volume of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium. After a 20-minute incubation period, the final mixture was added to each well of the plate to achieve a final siRNA concentration of 2 to 100 nM.

Total RNA extraction and reverse transcription PCR analysis

Using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from OSCC cell lines. After cDNA synthesis, 1X SYBR-Green Master Mix (Applied Biosystems) with 10 pmol of each primer and 2 μl of cDNA was utilized for quantitative real-time PCR analysis using a Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The PCR was run with the following parameters: 40 cycles of amplification, 20 seconds at 95°C, 30 seconds at 50°C, and 45 seconds at 72°C, were conducted after a 10-minute initial denaturation phase at 95°C. A list of the specific oligonucleotide primers utilized in the PCR can be found in **Table 2**.

Table 2. List of primers

Primer	5′ to 3′
GAPDH	Forward:5'-GGCACAGTCAAGGCTGAGAATG-3'
	Reverse:5'-ATGGTGGTGAAGACGCCAGTA-3'
TNF-α	Forward:5'-CAGAGGGCCTGTACCTCATC-3'
	Reverse:5'-GGAAGACCCCTCCCAGATAG-3'
IL-1α	Forward:5'-AGCCATGGCAGAAGTACCTG-3'
	Reverse:5'-CCTGGAAGGAGCACTTCATCT-3'
IL-1β	Forward:5'-TGCCTTCAGCAGAGTGAAGA-3'
	Reverse:5'-GGTCTTGGTTCTCAGCTTGG-3'
IL-6	Forward:5'-ATTGGGATCATCTTGCTGGT-3'
	Reverse:5'-CCTGCTGTTCACAGTTGCC-3'
IL-8	Forward:5'-CCTCCATGGGCATCATCGTT-3'
	Reverse:5'-TGCAGCTTCTCGGTTGCATA-3'

Ki67	Reverse:5'-TGCAGCAGCAGCGGGTTTAT-3'
	Reverse:5'-GAGAGGCGCCTGATCTCTTC-3

Transwell invasion assay

The cell invasion ability was assessed using Matrigel-coated invasion chambers (BD Bioscience, Woburn, MA, USA). Matrigel was added to the upper chambers after being diluted in media containing 0% FBS and left to gel at room temperature after 6–8 hours. Then, a full medium with 20% FBS was introduced to the lower chamber as a chemoattractant and 2 × 10^5 cells were seeded into the upper chamber in a serum-free medium. After 36 hours of incubation, cells that had invaded through the Matrigel and reached the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Following that, cotton wool was used carefully to remove any non-invading cells from the upper membrane surface. The amount of invasion was then determined by counting the stained cells.

Statistical analysis

For statistical analysis, the study used SPSS version 26.0 (IBM Corp., Armonk, NY, USA). The tumor nodule volumes were compared between groups using the Mann-Whitney U test. The relationships between clinicopathological characteristics and protein expression were examined using the chi-square test. Kaplan-Meier and Cox regression

analyses were performed to investigate the impact of protein expression on OSCC recurrence. At $P < 0.05$, the results were considered statistically significant.

RESULTS

Characteristics of the patients in the OSCC cohort

A total of 202 OSCC patients were included in this study. This cohort included 47 OSCC patients with local recurrence and 155 OSCC patients did not occur recurrence during follow-up (**Figure 1**)

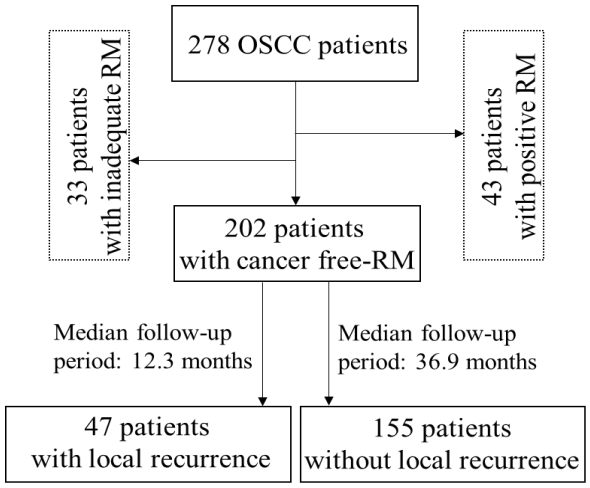


Figure 1. Flow diagram and follow-up period for OSCC cohort

Clinicopathologic significance of ARID1A and ARID1B expression in resection margins of OSCC

Both ARID1A and ARID1B expression was found in the nucleus of epithelial cells in resection margins of OSCC. ARID1A expression was high in 155 (ARID1A-high, 76.7%) and low in 47 (ARID1A-low, 23.3%) in resection margin of OSCC patients. ARID1B expression was high in 152 (ARID1B-high, 75.2%) and low in 50 (ARID1B-low, 24.8%) resection margins of OSCC patients. Representative expression patterns for ARID1A and ARID1B expression in resection margins of OSCC were shown in Figure 2. In our cohort, both ARID1A and ARID1B expression did not show a significant association with the presence of dysplasia in resection margins of OSCC (Table 3, 4). Kaplan-Meier analysis showed that patients with high-ARID1A/B expression in resection margins showed favorable prognosis in our cohort (all $p<0.001$) (Figure 3).

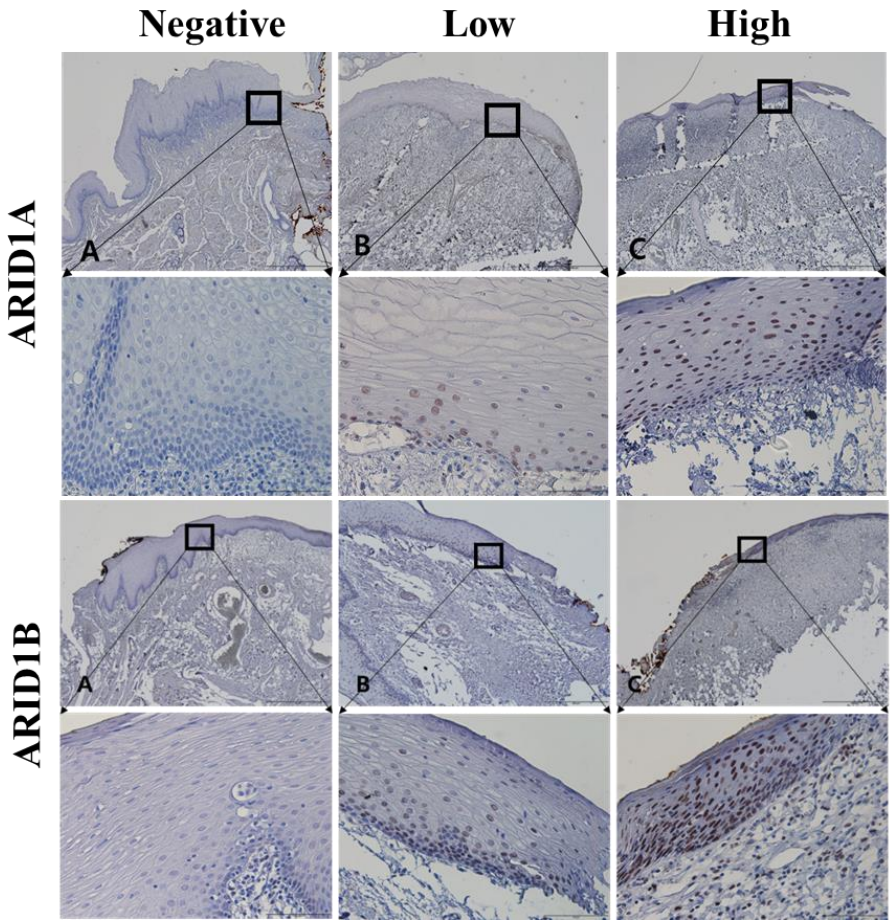


Figure 2. Representative expression patterns for ARID1A and ARID1B expression in resection margins of OSCC (Original magnification, x40; Scale bar, 50 μm).

Table 3. Association between ARID1A expression and characteristics of resection margins in OSCC patients.

Variables	N（%）	ARID1A		*P*
		Low	High	
Dysplasia in RM				0.311
Absent	145 (71.8)	31 (21.4)	114 (78.6)	
Present	57 (28.2)	16 (28.1)	41 (71.9)	
Width of RM				0.769
<1mm	16 (7.9)	5(31.3)	11 (68.8)	
≥1mm to <3mm	94 (46.5)	21 (22.3)	73 (77.7)	
≥3mm to <5mm	69 (34.2)	17 (24.6)	52 (75.4)	
≥5mm	23 (11.4)	4 (17.4)	19 (82.6)	

Table 4. Association between ARID1B expression and characteristics of resection margins in OSCC patients.

Variables	N（%）	ARID1B		*P*
		Low	High	
Dysplasia in RM				0.969
Absent	145 (71.8)	36 (24.8)	109 (75.2)	
Present	57 (28.2)	14 (24.6)	43 (75.4)	
Width of RM				0.948
<1mm	16 (7.9)	3 (18.8)	13 (81.3)	
≥1mm to <3mm	94 (46.5)	24 (25.5)	70 (74.5)	
≥3mm to <5mm	69 (34.2)	17(24.6)	52 (75.4)	
≥5mm	23 (11.4)	6 (26.1)	17 (73.9)	

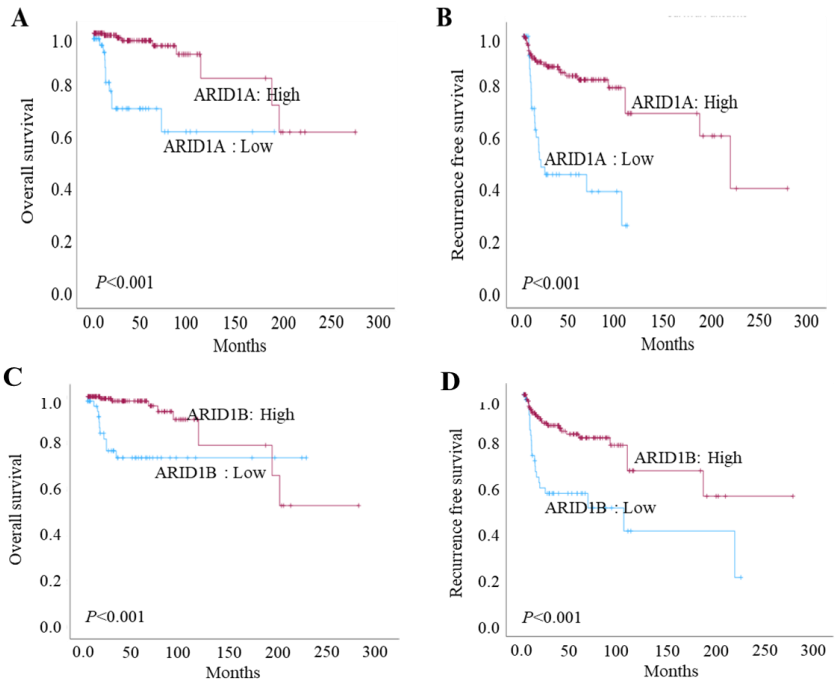
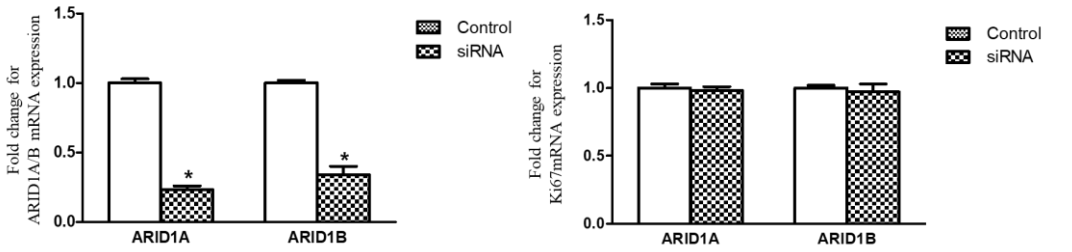


Figure 3. Kaplan-Meier analysis showed that patients with high-ARID1A/B expression in resection margins showed favorable prognoses in our cohort.

Influence of ARID1A/B downregulation in proliferation and invasion abilities of IHOK cells in vitro

To investigate the influence of ARID1A/B downregulation in proliferation and invasion abilities of IHOK cells, Ki67mRNA expression was detected in each group of cells. We found no significant difference between control and siRNA group of IHOK cells in Ki67mRNA expression. Invasion ability was significantly increased after ARID1B downregulation in IHOK cells ($P<0.001$). By contrast, no significant difference was found after ARID1A downregulation in invasion ability of IHOK cells (**Figure 4**).

(i)



(ii)

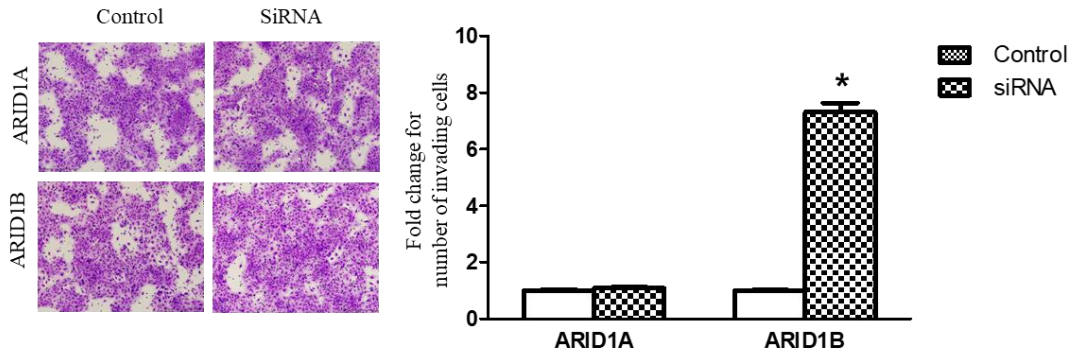
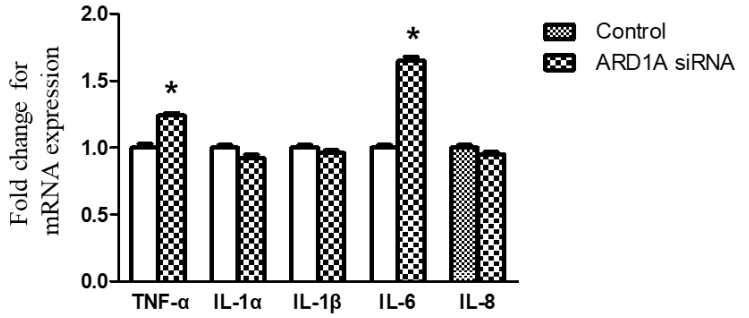


Figure 4. Influence of ARID1A/B downregulation in proliferation (i) and invasion (ii) abilities of IHOK cells *in vitro* (*P<0.001).

Influence of ARID1A/B downregulation in cytokine expression of cells in vitro

Cytokines related to immune escape were determined by real time PCR analysis in each group of cells. In IHOK cells, ARID1A downregulation can mediate significantly increased TNF-α (1.24 ± 0.02 fold), IL-6 (1.65 ± 0.03 fold) mRNA expression and ARID1B downregulation can mediate significantly increased TNF-α (2.23±0.04 fold), IL-8 (1.27 ±0.03 fold) mRNA expression (Figure 5) (*P<0.001).

(i)



(ii)

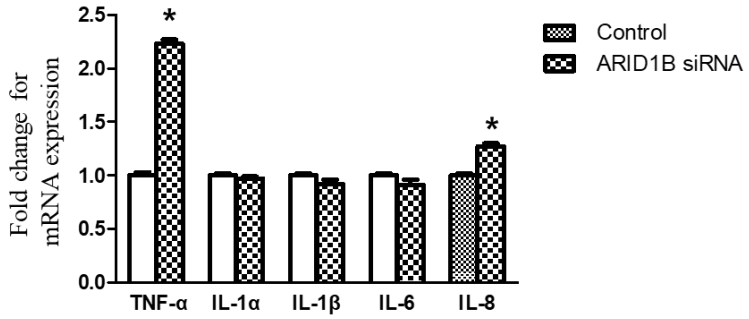


Figure 5. Influence of ARID1A/B downregulation in cytokine expression of cells *in vitro* (*P<0.001).

Clinicopathological significance of distribution of a-SMA positive fibroblasts in resection margins of OSCC patients.

a-SMA positive fibroblasts were frequently found in resection margins of OSCC. Distribution of a-SMA positive fibroblasts was high in 72 (α-SMA-high, 39.1%) and low in 112 (α-SMA-low, 60.9%). Representative expression patterns for a-SMA expression in resection margins of OSCC are shown in Figure 6.

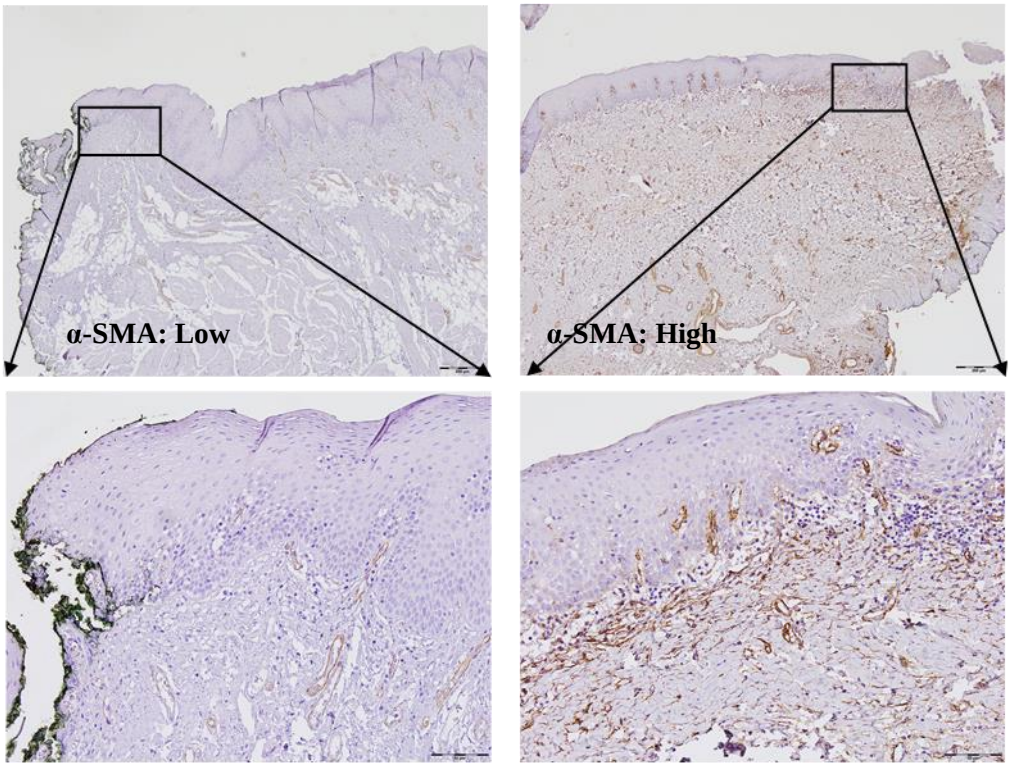


Figure 6. Representative expression patterns for α-SMA expression in resection margins of OSCC (Magnification, x40 and x200; Scale bar, 50 μm and 100μm).

In our cohort, no significant association was found between width or presence of dysplasia in resection margins and status of distribution of α-SMA positive fibroblast in resection margins (Table 5). Kaplan-Meier analysis showed that patients with α-SMA-high in resection margins showed poor prognosis in our cohort (Figure 7).

Table 5. Association between the distribution of α-SMA positive fibroblast and characteristics of resection margins in OSCC patients.

Variables	N（%）	α-SMA positive fibroblast		*P*
		Low	High	
Dysplasia in RM				0.311
Absent	127 (69.0)	82 (64.6)	45 (35.4)	
Present	57 (30.9)	30 (52.6)	27 (47.3)	
Width of RM				0.769
<1mm	16 (8.7)	7(43.8)	9 (56.3)	
≥1mm to <3mm	94 (51.1)	52 (55.3)	29 (30.9)	
≥3mm to <5mm	69 (37.5)	38 (55.1)	26 (37.7)	
≥5mm	23 (12.5)	15 (65.2)	8 (34.8)	

RM: Resection margin

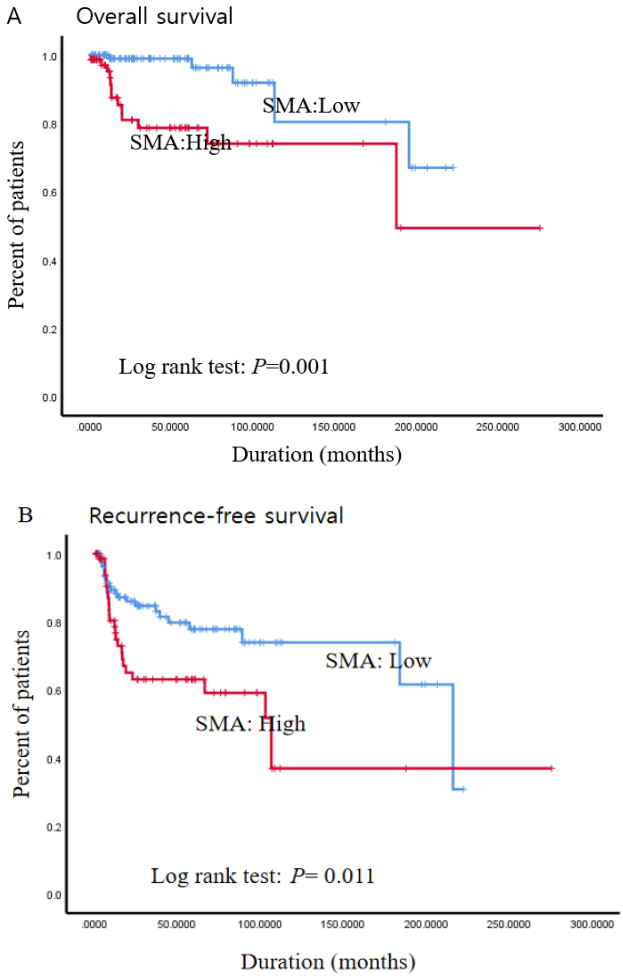
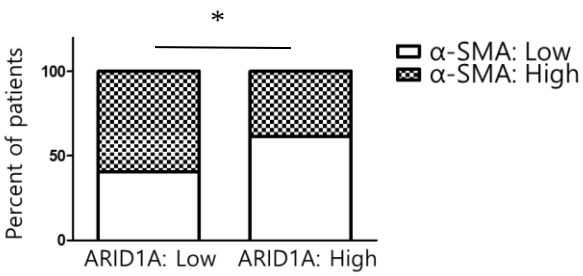


Figure 7. Kaplan-Meier analysis showed that patients with α-SMA-high in resection margins showed poor prognosis in our cohort.

Association between ARID1A/B expression and distribution of α-SMA positive fibroblasts in resection margins of OSCC

In OSCC patients, high distribution of α-SMA positive fibroblasts was detected more often in patients with low ARID1A/B expression (53.6%, 61.7%) than patients with high ARID1A/B expression (32.8%, 23.4%) (Figure 8).

(i)



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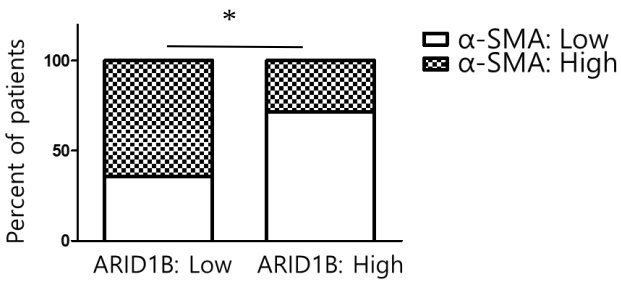


Figure 8. Association between ARID1A/B expression and distribution of α-SMA positive fibroblasts in resection margins of OSCC (*p<0.05).

Clinicopathological significance of the distribution of IL-6 positive fibroblasts in resection margins of OSCC patients.

IL-6 positive fibroblasts were frequently found in resection margins of OSCC. The distribution of IL-6 positive fibroblasts was high in 58 (IL-6-high, 31.5%) and low in 126 (IL-6-low, 68.5%). Representative expression patterns for IL-6 expression in resection margins of OSCC are shown in Figure 9.

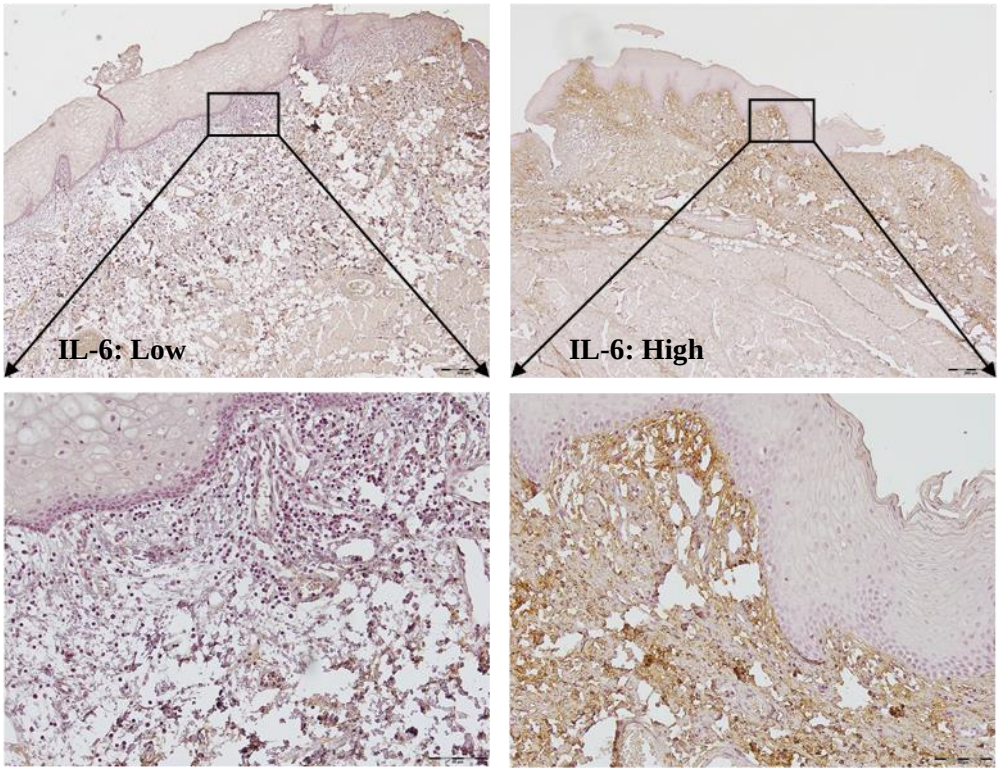


Figure 9. Representative expression patterns for IL-6 expression in resection margins of OSCC (Magnification, x40 and x200; Scale bar, 50 μm and 100μm).

In our cohort, the distribution of IL-6 positive fibroblast did not show a significant association with width or the presence of dysplasia in resection margins (Table 6). Kaplan-Meier analysis showed that patients with IL-6 in resection margins showed poor prognosis in our cohort (Figure 10).

Table 6. Association between the distribution of IL-6 positive fibroblast and characteristics of resection margins in OSCC patients.

Variables	N（%）	IL-6 positive fibroblast		
		Low	High	*P*
Dysplasia in RM				0.723
Absent	127 (69.0)	88 (69.3)	39 (30.7)	
Present	57 (30.9)	38 (66.7)	19 (33.3)	
Width of RM				0.232
<1mm	16 (8.7)	12(75.0)	4 (25.0)	
≥1mm to <3mm	94 (51.1)	49 (60.5)	32 (39.5)	
≥3mm to <5mm	69 (37.5)	48 (75.0)	16 (25.0)	
≥5mm	23 (12.5)	17 (73.9)	6 (26.1)	

RM: Resection margin

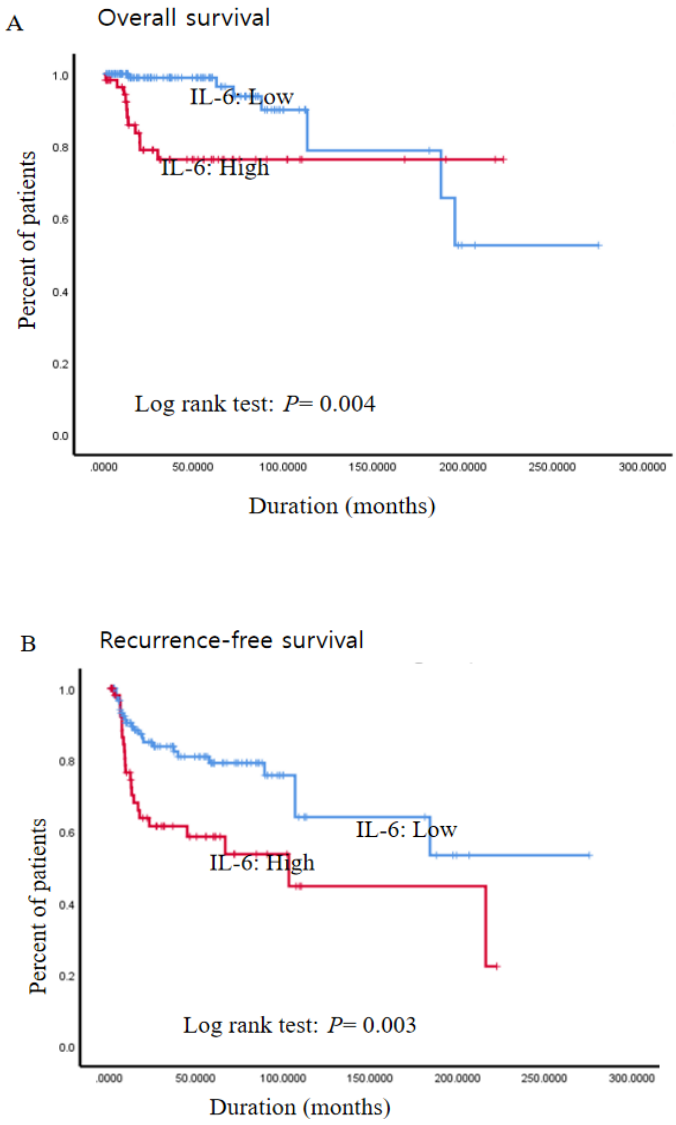
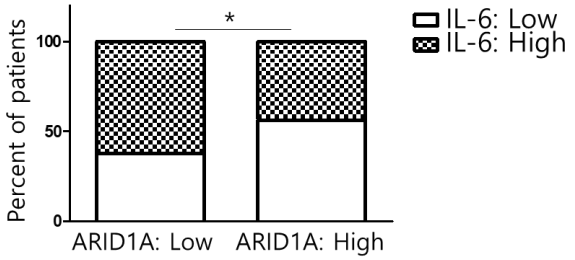


Figure 10. Kaplan-Meier analysis showed that patients with IL-6-high in resection margins showed poor prognosis in our cohort.

Association between ARID1A/B expression and distribution of IL-6 positive fibroblasts in resection margins of OSCC

In OSCC patients, high distribution of IL-6 positive fibroblasts was detected more often in patients with low ARID1A/B expression (60.3%, 70.2%) than patients with high ARID1A/B expression (39.6%, 27.2%) (Figure 11).

(i)



(ii)

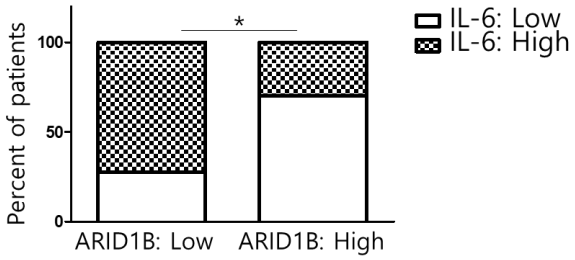


Figure 11. Association between ARID1A/B expression and distribution of IL-6 positive fibroblasts in resection margins of OSCC (*p<0.05).

DISCUSSION

Genetic alterations can be frequently detected in resection margins, however they often can not be detected by routine pathologic processes when the cells without typical histological change. So, many researchers aimed to investigate the molecular characteristics of resection margins. However it remains largely unknown in OSCC.

The presence study shows for the first time that ARID1A and ARID1B are candidate molecular biomarkers for highlighting resection margin with genetic alteration in OSCC. ARID1A and ARID1B subunits members of BRG1/BRM-associated factor complex (BAF), can link the subunits that have ATPase activity and BAF core module(Mashtalir et al., 2018). Both ARID1A and ARID1B showed mutually exclusive interaction with SWItch/Sucrose Non-Fermentable (SWI/SNF) complex in mammalian cells(Raab et al., 2015).

Board spectrum of SWI/SNF complex was found in mammalian cells, and the functional specificity was depended on the subunit combination(Wu, 2012). Studies found that mutation was detected in 29 genes from SWI/SNF complex in all cancer types(Helming et al., 2014). Genetic alteration in SWI/SNF complex may be an important trigger in cancer progression(Kadoch et al., 2013; Shain and Pollack, 2013). As the most common mutated genes in SWI/SNF complex, ARID1A is altered in various types of cancers and known to play an important role in cancer progression(Jiang et al., 2020). ARID1B loss is also frequently detected in various types of cancers, and its critical role in

cancer progression has also received much attention(Cajuso et al., 2014; Fontana et al., 2023; Gao et al., 2013; Oike et al., 2013; Sausen et al., 2013; Shain et al., 2012).

In this study, we found that OSCC patients with decreased ARID1A and ARID1B expression in resection margins showed poor prognosis in our cohort. Moreover, ARID1A or ARID1B downregulation can mediate increased TNF-α and IL-6 expression, cytokines related to immune escape(Groeger and Meyle, 2019), in IHOK cells. Moreover, increased invasion ability was also found after ARID1B downregulation in IHOK cells. All of those implied that loss of ARID1A and ARID1B expression can promote cancer progression in resection margins via control both biologic behavior and microenvironment of keratinocytes in resection margins.

The tumor microenvironment has been considered a critical regulator in cancer progression(Arneth, 2019). In resection margins, carcinogenesis may also largely depend on characteristics of the microenvironment of epithelial cells with genetic changes.

Fibroblasts are the main cell types of stroma tissues in oral mucosa, and in this study, a-SMA or IL6 positive fibroblasts were found in resection margins of OSCC. A-SMA and IL6 were known as cancer-associated fibroblasts (CAFs) biomarkers. CAFs show heterogeneity in many aspects, including cellular precursors, molecular characteristics, and functions(Bu et al., 2019; Liu et al., 2019). Cellular precursors of CAFs include resident fibroblasts, endothelial cells, epithelial cells, pericytes, smooth muscle cells, and bone marrow-derived mesenchymal stem cells (Costa et al., 2018). Derived from many types of cell precursors, CAFs exhibit molecular and functional diversity during cancer progression.

In addition to internal factors, external factors, such as crosstalk between cancer cells and CAFs, are also involved in triggering CAF heterogeneity. The interaction between cancer cells and CAFs has been reported in various cancer types (Bae et al., 2014; Fan et al., 2021; Novak et al., 2021). Some investigators have shown that microRNA-375 secreted by merkel cell carcinoma (MCC) can polarize fibroblasts toward a CAF-like phenotype via inhibiting the expression of target genes, such as recombination signal binding protein for immunoglobulin kappa J region and TP53 (Fan et al., 2021). Furthermore, expression of recombination signal binding protein for immunoglobulin kappa J region and TP53 is reportedly related to fibroblast polarization in different cancer types such as lung cancer, CSCC, and MCC (Arandkar et al., 2018; Fan et al., 2021; Goruppi et al., 2017). Generating CAF heterogeneity is a complex process, further studies are required to clarify the molecular mechanisms underlying CAF heterogeneity.

Originally, the pro-tumorigenic roles of CAFs have been highlighted in many types of cancers (Kojima et al., 2010; Su et al., 2018). Recently, tumor suppressive functions of CAFs were also reported by some investigators (Mizutani et al., 2019), and the heterogeneous subpopulation of CAFs has emerged as a new research topic. Although several molecules have been proposed as CAFs biomarkers(Fan et al., 2021; Ohashi et al., 2022), investigators have identified two distinct subsets of CAFs, myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs), based on their molecular characteristics, spatial distributions, and functions in different cancer types, such as breast and pancreatic cancers (Ohlund et al., 2017; Sebastian et al., 2020). In pancreatic cancer, myCAFs are

distributed in direct proximity to the tumor cell bed and show high immunoreactivity against classic markers of fibroblast activation such as α-SMA(Elyada et al., 2019). MyCAFs have been implicated in various biological behaviors of cancer cells, including proliferation, migration, invasion, and ECM remodeling (Elyada et al., 2019; Sebastian et al., 2020). In contrast, iCAFs were distributed relatively far from the tumor bed and showed high immunoreactivity to various cytokines, including IL6. iCAFs are known to contribute to immune escape and chemoresistance (Elyada et al., 2019; Lakins et al., 2018; Ohlund et al., 2017). In this study, a-SMA positive or IL6 positive fibroblasts were frequently found in resection margins, and patients with high distribution of a-SMA or IL6 positive fibroblasts in resection margins showed poor prognosis in our cohort.

As a common cytokine detected in the tumor microenvironment, IL6 can regulate various key cellular components of TIME, such as tumor-associated macrophages (TAMs), dendritic cells (DCs), tumor-associated neutrophils, and myeloid-derived suppressor cells. IL6 can promote M2-type macrophage transformation during the crosstalk between CAFs and TAMs (Lakins et al., 2018). M2-type TAMs contribute to immune evasion by tumor cells via their secretomes with immunosuppressive functions, such as TGF-β (Van Ginderachter et al., 2006). DCs, the major antigen-presenting cells, play crucial roles in anticancer immunity. Under normal conditions, most DCs exist in an immature state, which plays an important role in maintaining tolerance to self-antigens. IL6 can abrogate dendritic cell maturation in vivo, which may further contribute to the formation of an immunosuppressive microenvironment (Park et al., 2004; Xu et al., 2022). Moreover, IL6

can mediate the recruitment of PDL1-positive neutrophils in the tumor microenvironment, thereby impairing T cell function and further inducing an immunosuppressive microenvironment via the IL6-STAT3-PDL1 axis in hepatocellular carcinoma(Cheng et al., 2018). In addition, the synergistic effect of IL6 and exosomepacked microRNA-21 can promote STAT3 activation, thereby inducing monocytic myeloid-derived suppressor cell generation in esophageal squamous cell carcinoma (Zhao et al., 2021). IL6 positive fibroblast may affect the immune microenvironment of resection margins by influencing the function of various effector cells in the immune microenvironment, thereby resulting in poor patient prognosis.

CONCLUSION

This study supports the idea that genetic alterations may occur in both epithelial cells and stroma components in resection margins of OSCC. Combined factors representing epithelial and stroma changes may need risk assessment for resection margins in OSCC. ARID1A/B, α-SMA/IL-6 positive fibroblasts may contribute to carcinogenesis of resection margins in OSCC, and it can serve as a novel molecular marker for risk assessment of resection margins in OSCC.

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ABSTRACT (in Korean)

구강암 환자의 수술절제연에서 유전적 변화의 임상병리학적 중요성

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해 호 연

상피 조직은 상피 세포와 기질 조직(stromal tissue)으로 구성되어 있어, 유전적 변형은 상피 및 기질 요소 모두에서 발생할 수 있다. 그러나 구강 편평세포암 (oral squamous cell carcinoma, OSCC)의 절제 변연 기질 세포의 유전적 변형에 대해서는 거의 알려진 바가 없다. 이 연구에서는 절제 변연의 상피 세포와 기질에서 일부 유전 인자의 변형을 평가하고, OSCC 환자에서의 임상병리학적 관련성을 조사하였다.

이 연구는 202명의 OSCC 환자를 대상으로 하였으며 절제 변연에서 ARID1A (AT-Rich Interaction Domain 1A), ARID1B(AT-Rich Interaction Domain 1B), 알파-평활근 액틴(α-SMA) 및 인터류킨-6 (IL-6)의 발현을 면역조직화학적으로 확인하였다. ARID1A와 ARID1B 발현이 자발적으로 불멸화된 인간 구강각화세포 (immortalized human oral keratinocytes, IHOK)의 생물학적 역할에 미치는 영향 또한 다양한 세포 실험 기법으로 조사하였다.

47명(23.3%)의 OSCC 환자에서 ARID1A 발현 감소 및 50명(24.8%)의

OSCC 환자에서 ARID1B 발현 감소를 확인하였다. Kaplan-Meier 분석에서는 절제 변연에서 ARID1A/B 발현이 높은 환자가 더 좋은 예후를 나타냈다. 또한 ARID1B 발현이 감소(downregulation)된 IHOK 세포에서 침윤 능력(invasion ability) 증가와 TNF-α 및 IL-8 mRNA 발현 증가를 확인하였다. 이와 유사하게 ARID1A 발현이 감소된 IHOK 세포에서 TNF-α 및 IL-6 mRNA 발현이 증가함을 확인하였다. 추가로 α-SMA/IL-6 양성 섬유아세포(α-SMA/IL-6 positive fibroblast)가 각각 72명(39.1%)과 58명(31.5%)의 OSCC 환자에서 증가함을 확인하였다. Kaplan-Meier 분석에서는 절제 변연에서 α-SMA/IL-6 양성 섬유아세포가 높은 분포를 보이는 환자가 불량한 예후를 보였다.

결론적으로, 이 연구를 통해 OSCC 절제 변연에서 상피 세포와 섬유아세포 모두에서 유전적 변형이 발생할 수 있을 것으로 생각되며 상피 및 기질 세포의 변화를 모두 나타낼 수 있는 결합 요소가 OSCC 절제 변연의 위험(risk) 평가에 필요할 것으로 생각된다. 즉, ARID1A/B의 발현과 α-SMA/IL-6 양성 섬유아세포의 분포 특징은 OSCC 절제 변연의 발암에 기여할 수 있으며, OSCC 절제 변연의 위험 평가를 위한 새로운 분자 마커로서의 사용이 기대된다.

핵심 되는 말: 구강 편평세포암, 절제 변연, 재발, ARID1A, ARID1B, α-SMA/IL-6 양성 섬유아세포