Study of *MYB-NFIB* chimeric gene expression, tumor angiogenesis, and proliferation in adenoid cystic carcinoma of salivary gland

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Abstract Adenoid cystic carcinoma (ACC) is one of the common malignant tumors in salivary glands, and the clinical prognosis is poor with frequent distant metastasis which may lead to death. Expression of the *MYB-NFIB* chimeric gene in ACC has been reported recently. *MYB* is an oncogene with transcription regulating functions, and *NFIB* encodes nuclear transcription factor although detailed functions are unknown. This study investigated whether *MYB-NFIB* chimeric gene expression affects tumor angiogenesis and proliferation in salivary gland ACC. In 26 salivary gland ACC cases, *MYB-NFIB* chimeric gene expression was analyzed by RT-PCR and direct sequencing. Immunohistochemical studies for CD31, vascular endothelial growth factor (VEGF) and Ki-67 were performed. Tumor angiogenesis was evaluated by blood vessel (CD31-positive) density and tumor proliferation by Ki-67 labeling index, and the relationship with *MYB-NFIB* chimeric gene expression was analyzed. *MYB-NFIB* chimeric gene expression was detected in nine of 26 ACC cases. Blood vessel density was significantly higher in chimeric gene-expressing cases compared to non-expressing cases. VEGF score tended to be higher in chimeric gene-expressing cases than in non-expressing cases, while Ki-67 labeling index was not significantly different. The number of chimeric gene-expressing cases increased with age, peaking in the sixties age group and declining thereafter, while the number of non-expressing cases increased with age continuously. In ACC, blood vessel density was significantly higher in *MYB-NFIB* chimeric gene-expressing cases compared to non-expressing cases, which may be due to higher VEGF production capability. *MYB-NFIB* chimeric gene expression may also be related to the onset age of ACC.

Key Words: *MYB-NFIB* chimeric gene, adenoid cystic carcinoma, salivary gland, tumor angiogenesis, tumor proliferation

Introduction

Adenoid cystic carcinoma (ACC) is one of the common malignant tumors of the salivary gland. The prognosis of ACC is poor, and distant metastases are often observed, which may have fatal outcome. Histopathologically, ACC has a biphasic cells pattern composed of luminal ductal cells and abluminal modified myoepithelial cells. The histopathological appearance may show a cribriform pattern, tubular pattern, or solid pattern, characterized by perineural invasion [1-6]. In recent years, ACC has been reported to harbor the *MYB-NFIB* chimeric gene formed by mutual translocation of the long arm of chromosome 6 and the short arm of chromosome 9 [t(6;9)(q22-23;p23-24)] [7, 8]. *MYB* is an oncogene with transcription regulatory function, and plays important roles in cell proliferation and differentiation [9]. *NFIB* is known to encode nuclear transcription factor [10, 11]. Previous reports have shown that the *MYB-NFIB* chimeric gene is expressed not only in ACC of salivary gland, but also in ACC of other organs and tissues such as sinonasal tract, tracheobronchial tree, breast, vulva, and skin [5, 12, 13]. The *MYB-NFIB* chimeric gene has been investigated as a biomarker and regarding its involvement in prognosis and carcinogenesis [12, 14-18]. Some studies have shown no relationship between *MYB-NFIB* chimeric gene expression and clinical outcome of ACC [14, 16]. However, the detailed functions of this gene are not known. In mucoepidermoid carcinoma of the salivary gland, expression of the chimeric gene has been reported to be related to the age of tumor onset [19], but there are few detailed studies of the association between *MYB-NFIB* chimeric gene expression and age of tumor onset in ACC [14].

Salivary gland carcinomas have been reported to demonstrate higher vascular activity and higher VEGF expression [20, 21] than benign tumors, and overexpression of vascular endothelial growth factor (VEGF) and nuclear antigen Ki-67 has been reported to be a prognostic factor of poor survival in ACC [22]. However, there is no report on the association of *MYB-NFIB* chimeric gene expression with tumor angiogenesis and tumor proliferation, which are generally considered to affect the prognosis.

In the present study, we analyzed *MYB-NFIB* chimeric gene expression by reverse transcription polymerase chain reaction (RT-PCR) and direct sequence method, and evaluated CD31, VEGF and Ki-67 by immunohistochemistry in salivary gland ACC, aiming to elucidate the relationship of *MYB-NFIB* chimeric gene expression with tumor angiogenesis and proliferation. We also examined the impact of age of tumor onset on molecular histopathological diagnosis.

Patients and methods

Case selection

The paraffin-embedded blocks of 26 cases of salivary gland ACC archived in our department were used in this study. The patients comprised 13 males and 13 females, aged from 44 to 81 years (mean 64.6 years). The tumor sites were the oral floor in 10 cases, the maxilla (hard palate) in 7 cases, the

submandibular gland in 4 cases, buccal mucosa in 3 cases, the sublingual gland in 1 case, and upper lip in 1 case. This study was approved by the Ethics Committee of The Nippon Dental University School of Life Dentistry at Niigata (ECNG-H-215).

Detection of *MYB-NFIB* transcripts

Ten-μm sections were cut from a paraffin-embedded block. After deparaffinization, RNA was extracted using ISOGEN-II (Wako, Osaka, Japan). Then cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). *Beta-actin* was used as the housekeeping gene (Table 1). PCR was performed with four primers (Table 1) using a MiniOpticon System (Bio-Rad, Hercules, CA).

A 2% agarose gel was prepared using agarose (NuSieve™ 3:1 Agarose; Lonza, Basle, Switzerland) and TBE buffer containing 0.089 M TBE base (Trizma®; Sigma-Aldrich, St. Louis, MO), 0.002 M EDTA (Sigma-Aldrich) and 0.089 M boric acid (Wako). PCR products were loaded on the gel and electrophoresed. Thereafter, the gel was stained with ethidium bromide (Nippon Gene, Tokyo, Japan) and DNA bands were photographed using a FAS-III System (Nippon Genetics, Tokyo, Japan).

Nucleotide sequence analyses

After purification, PCR products were analyzed using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) to confirm the DNA sequences.

Immunohistochemical staining

From paraffin-embedded tissues, serial sections of 3-μm thickness were prepared. After deparaffinization, the sections were incubated with Immunosaver (Nissin EM, Tokyo, Japan) at 98˚C for 45 min to retrieve antigen, and then treated with ethanol containing 0.3% H_2O_2 to block endogenous peroxidase. The sections were incubated with 5% normal goat serum (Dako, Glostrup, Denmark) for 10 min at room temperature, and then incubated with the primary antibodies overnight at 4˚C. Then the sections were treated with secondary antibodies (Histofine Simple Stain MAX-PO MULTI; Nichirei Bioscience Inc., Tokyo, Japan) for 30 min at room temperature. Color was developed using 3,3'-diaminobenzidine・4HCl (DAB Substrate Kit; Nichirei Bioscience Inc.). After nuclear staining with hematoxylin, the slides were observed under a light microscope (BX53; Olympus, Tokyo, Japan) and evaluated by two investigators. Antibodies against CD31 (clone 1A10, 1:100; Novocastra, Newcastle upon Tyne, UK), vascular endothelial growth factor (VEGF) (clone VG-1, 1:250; Abcam, Cambridge, UK) and Ki-67 (clone MIB-1, 1:1,000; Dako) were used as primary antibodies.

Angiogenesis was evaluated by counting the number of CD31-immunostained blood vessels within the cancer cell nest at the invasive front per 200× field observed under the light microscope according to the method of Okada [23]. The mean number of six randomly selected fields was

evaluated as blood vessel density.

Tumor proliferation was evaluated by calculating the Ki-67 (MIB-1) labeling index of cancer cells per 400× field under the light microscope according to the method of Mohtasham et al. [24]. The mean value of six randomly selected fields was evaluated.

VEGF was evaluated by calculating the immunostaining score of six randomly selected 200× fields according to the method of Stárek et al. [25]. The staining intensity for VEGF in the cytoplasm and/or nuclei of the tumor and normal parotid parenchymal cells was graded according to Moriyama et al. [26]. Intensity equal to that of lymphatic endothelial cells in adjacent normal salivary tissue was determined as grade 2. Absent, weaker, and stronger staining intensities were graded as 0, 1, and 3, respectively. The percentage of positive tumor cells was scored according to Lim et al. [27]. as 0 (0–19%), 1 (20–39%), 2 (40–59%), and 3 (60–100%). The sum of intensity and percent score provided a semi-quantitative histoscore (ranging from 0 to 6).

Statistical analysis

Mann-Whitney *U* test was used to analyze the association of *MYB-NFIB* chimeric gene expression with blood vessel density, VEGF expression score, and Ki-67 labeling index. Statistical analyses were conducted using IBM SPSS (version 21). A *p* value less than 0.05 was considered significant.

Results

MYB-NFIB chimeric gene expression

Gene expression analysis using PCR detected the *MYB-NFIB* chimeric gene in 9 of 26 (34.6%) cases of salivary gland ACC (Table 2, Fig. 1A and B). Eight cases were detected by primer A (Fig. 1A) and 1 case was detected by primer B (primer covering exon 14 and exon 9; Fig. 1B). The 9 cases comprised 5 males and 4 females, with ages ranging from 45 to 81 years (mean 63.7 years). Among the cases expressing the *MYB-NFIB* chimeric gene, 1 case was in the forties age group, 2 cases were in the fifties age group, 3 cases were in the sixties age group, 2 cases were in the seventies age group, and 1 case was in the eighties age group, with the largest number in the sixties age group. On the other hand, among cases not expressing the chimeric gene, 1 case was in the forties age group, 4 cases were in the fifties age group, 5 cases were in the sixties age group, and 7 cases were in the seventies age group. In this group, the number of cases increased with age, with the largest number in the seventies age group (Fig. 2). In the group showing chimeric gene expression, the cancer was located in the maxilla (hard palate) in 3 cases, the submandibular gland in 2 cases, oral floor in 2 cases, and buccal mucosa in 2 cases.

Direct sequence analysis identified the PCR product amplified from primer A as the fusion site equivalent to NCBI database accession No. FJ969915 (breakpoint 1593-1594) (Fig. 1C), and the PCR product amplified from primer B as the fusion site equivalent to accession No. FJ969916 (breakpoint 1593-1594) (Fig. 1D).

Immunohistochemical studies

The blood vessel density evaluated by CD31 (mean ± SD) was 11.2 ± 3.4 in the group expressing the *MYB-NFIB* chimeric gene and 7.9 ± 1.5 in the group not expressing the gene, and was significantly higher ($P < 0.05$) in the chimeric gene-expressing group (Table 3, Fig. 3B).

The VEGF immunostaining score (mean ± SD) calculated according to Stárek et al. [25] was 4.5 ± 1.2 in the *MYB-NFIB* chimeric gene-expressing group and 3.9 ± 1.5 in the non-expressing group. Although the score was apparently higher in the chimeric gene-expressing group than in the non-expressing group, no significant difference was observed (Table 3, Fig. 3C).

When tumor proliferation capability was evaluated by Ki-67 labeling index, the index (mean ± SD) was 12.6 ± 14.6 in the *MYB-NFIB* chimeric gene-expressing group and 15.3 ± 9.3 in the non-expressing group. Although the index appeared slightly higher in the non-expressing group than in the chimeric gene-expressing group, there was no significant difference (Table 3, Fig. 3D).

Discussion

The functions of the *MYB-NFIB* chimeric gene in ACC of salivary gland have not been fully elucidated [7, 12, 14-18]. Therefore, we investigated the effect of *MYB-NFIB* chimeric gene expression in salivary gland ACC on tumor angiogenesis and tumor proliferation, which are among the factors regarded to be related to prognosis of tumors in general, and also examined the impact of age of tumor onset on molecular pathological diagnosis.

In this study, we analyzed *MYB-NFIB* chimeric gene expression by RT-PCR using four primers (A to D). *MYB-NFIB* chimeric gene expression was detected in a total of 9 of 26 cases (34.6%), consistent with previous reports (28 to 44%) [12, 14]. Using four primers, the chimeric gene expression was detected in 8 cases by primer A, and in 1 case by primer B located in exon 14 and exon 9. At first, when we used the primer *MYB*-1910F 5'-AGCTCC GTTTTAATGGCACC-3' (located in exon 14) and the primer *NFIB*-1096R 5'-GGGTATAAATGCCTGCCGTT-3' (located in exon 8c) reported by Brill et al. [12], we found abnormal expression of primer dimer. Consequently, we changed only the *MYB* primer in primer A to *MYB*-1513 5'-GCACCAGCATCAGAAGATGA-3', and were able to obtain clear bands. Therefore, primer A used in the present study appears to be useful for the detection of the *MYB-NFIB* chimeric gene. In addition, this chimeric gene has multiple variants. In the present study, gene expression was detected by primer A in 8 cases and by primer B in 1 case, indicating the need to use multiple primers for detection.

Among 58 patients followed for at least three years in the study of Mitani et al. [14], 16 patients expressed the *MYB-NFIB* chimeric gene and 42 did not. Of 16 patients who were positive for *MYB-NFIB* chimeric gene, only three patients were younger than 50 years, while the majority of 13 patients were aged 50 years or older. Among their cases not expressing the chimeric gene, there were 21 cases each in the group younger than 50 years and the group aged 50 years or older. In the

present study, the number of cases expressing the *MYB-NFIB* chimeric gene increased gradually with age, reaching a peak in the sixties age group and declining thereafter. On the other hand, the number of cases not expressing the chimeric gene increased with age continuously and was the highest in the seventies age group. These findings suggest an association between *MYB-NFIB* chimeric gene expression and the age of ACC onset. However, the number of the present study was relatively small, which limits the interpretation of the results. Further larger scale study is required to validate the present findings.

Regarding the relationship between *MYB-NFIB* chimeric gene expression and blood vessel density measured by CD31 immunostaining, the density was significantly higher in the chimeric gene-expressing group than in the non-expressing group. This finding may be explained by the high total score of VEGF immunostaining in the chimeric gene-expressing group, indicating a tendency of high VEGF production capability in this group. Our finding is consistent with the result of Persson et al. [7] showing high VEGF production in cases expressing the *MYB-NFIB* chimeric gene. Histopathologically, a high blood vessel density increases the contact of tumor with blood vessels, and has been reported to increase the frequency of vascular invasion (intravascular infiltration) and consequently increase the risk of distant metastasis [28]. This finding suggests an increased possibility of distant metastasis in ACC expressing the chimeric gene. However, assessment of distant metastasis requires observation periods of 5 to 10 years [2,3] Further long-term follow-up study on a large number of cases is required to verify this association.

We also examined the relationship between *MYB-NFIB* chimeric gene expression and tumor proliferation capability indicated by Ki-67 immunostaining. Contrary to the result of blood vessel density, Ki-67 labeling index appeared slightly higher in the chimeric gene non-expressing group, although the difference was not statistically significant. Because ACC is a slow-growing tumor, a significant difference in proliferation capability depending on chimeric gene expression is probably difficult to demonstrate. Furthermore, previous studies have indicated that *MYB* is related to suppression of cell proliferation [29]. These complex associations have to be further investigated in a larger series.

Regarding the relationship between *MYB-NFIB* chimeric gene expression and prognosis, some reports have indicated that expression of the chimeric gene is unrelated to prognosis [14,16]. Prognosis is greatly affected by clinical factors including tumor size, metastasis to cervical lymph nodes, treatment, and recurrence. Therefore, simply comparing the status of *MYB-NFIB* chimeric gene expression with prognosis is not appropriate, and further study should be undertaken to analyze the relationship with prognosis under the same conditions for the above-mentioned clinical factors.

In conclusion, the present study with a relatively small sample size demonstrated that salivary gland ACC cases expressing the *MYB-NFIB* chimeric gene showed significantly higher blood vessels density compared to non-expressing cases, and suggested that higher VEGF production capability in the former cases may be the cause. The findings also suggested that *MYB-NFIB*

chimeric gene expression may be related to the onset age of ACC.

Conflicts of Interest

The authors declare that they have no conflict of interest.

References

1. Fonseca FP, Carvalho MV, Almeida OP, Rangel AL, Takizawa MC, Bueno AG, Vargas PA. Clinicopathologic analysis of 493 cases of salivary gland tumors in a Southern Brazilian population. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012;114:230-9.
2. Gao M, Hao Y, Huang MX, Ma DQ, Luo HY, Gao Y, Peng X, Yu GY. Clinicopathological study of distant metastases of salivary adenoid cystic carcinoma. Int J Oral Maxillofac Surg. 2013;42:923-8.
3. Weert SV, Bloemena E, Waal IVD, de Bree R, Rietveld DH, Kuik JD, Leemans CR. Adenoid cystic carcinoma of the head and neck: A single-center analysis of 105 consecutive cases over a 30-year period. Oral Oncol. 2013;49:824-9.
4. Perzin KH, Gullane P, Clairmont AC. Adenoid cystic carcinomas arising in salivary glands: a correlation of histologic features and clinical course. Cancer. 1978;42:265-82.
5. Stenmam G, Licitra L, Said-Al-Naief N, et al. Adenoid cystic carcinoma. In: El-Naggar AK, Chan JKC, Grandis JR, et al. eds. World Health Organization Classification of Tumours: WHO Classification of Head and Neck Tumours. Lyon: IARC Press; 2017:164-5.
6. Moskaluk CA. Adenoid cystic carcinoma: Clinical and molecular features. Head Neck Pathol. 2013;7:17-22.
7. Persson M, Andrén Y, Mark J, Horlings HM, Persson F, Stenman G. Recurrent fusion of *MYB* and *NFIB* transcription factor genes in carcinomas of the breast and head and neck. Proc Natl Acad Sci U S A. 2009;106:18740-4.
8. Stenman G, Andersson MK, Andrén Y. New tricks from an old oncogene: gene fusion and copy number alterations of *MYB* in human cancer. Cell Cycle. 2010;9:2986-95.
9. Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. Nat Rev Cancer. 2008;8:523-34.
10. Qian F, Kruse U, Lichter P, Sippel AE. Chromosomal localization of the four genes (NFIA, B, C, and X) for the human transcription factor Nuclear Factor I by FISH. Genomics. 1995;28:66-73.
11. Murtagh J, Martin F, Gronostajski RM. The Nuclear Factor I (NFI) gene family in mammary gland development and function. J Mammary Gland Biol Neoplasia. 2003;8:241-54.
12. Brill LB II, Kanner WA, Fehr A, Andrén Y, Moskaluk CA, Löning T, Stenman G, Frierson HF Jr. Analysis of MYB expression and *MYB-NFIB* gene fusions in adenoid cystic carcinoma and other salivary neoplasms. Mod Pathol. 2011;24:1169-76.
13. North JP, McCalmont TH, Fehr A, van Zante A, Stenman G, LeBoit PE. Detection of MYB Alterations and other immunohistochemical markers in primary cutaneous adenoid cystic carcinoma. Am J Surg Pathol. 2015;39:1347-56.
14. Mitani Y, Li J, Rao PH, Zhao YJ, Bell D, Lippman SM, Weber RS, Caulin C, El-Naggar AK. Comprehensive analysis of the *MYB-NFIB* gene fusion in salivary adenoid cystic carcinoma:

incidence, variability, and clinicopathologic significance. Clin Cancer Res. 2010;16:4722-31.
15. Mitani Y, Rao PH, Futreal PA, Roberts DB, Stephens PJ, Zhao YJ, Zhang L, Mitani M, Weber RS, Lippman SM, Caulin C, El-Naggar AK. Novel chromosomal rearrangements and breakpoints at the t(6;9) in salivary adenoid cystic carcinoma: association with *MYB-NFIB* chimeric fusion, *MYB* expression, and clinical outcome. Clin Cancer Res. 2011;17:7003-14.
16. Rettig EM, Tan M, Ling S, Yonescu R, Bishop JA, Fakhry C, Ha PK. MYB rearrangement and clinicopathologic characteristics in head and neck adenoid cystic carcinoma. Laryngoscope. 2015;125:292-9.
17. West RB, Kong C, Clarke N, Gilks T, Lipsick JS, Cao H, Kwok S, Montgomery KD, Varma S, Le QT. *MYB* expression and translocation in adenoid cystic carcinomas and other salivary gland tumors with clinicopathologic correlation. Am J Surg Pathol. 2011;35:92-9.
18. Fehr A, Kovács A, Löning T, Frierson HF Jr, van den Oord J, Stenman G. The *MYB-NFIB* gene fusion–a novel genetic link between adenoid cystic carcinoma and dermal cylindroma. J Pathol. 2011;224:322-7.
19. Nakayama T, Miyabe S, Okabe M, Sakuma H, Ijichi K, Hasegawa Y, Nagatsuka H, Shimozato K, Inagaki H. Clinicopathological significance of the *CRTC3-MAML2* fusion transcript in mucoepidermoid carcinoma. Mod Pathol. 2009;22:1575-81.
20. Moghadam SA, Abadi AM, Mokhtari S. Immunohistochemical analysis of CD34 expression in salivary gland tumors. J Oral Maxillofac Pathol. 2015;19:30-3.
21. Faur AC, Lazar E, Cornianu M. Vascular endothelial growth factor (VEGF) expression and microvascular density in salivary gland tumours. APMIS. 2014;122:418-26.
22. Park S, Nam SJ, Keam B, Kim TM, Jeon YK, Lee SH, Hah JH, Kwon TK, Kim DW, Sung MW, Heo DS, Bang YJ. VEGF and Ki-67 overexpression in predicting poor overall survival in adenoid cystic carcinoma. Cancer Res Treat. 2016;48:518-26.
23. Okada Y. Relationships of cervical lymph node metastasis to histopathological malignancy grade, tumor angiogenesis, and lymphatic invasion in tongue cancer. Odontology. 2010;98:153-9.
24. Mohtasham N, Babakoohi S, Shiva A, Shadman A, Kamyab-Hesari K, Shakeri MT, Sharifi-Sistani N. Immunohistochemical study of p53, Ki-67, MMP-2 and MMP-9 expression at invasive front of squamous cell and verrucous carcinoma in oral cavity. Pathol Res Pract. 2013;209:110-4.
25. Stárek I, Salzman R, Kučerová L, Skálová A4, Hauer L. Expression of VEGF-C/-D and lymphangiogenesis in salivary adenoid cystic carcinoma. Pathol Res Pract. 2015;211:759-65.
26. Moriyama M, Kumagai S, Kawashiri S, Kojima K, Kakihara K, Yamamoto E. Immunohistochemical study of tumour angiogenesis in oral squamous cell carcinoma. Oral Oncol. 1997;33:369-74.
27. Lim JJ, Kang S, Lee MR, Pai HK, Yoon HJ, Lee JI, Hong SP, Lim CY. Expression of vascular endothelial growth factor in salivary gland carcinomas and its relation to p53, Ki-67 and

prognosis. J Oral Pathol Med. 2003;32:552-61.
28. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis– correlation in invasive breast carcinoma. N Engl J Med. 1991;324:1-8.
29. Oh IH, Reddy EP. The *myb* gene family in cell growth, differentiation and apoptosis. Oncogene. 1999;18:3017-33.

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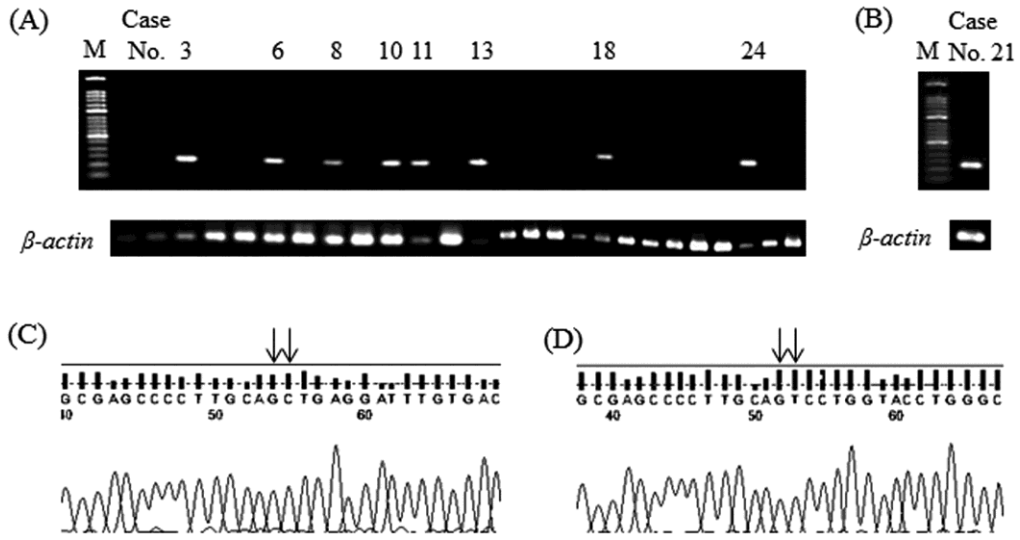
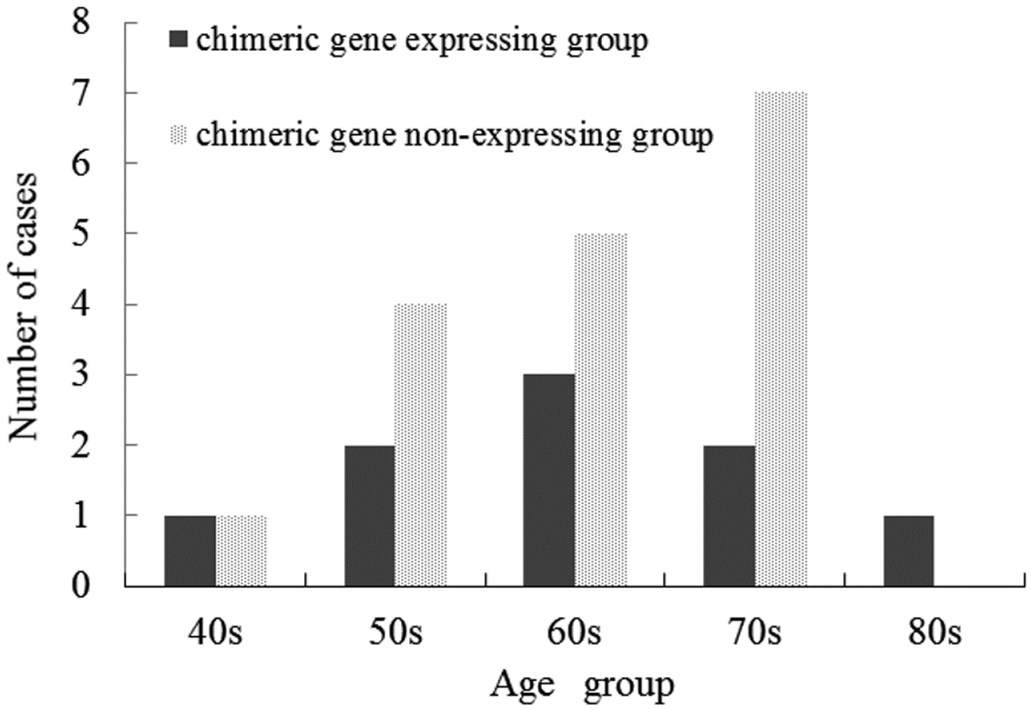

(A)
Case
M No. 3 6 8 10 11 13 18 24
β-actin
(B)
Case
M No. 21
β-actin
(C)
G C G A G C C C C T T G C A G C T G A G G A T T T G T G A C
10 50 60
(D)
G C G A G C C C C T T G C A G T C C T G G T A C C T G G G C
40 50 60


chimeric gene expressing group
chimeric gene non-expressing group
Number of cases
0
1
2
3
4
5
6
7
8
40s
50s
60s
70s
80s
Age group

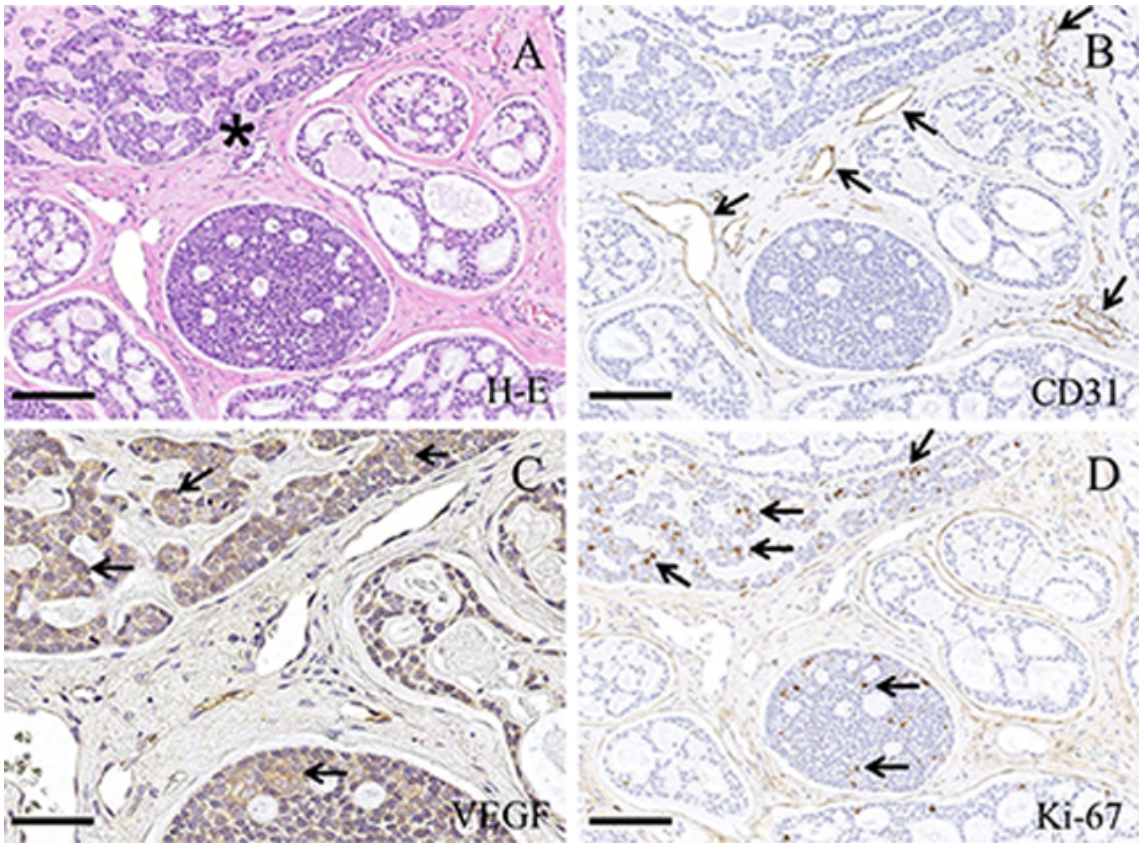

A
H-E
B
CD31
C
VEGF
D
Ki-67

Figure Legends

Fig. 1 *MYB-NFIB* chimeric gene expression in 26 cases of adenoid cystic carcinoma of salivary gland. (A) RT-PCR using primer A: chimeric gene expression is detected in 8 cases. (B) RT-PCR using primer B: chimeric gene expression is detected in case No. 21. (C) Direct sequencing of PCR product amplified by primer A: breakpoints are shown by arrows. (D) Direct sequencing of PCR product amplified by primer B: breakpoints are shown by arrows. M: molecular markers.

Fig. 2 Number of cases expressing the *MYB-NFIB* chimeric gene according to age group.

Fig. 3 A representative case of adenoid cystic carcinoma expressing the *MYB-NFIB* chimeric gene (Case No. 10, a 76 year-old male). (A) Hematoxylin and eosin (H-E) staining showing a cribriform structure. Scale bar: 100 μm. (B) Serial section of (A) immunostained for CD31 showing blood vessels among tumor cell nests (arrows). Scale bar: 100 μm. (C) Serial section of (A) immunostained for VEGF [magnified image of the portion marked "*" in (A)] showing VEGF expression in tumor cells (arrows). Scale bar: 50 μm. (D) Serial section of (A) immunostained for Ki-67 (arrows). Scale bar: 100 μm.

Table 1 Sequences of the Four Primers Used in for the Detection of *MYB-NFIB* Chimeric Gene Expression and *β-actin*

Primer		Primer Sequence
A	*MYB*	5’-GCACCAGCATCAGAAGATGA-3’
	NFIB	5’-GGGTATAAATGCCTGCCGTT-3’
B	*MYB*	5’-GCACCAGCATCAGAAGATGA-3’
	NFIB	5’-CCGGTAAGATGGGTGTCCTA-3’
C	*MYB*	5’-TTTTAATGGCACCAGCATCA-3’
	NFIB	5’-TTCCCAATGTATCCTCACTGG-3’
D	*MYB*	5’-TTTTAATGGCACCAGCATCA-3’
	NFIB	5’-CCGGTAAGATGGGTGTCCTA-3’
β-actin		5'-GATGCAGAAGGAGATCACTGC-3'
		5'-TACTCCTGCTTGCTGATCCA-3'

Table 2 Number of Adenoid Cystic Carcinomas Expressing the *MYB-NFIB* Chimeric Gene

Primer	*MYB-NFIB* Chimeric Gene Expression
A	8
B	1
C	0
D	0

Table 3 Relationship of *MYB-NFIB* Chimeric Gene Expression with Blood Vessel Density, VEGF Immunostaining Score, and Tumor Proliferation Ability (Ki-67 labeling index)

	MYB-NFIB chimeric gene expression		
	Positive	Negative	*p* value
Blood vessel density	11.2 ± 3.4	7.9 ± 1.5	*<0.05*
VEGF immunostaining score	4.5 ± 1.2	3.9 ± 1.5	NS
Ki-67 labeling index	12.6 ± 14.6	15.3 ± 9.3	NS

VEGF: vascular endothelial growth factor

Data are expressed in mean ± standard deviation. NS: not significant

p value by Mann-Whitney *U* test