Possible Relationship Between *MYBL1* Alterations and Specific Primary Sites in Adenoid Cystic Carcinoma: A Clinicopathological and Molecular Study of 36 Cases

Yukari Endo,*† Satoshi Kuwamoto,* Takahito Ohira,‡ Michiko Matsushita,† Takahiro Matsushige,* Takahiro Fukuhara,§ Shu Nakamoto,‖ Kazuhiko Hayashi,¶ Hiroyuki Kugoh,‡ and Yasuaki Hirooka†

**Department of Pathology, Tottori University Hospital, Yonago 683-8504, Japan, †Department of Pathobiological Science and Technology, School of Health Sciences, Tottori University Faculty of Medicine, Yonago 683-8503, Japan, ‡Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Sciences, Tottori University, Yonago 683-8503, Japan, §Division of Otolaryngology, Head and Neck Surgery, Department of Sensory and Motor Organs, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8504, Japan, ‖Department of Pathology, Tottori Prefectural Central Hospital, Tottori 680-0901, Japan, and ¶Division of Molecular Pathology, Department of Pathology, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8503, Japan*

ABSTRACT

Background Adenoid cystic carcinoma (ACC) is a relatively rare malignant neoplasm that occurs in salivary glands and various other organs. Recent studies have revealed that a significant proportion of ACCs harbor gene alterations involving *MYB* or *MYBL1* (mostly fusions with *NFIB*) in a mutually-exclusive manner. However, its clinical significance remains to be well-established.

Methods We investigated clinicopathological and molecular features of 36 ACCs with special emphasis on the significance of *MYBL1* alterations. Reverse-transcription polymerase-chain reaction (RT-PCR) and fluorescence *in-situ* hybridization (FISH) were performed to detect *MYB*/*MYBL1*–*NFIB* fusions and *MYBL1* alterations, respectively. Immunohistochemistry was performed to evaluate MYB expression in the tumors. The results were correlated with clinicopathological profiles of the patients.

Results RT-PCR revealed *MYB*–*NFIB* and *MYBL1*–*NFIB* fusions in 10 (27.8%) and 7 (19.4%) ACCs, respectively, in a mutually-exclusive manner. FISH for *MYBL1* rearrangements was successfully performed in 11 cases, and the results were concordant with those of RT-PCR. Immunohistochemically, strong MYB expression was observed in 23 (63.9%) tumors, none of which showed *MYBL1* alterations. Clinicopathologically, a trend of a better disease-specific survival was noted in patients with *MYBL1* alterations than in those with *MYB*–*NFIB* fusions and/or strong MYB expression; however, the difference was not significant. Interestingly, we found tumors with *MYBL1* alterations significantly frequently occurred in the mandibular regions ($P = 0.012$). Moreover, literature review revealed a similar tendency in a previous study.

Conclusion Our results suggest that there are some biological or etiological differences between ACCs with *MYB* and *MYBL1* alterations. Moreover, the frequent occurrence of *MYBL1*-associated ACC in the mandibular regions suggests that MYB immunohistochemistry is less useful in diagnosing ACCs arising in these regions. Further studies are warranted to verify our findings.

Key words adenoid cystic carcinoma; fluorescence *in-situ* hybridization; mandibular neoplasms; oncogene fusion; prognosis

Adenoid cystic carcinoma (ACC) is a relatively rare malignant neoplasm that occurs in various organs, including the major and minor salivary glands, nasal cavity, bronchial trees, and breast. ACC is characterized by a slow but relentless progression, showing an infiltrative growth along the peripheral nerves and frequent distant metastases. Histologically, this tumor comprises epithelial and myoepithelial neoplastic cells showing diverse growth patterns, which are roughly subclassified into tubular/cribriform and solid types based on the dominant pattern. Due to the aggressive nature of the tumor, long-term prognosis in patients with ACC is poor, with the 10-year survival rate of 50%–70%.[1] Currently, radical surgical resection with or without postoperative irradiation is the standard treatment for ACC; however, local recurrences are common with no effective chemotherapeutic regimens known. Accordingly, further understanding of the molecular basis of ACC that can lead to the development of newer effective therapies is urgently required.

Recently, a recurrent fusion of *MYB* and *NFIB*

Corresponding authors: Yukari Endo, Satoshi Kuwamoto
enyu@med.tottori-u.ac.jp (Y.E.)
s.quamoto@gmail.com (S.K.)
Received 2018 November 29
Accepted 2019 January 5
Abbreviations: ACC, adenoid cystic carcinoma; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence *in-situ* hybridization; FITC, fluorescein isothiocyanate; RT-PCR, reverse-transcription polymerase chain reaction; SSC, saline-sodium citrate

transcription factor genes resulting from a translocation t(6;9) was discovered in ACC, and it was found to be associated with the overexpression of the fusion transcript containing the 5′ end of *MYB*.[2] This *MYB–NFIB* fusion was later confirmed to be present in approximately 50% of ACCs, but not in non-ACC salivary gland tumors, which indicates that it is a specific and common driver of ACC tumorigenesis.[3–6] Moreover, the overexpression of full-length or the 5′ end of *MYB* was observed in 65%–80% of ACCs, including in those without *MYB–NFIB* fusion, which suggests the presence of additional mechanisms for MYB overexpression in ACC. Indeed, Drier et al. have recently demonstrated that chromosomal rearrangements that reposition super-enhancers in *NFIB* or other gene loci to the vicinity of *MYB* result in the formation of a positive feedback loop that sustains MYB expression and suggested that these rearrangements are the unifying features of ACCs overexpressing MYB with or without *MYB–NFIB* fusion.[7]

Meanwhile, several studies have revealed that *MYBL1*—another member of the *MYB* gene family—is also involved in ACC tumorigenesis. In fact, in 2015, two research groups independently reported that alterations of this gene—mostly the formation of a *MYBL1–NFIB* fusion—were noted in a small subset of ACCs and were associated with the overexpression of the 5′ end of *MYBL1*.[8, 9] In addition, the genetic alterations involving *MYB* and *MYBL1* were found to occur in a mutually exclusive manner, which further supports that they are crucial events that occur early in the ACC tumorigenesis. Moreover, although previous studies observed another subset of ACCs that apparently did not possess structural aberrations in both *MYB* and *MYBL1* loci, Togashi et al. have recently suggested that nearly all ACCs actually harbor chromosomal rearrangements involving either of these two genes.[10] Using multiple methods including fluorescence *in-situ* hybridization (FISH) with conventional probe sets for *MYB* and *MYBL1* split as well as a wide-range *MYB* probe set covering a much broader region (approximately 20 Mb) around *MYB*, they found that 97 of the 100 ACCs harbored rearrangements in either *MYB* or *MYBL1* locus, and reported that 10 of them had atypical FISH patterns that were undetectable by ordinary FISH methods.

As described above, recent studies have rapidly uncovered molecular aberrations in ACC. However, their clinicopathological significance remains to be fully elucidated. Although some studies reported that cases with *MYB* rearrangements detected by conventional FISH showed a trend toward shorter disease-free survival,[5, 11] the results were not statistically significant. Moreover, there are few studies investigating the correlation between *MYBL1* rearrangements and patient outcome. In addition, although *MYB* and *MYBL1* rearrangements were detected in ACCs of various organs throughout the body, the association between these rearrangements and specific body sites remain to be fully characterized. In the present study, we investigated the clinicopathological and molecular features of 36 cases of ACCs, with an emphasis on the significance of the *MYBL1* rearrangement.

MATERIALS AND METHODS

Case selection

The present study was approved by the Institutional Review Board of Tottori University, Faculty of Medicine (1610A121) and Tottori Prefectural Central Hospital (2017-15). We examined 36 cases of ACC diagnosed at the Tottori University Hospital or Tottori Prefectural Central Hospital. Cases in which formalin-fixed paraffin-embedded (FFPE) specimens were available were included in the study. In all cases, the histological slides were reviewed by a board-certified pathologist of the Japanese Society of Pathology (S.K.) and the diagnosis was confirmed. Clinicopathological data of the patients were obtained from the data system of each facility. As for alcohol consumption status, the patients were classified into non/occasional drinkers or daily drinkers based on the patients' self-report. The histology of ACC was graded into two categories: grade 1 or 2 (the solid component was < 50%) and grade 3 (the solid component was ≥ 50%).[10] Tumor extent was classified as localized (which is confined to the primary organ at diagnosis) or advanced (which directly invades the neighboring organs and/or metastasizes to regional lymph nodes or distant organs at diagnosis).

Reverse-transcription polymerase chain reaction (RT-PCR) for *MYB–NFIB* and *MYBL1–NFIB* fusions

To detect *MYB–NFIB* and *MYBL1–NFIB* fusions, RT-PCR was performed using the following procedure: total RNA was extracted from each archival tissue using the PureLink FFPE RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) and was converted into cDNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). PCR was performed using the TaKaRa Ex Taq Hot Start Version (Takara Bio, Kusatsu, Shiga, Japan), where the program consisted of an initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation (94°C, 30 s), annealing (57°C, 30 s), and extension (72°C, 1 min). The primer sequences are shown in Table 1. The exon numbers of *MYB*, *MYBL1*, and *NFIB* were defined according to the GenBank accession numbers U22376.1,

Table 1. RT-PCR primers used in the present study

Primers		Direction	Nucleotide sequence (5′—> 3′)
MYB	Exon 8	Forward	ACCGAGAATGAGCTAAAAGGACA
	Exon 9	Forward	ACCTCTTAGAATTTGCAGAAACAC
	Exon 10	Forward	AGAGACCAGACTGTGAAAACTCA
	Exon 11	Forward	ACACTTGGAAAGGAACCAAGCTA
	Exon 12	Forward	AATGGACCACCCTTACTGAAGA
	Exon 13	Forward	ACACTTGGAAAGGAACCAAGCTA
	Exon 14	Forward	ACAGTACCTAAAAACAGGTCCC
MYBL1	Exon 8	Forward	GTCAGCTGAGAATGAAGTTAGAAGA
	Exon 14	Forward	AGAAGAATCAGGCACTCAACTG
NFIB	Exon 9	Reverse	ACACTTGGAAAGGAACCAAGCTA

RT-PCR, reverse-transcription polymerase chain reaction.

NM_001080416.3, and NM_001190738.1, respectively.[12] A 98-bp partial cDNA of β-actin was amplified as an internal control. The PCR products were electrophoresed on 2.0% agarose gels, stained with ethidium bromide, and visualized under UV illumination. The results were interpreted based on the product size. In some cases, Sanger sequencing was also performed for confirming that the fusion products were obtained as expected.

Immunohistochemistry

Immunohistochemical staining for MYB was performed on an automatic immunostainer (Autostainer Link 48, Dako, Glostrup, Denmark) using an anti-MYB rabbit monoclonal antibody (clone EP769Y, 1:200 dilution, Abcam, Cambridge, UK). Antigen retrieval was performed by incubating the slides at 97°C for 20 min in citrate buffer (pH 6.0). The staining results was recorded as strong when ≥ 5% of myoepithelial cells in the tumor showed a distinct nuclear immunoreactivity, and as weak/negative when no or only weak immunoreactivity was observed throughout the tumor specimen. Because a weak immunoreactivity was observed in a subset of various non-ACC tumors,[5] we considered that only a strong MYB immunoreactivity was significant. Moreover, as MYB immunoreactivity in ACC is known to be confined to myoepithelial cells,[5] we did not evaluate immunoreactivity in luminal epithelial cells in tumors.

Fluorescence *in-situ* hybridization (FISH) for *MYBL1* rearrangements

FISH analysis for detecting *MYBL1* rearrangements was performed using bacterial artificial chromosome clone-derived probes covering the 3′ region (RP11-346I3) and the 5′ region (RP11-110J18) of the *MYBL1* locus, where the probes were labeled with biotin and digoxigenin, respectively. The procedure was conducted as previously described.[13, 14] Briefly, after sections were deparaffinized, heat-treated, and digested with pepsin, denaturation was performed by placing the sections in 70% formamide/2X SSC solution. Then, hybridization was conducted by incubating the sections with the above probes at 37°C for approximately 48 h. After the post-hybridization washing, the biotin-labeled and digoxigenin-labeled probes were detected using avidin-FITC (green) and anti-digoxigenin-rhodamine (red), respectively. The results were interpreted as positive (*MYBL1* rearranged) when > 10% of cells that were observed exhibited at least one break-apart signal or a separation between green and red signals of more than two signal widths.[15]

Statistical analysis

According to the results of the above analysis, 31 of the 36 cases were classified as either *MYBL1* or *MYB* genetic group (see Results for detail), and then clinicopathological factors were compared between these groups. Fisher's exact test, chi-square test, and Wilcoxon rank sum test were used, as appropriate. Difference in the disease-specific survivals between the two groups was investigated using the Kaplan–Meier method and the log-rank test. In addition, using similar methods, we also investigated the significance of the "length" of *MYB*/*MYBL1*–*NFIB* fusion transcripts among *MYB*/*MYBL1*–*NFIB*-positive cases (see Results for detail). All statistical analyses were performed using SPSS version 24 (IBM Corp., Armonk, NY). The results were considered statistically significant at $P < 0.05$.

RESULTS

Clinicopathological characteristics

The subjects included 9 men and 27 women of a median age at the time of diagnosis of 60.5 years (age range: 32–86 years). Tumors occurred in the major salivary glands in 16 cases (parotid = 7, sublingual = 5 and submandibular

Table 2. Results of immunohistochemistry and molecular genetic analysis of 36 cases of adenoid cystic carcinoma

Case No.	Age	Gender	Location	IHC	RT-PCR*		FISH†	Genetic group‡
				MYB	Fusion	Fusion pattern Exon–Exon	*MYBL1* split	
ACC1	35	F	Nasal cavity	Strong	–	–	Negative	*MYB*
ACC2	48	F	Parotid gland	Strong	*MYB–NFIB*	Exon 12–Exon 9	n.d.	*MYB*
ACC3	82	F	Ear canal	Strong	–	–	Negative	*MYB*
ACC4	61	F	Sublingual gland	None	*MYBL1–NFIB*	Exon 8–Exon 8C Exon 8–Exon 9	n.d.	*MYBL1*
ACC5	32	F	Paranasal cavity	Strong	*MYB–NFIB*	Exon 14–Exon 8C Exon 14–Exon 9	n.d.	*MYB*
ACC6	49	F	Nasal cavity	Strong	*MYB–NFIB*	Exon 14–Exon 8C	n.d.	*MYB*
ACC7	66	M	Sublingual gland	Weak	*MYBL1–NFIB*	Exon 8–Exon 8C Exon 8–Exon 9	Positive	*MYBL1*
ACC8	48	M	Parotid	Strong	*MYB–NFIB*	Exon 9–Exon 8C Exon 9–Exon 9	n.d.	*MYB*
ACC9	37	F	Nasal cavity	Strong	–	–	Negative	*MYB*
ACC10	62	F	Paranasal cavity	None	*MYBL1–NFIB*	Exon 14–Exon 8C Exon 14–Exon 9	n.d.	*MYBL1*
ACC11	69	F	Sublingual gland	None	*MYBL1–NFIB*	Exon 8–Exon 8C	n.d.	*MYBL1*
ACC12	50	M	Sublingual gland	Weak	*MYBL1–NFIB*	Exon 8–Exon 8C Exon 8–Exon 9	n.d.	*MYBL1*
ACC13	63	F	Trachea	Strong	*MYB–NFIB*	Exon 10–Exon 9	n.d.	*MYB*
ACC14	54	F	Paranasal cavity	Strong	–	–	failed	*MYB*
ACC15	81	F	Submandibular gland	Strong	–	–	failed	*MYB*
ACC16	53	F	Nasal cavity	Strong	*MYB–NFIB*	Exon 12–Exon 9	n.d.	*MYB*
ACC17	39	F	Oropharynx	Weak	–	–	failed	unknown
ACC18	84	M	Trachea	Weak	–	–	failed	unknown
ACC19	79	F	Sublingual gland	None	*MYBL1–NFIB*	Exon 8–Exon 9	n.d.	*MYBL1*
ACC20	45	M	Submandibular gland	None	–	–	failed	unknown
ACC21	57	F	Submandibular gland	Weak	*MYB–NFIB*	Exon 12–Exon 9	n.d.	*MYB*
ACC22	55	F	Parotid gland	None	–	–	failed	unknown
ACC23	77	F	Submandibular gland	Strong	–	–	Negative	*MYB*
ACC24	62	F	Orbital	Strong	–	–	failed	*MYB*
ACC25	71	F	Parotid gland	Strong	–	–	Negative	*MYB*
ACC26	73	F	Nasal cavity	Strong	–	–	failed	*MYB*
ACC27	63	M	Floor of mouth	Strong	–	–	Negative	*MYB*
ACC28	60	F	Palate	Strong	*MYB–NFIB*	Exon 14–Exon 9	n.d.	*MYB*
ACC29	73	M	Nasal cavity	Weak	–	–	Negative	unknown
ACC30	33	M	Nasal cavity	Strong	–	–	Negative	*MYB*
ACC31	66	M	Lung	Strong	*MYB–NFIB*	Exon 14–Exon 8C Exon 14–Exon 9	n.d.	*MYB*
ACC32	69	F	Parotid gland	Strong	*MYB–NFIB*	Exon 14–Exon 8C Exon 14–Exon 9	n.d.	*MYB*
ACC33	86	F	Parotid gland	Strong	–	–	Negative	*MYB*
ACC34	34	F	Palate	Weak	*MYBL1–NFIB*	Exon 8–Exon 8C Exon 8–Exon 9	n.d.	*MYBL1*
ACC35	59	F	Parotid gland	Strong	–	–	Negative	*MYB*
ACC36	74	F	Trachea	Strong	–	–	n.d.	*MYB*

*"–" indicates that no fusions were detected. †Failed indicates that the FISH result was uninterpretable (no signals observed, etc.). ‡Genetic group was determined according to the results of IHC, RT-PCR and FISH (see Results for detail). Unknown indicates that the results were insufficient to subclassify the case. ACC, adenoid cystic carcinoma; F, female; FISH, fluorescence *in-situ* hybridization; IHC, Immunohistochemistry; M, male; n.d., not done; RT-PCR, reverse-transcription polymerase chain reaction.

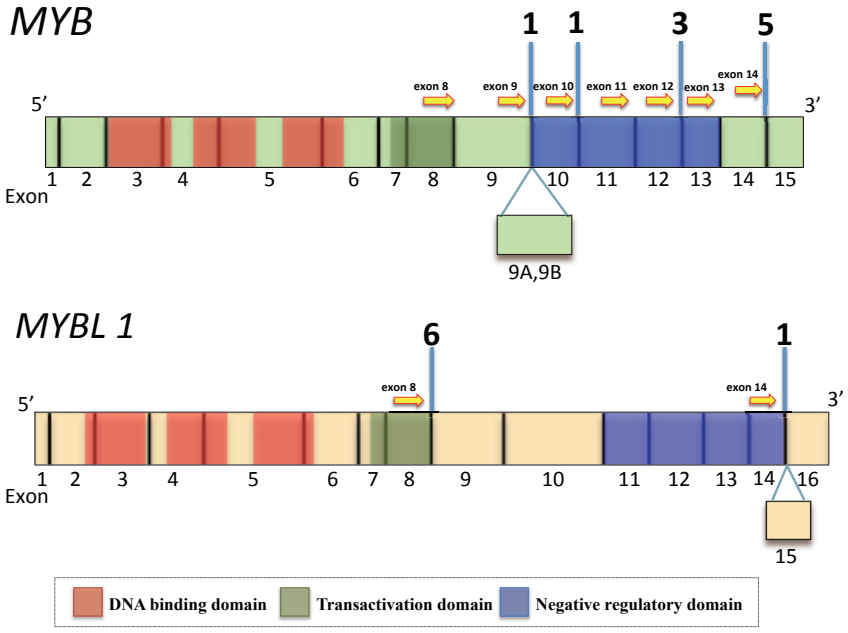

Fig. 1. Breakpoints of *MYB* and *MYBL1* within the *MYB–NFIB* and *MYBL1–NFIB* fusion transcripts detected by reverse-transcription polymerase chain reaction. Yellow arrows indicate the positions of the primers used in the present study (not to scale). Blue vertical bars indicate the positions of the breakpoints, with the number of cases being indicated above them.

= 4); nasal/paranasal cavity in 10; bronchial trees in 4; palate in 2, and the floor of mouth, ear, oropharynx and orbital cavity in 1 each. Of these, 25 patients had localized disease, and the remaining 11 had advanced disease at the time of diagnosis. Surgical removal of the tumor was performed in 26 cases, with 11 of them receiving postoperative radiation therapy. Eight patients received radiation therapy alone, and the other 2 received only palliative therapy. Histologically, 30 (83.3%) tumors were classified as grade 1/2 and 6 (16.6%) as grade 3. Seven patients died during the follow-up period, and six of them died of ACC. The median follow-up period for patients who were alive at the last follow-up was 90.0 months (range: 13–233 months).

RT-PCR

Internal control cDNA (β-actin) was successfully amplified in all 36 cases. Among them, fusion transcripts of *MYB–NFIB* and *MYBL1–NFIB* were detected in 10 (27.8%) and 7 (19.4%) cases, respectively, and they were mutually exclusive. The fusion patterns differed from one case to another in both *MYB–NFIB*- and *MYBL1–NFIB*-positive groups (Table 2, Fig. 1). In nine cases, two amplification products with different lengths were detected, where the longer products corresponded to alternatively spliced transcript variants that contained *NFIB* exon 8C. In the remaining 19 cases, no fusion transcripts were detected in this assay.

Immunohistochemistry

Immunohistochemically, a strong expression of MYB was observed in 23 (63.9%) of 36 cases, whereas no or only weak expression was noted in the remaining 13 cases (Table 2, Fig. 2). Of the 10 cases in which *MYB–NFIB* fusion transcripts were detected by RT-PCR, a strong expression of MYB was observed in 9 (90%) cases. On the other hand, among the seven cases in which *MYBL1–NFIB* fusion transcripts were detected by RT-PCR, none showed strong MYB expression. In many MYB-positive cases, the staining was often limited to the periphery of the tumors. It was judged from the previous reports that this occurred because formalin fixation was delayed and the half-life of MYB protein was short.[6] However, in these peripheral areas, strong MYB expression was observed at least 40% of the tumor myoepithelial cells in all MYB-positive ACCs.

FISH analysis

FISH analysis for *MYBL1* rearrangements was performed in 18 of the 19 cases in which no fusions were detected in RT-PCR (in the remaining 1 case, no specimen was available for FISH analysis). In addition, a selected *MYBL1–NFIB*-positive case determined by RT-PCR (ACC7) was included in the analysis as a positive control. Of these 19 cases, the results were interpretable in 11 cases, and *MYBL1* rearrangements were detected only in the *MYBL1–NFIB*-positive case (Table 2; Figs.

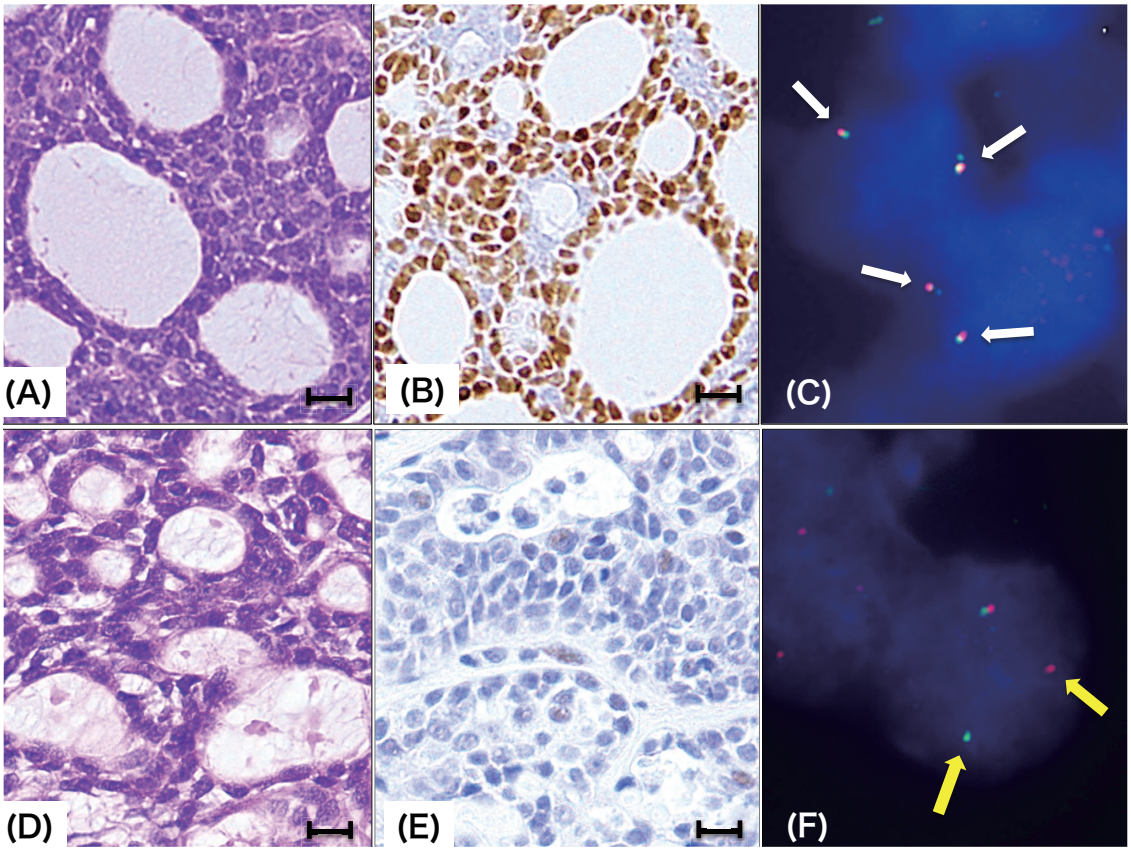

Fig. 2. Representative images for histology (**A** and **D**, hematoxylin-eosin), MYB immunohistochemistry (**B** and **E**), and *MYBL1* break-apart FISH (**C** and **F**) of adenoid cystic carcinomas. **A**–**C**: ACC3, in which neither *MYB–NFIB* nor *MYBL1–NFIB* were detected by RT-PCR, showing a typical cribriform histology (**A**), a strong immunoreactivity for MYB (**B**), and no evidence of *MYBL1* rearrangement (white arrows) (**C**). **D**–**F**: ACC7, in which *MYBL1–NFIB* fusion was detected by RT-PCR, showing a typical cribriform histology (**D**), only a weak immunoreactivity for MYB (**E**), and *MYBL1* rearrangement (yellow arrows; red, 5′ *MYBL1*; green, 3′ *MYBL1*) (**F**). Bars = 10 μm. ACC, adenoid cystic carcinoma; FISH, fluorescence in-situ hybridization; RT-PCR, reverse-transcription polymerase chain reaction.

Table 3. Clinicopathological comparison between *MYBL1* and *MYB* genetic groups (Analysis of 31 cases)

		Total	Genetic group		*P* value†
			MYBL1 (%)	*MYB* (%)	
Number of cases		31	7 (22.6)	24 (77.4)	
Age		59.5 ± 14.7	64.6 ± 15.6	58.0 ±14.7	0.228
Gender	Male	6	1 (16.7)	5 (83.3)	1.000
	Female	25	6 (24.0)	19 (76.0)	
Size (cm)		2.8 ± 1.0	2.9 ± 0.6	2.8 ± 1.1	0.448
Smoking	No	21	5 (23.8)	16 (76.2)	0.599
	Yes	10	2 (20.0)	8 (80.0)	
Drinking	Occasional/non	24	5 (20.8)	19 (79.2)	1.000
	Daily	4	1 (25.0)	3 (75.0)	
Primary tumor site (1)	Major salivary glands	14	5 (35.7)	9 (64.3)	0.197
	Minor salivary glands	11	2 (18.2)	9 (81.8)	
	Others‡	6	0 (0.0)	6 (100.0)	
Primary tumor site (2)	Mandibular regions§	9	5 (55.6)	4 (44.4)	0.012*
	Others	22	2 (9.1)	20 (90.9)	
Histological grade	Grade 1/2	27	6 (22.2)	21 (77.8)	0.662
	Grade 3	4	1 (25.0)	3 (75.0)	
Tumor extent	Advanced	9	2 (22.2)	7 (77.8)	1.000
	Localized	22	5 (22.7)	17 (77.3)	

*Statistically significant. †Fisher's exact test, chi-square test, and Wilcoxon rank sum test. ‡Include external auditory canal, pharynx, and orbital. §The mandibular regions contain the following parts: Submandibular gland, Sublingual gland, and Floor of mouth.

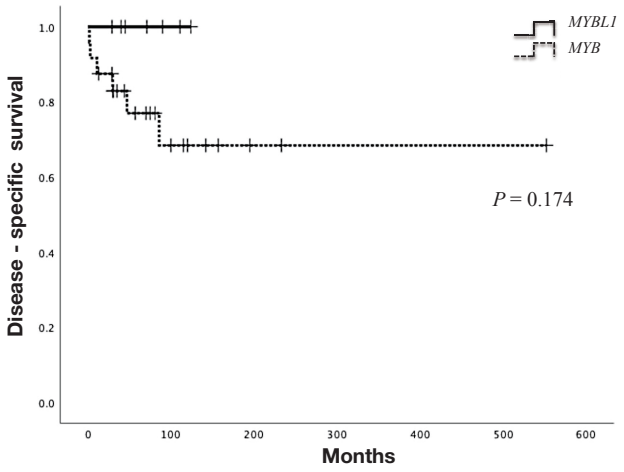

Fig. 3. Kaplan–Meier curves depicting disease-specific survival for patients with adenoid cystic carcinoma stratified by genetic group (*MYBL1*, $n = 7$; *MYB*, $n = 24$). Log-rank test, $P = 0.174$.

2C and F). The other 10 cases showed no evidence of *MYBL1* rearrangement.

Correlation between gene alterations and clinicopathological factors

From the recent observation that almost all ACCs harbor chromosomal rearrangements involving either *MYB* or *MYBL1*,[10] we hypothesized that most tumors in the present study also actually harbored genetic alterations involving either of these 2 genes. Moreover, as previous studies revealed that the overexpression of *MYB* and *MYBL1* in ACCs was mutually exclusive and highly correlated with genetic alterations involving *MYB* and *MYBL1*, respectively,[9, 10] we considered that tumors showing strong MYB expression in immunohistochemistry can be regarded as those that actually harbor genetic alterations involving *MYB*. Accordingly, we compared the clinicopathological factors between cases with *MYBL1* alterations (*MYBL1* group, $n = 7$) and those with *MYB–NFIB* fusions and/or strong MYB expression (*MYB* group, $n = 24$). As a result, however, we found no significant differences between these two groups with regard to patient age, gender, smoking and drinking status, tumor size, primary tumor site, histological grade, and tumor extent at the time of diagnosis (Table 3). Disease-specific survival was also not significantly different between these 2 groups, although there was a trend toward a more favorable prognosis in patients with *MYBL1* alterations ($P = 0.174$, log-rank test) (Fig. 3).

Detailed analysis of the association between gene alterations and tumor primary site

During analysis of the correlation between gene alterations and clinicopathological factors, we noticed that tumors with *MYBL1* alterations predominantly occurred in the mandibular regions (i.e., on the floor of the mouth, sublingual glands, and submandibular glands). Therefore, we decided to investigate this point in greater detail and found that tumors with *MYBL1* alterations occurred significantly frequently in these regions as than in those with *MYB–NFIB* fusions and/or strong MYB expression ($P = 0.012$) (Table 3). Moreover, review of the literature from this perspective revealed a similar tendency in a previous study,[9] with the difference being statistically significant. However, in the other studies, such a tendency was not observed.[8, 10, 14, 16]

Impact of the length of *MYB/MYBL1–NFIB* fusion transcripts on clinicopathological factors

Previous studies revealed that the gene expression profiles in ACCs with *MYB*/*MYBL1* rearrangements were different between those with "long fusions" containing the C-terminal negative regulatory domains of *MYB*/*MYBL1* and those with "short fusions" that lacked such domains.[9] Moreover, it has also been demonstrated in a reporter gene assay that *MYB*/*MYBL1–NFIB* fusion constructs that lack the negative regulatory domains displayed significantly higher transcriptional activity than those containing the domains.[8] Therefore, we additionally investigated clinicopathological differences between ACCs with "long fusions" containing breakpoints after exon 12 of *MYB*/*MYBL1* ($n = 9$) and those with "short fusions" containing breakpoints before exon 12 ($n = 8$). Consequently, however, we observed no significant differences between cases with long and short fusions in any of the clinicopathological factors, including prognosis (data not shown).

DISCUSSION

In the present study, we confirmed several previous observations regarding molecular aberrations in ACCs, as follows: i) a substantial subset of ACCs harbor *MYB–NFIB* or *MYBL1–NFIB* fusions, ii) gene alterations involving *MYB* and *MYBL1* occur in a mutually exclusive manner, and iii) overexpression of MYB protein is well-correlated with *MYB* alterations and not observed in tumors with *MYBL1* alterations. These findings clearly indicate that *MYB* and *MYBL1* alterations are the crucial events that occur early in the ACC tumorigenesis. Furthermore, recent studies demonstrated that chromosomal rearrangements involving *MYB* in ACC create a positive feedback loop that sustains MYB expression,[7] which suggests that the overexpression of MYB (and probably also MYBL1) is indispensable for the growth and maintenance of ACC cells. Therefore, the inhibition

of MYB/MYBL1 or their downstream signaling pathways is a promising novel treatment of ACC, and such molecular targeted therapies may contribute to the improvement of the survival and quality of life of patients with unresectable/metastatic ACCs.

It is notable that *MYBL1* alterations were significantly frequently noted in ACCs that arose from the mandibular regions in our cohort. To the best of our knowledge, this is the first study in which the relationship of genetic alterations and specific body sites was suggested in ACCs. Inspired by this observation, we conducted a literature review from this perspective and found a similar result in one past study,[9] although such a difference was not observed in other studies.[8, 10, 14, 16] The reason for this discrepancy is unclear and may be attributable to chance or sampling bias. However, the correlation of specific gene fusions and body sites has been suggested in several other tumors. For example, the *EML4-ALK* fusion is known to be found in approximately 4% of lung adenocarcinomas, but is generally not found in tumors of other organs,[17] although it has been reported in a few colorectal and breast cancers and renal cell carcinomas.[18, 19] Similarly, in inflammatory myofibroblastic tumors, most cases are shown to harbor gene fusions involving *ALK* or *ROS1*, where the *EML4-ALK* is predominantly found in tumors arising in the lungs.[20, 21] Therefore, similar to the relationship between *EML4-ALK* fusion and lungs, there may be unknown mechanisms or etiological factors that result in the formation of *MYBL1*-rearranged tumors in the mandibular regions. Further studies are hence warranted to assess this point in greater detail.

The potential relationship of *MYBL1* alterations and the mandibular regions has some clinical significance. In a previous study,[22] an immunohistochemical panel including MYB was reported to be useful in distinguishing ACCs from pleomorphic adenomas and other salivary gland neoplasms. However, because ACCs with *MYBL1* alterations never exhibit a strong MYB expression, such an immunohistochemical panel may be less useful in assessing tumors arising in the mandibular regions. Accordingly, it is advisable for pathologists and cytopathologists to be aware of this potential pitfall, since the diagnosis of salivary gland tumors can sometimes be challenging, especially in fine-needle aspiration cytology or small biopsy specimens.

It is difficult to explain why ACCs with *MYBL1* alterations are frequent in the mandibular regions. This is because that mechanisms causing the formation of specific gene fusion remain largely unknown, although chromosome translocations in general are known to be caused by inappropriate religation of two DNA double-strand breaks generated by endogenous or exogenous sources.[23] Possible explanations for the association between *MYBL1* alterations and the mandibular regions include that the break in the *MYBL1* locus may be resulted from exposure to some kinds of exogenous agents that are taken orally. However, we did not find any correlations between *MYBL1* alterations and patients' smoking history and alcohol consumption status. Another explanation is that *MYBL1* may be actively expressed in specific cells in the mandibular regions, and the gene locus is loosely packed and thus is susceptible to physical DNA damage and subsequent erroneous repair.

Despite no statistically significant difference noted, we found a trend toward a more favorable prognosis in patients in the *MYBL1* group as compared with those in the *MYB* group. Notably, no patients in the *MYBL1* group died of ACC during the follow-up period (range: 29–124 months; median: 71 months), whereas 6 (28.6%) of the 21 patients in the *MYB* group died of ACC at 2–86 months after diagnosis. These results are in line with those of several previous studies. For example, Mitani et al. have noted a significantly shorter survival in patients with *MYB* alterations as compared to that in patients with *MYBL1* alterations.[9] Moreover, prior to the discovery of *MYBL1* alterations in ACCs, the same study group have reported that patients high MYB expression showed a significantly poorer prognosis compared to those with low MYB expression (presumably the latter group consisted mainly of patients with high MYBL1 expression).[4] Similarly, West et al. have reported that ACCs with *MYB* translocation showed a trend (not significant) toward higher local relapse rates as compared with those with no abnormalities detected in FISH.[5] Although data may be insufficient to draw a definitive conclusion, these results suggest that ACCs with *MYB* alterations behave more aggressively than those with *MYBL1* alterations, and that there are some differences in the biological effects between *MYB* and *MYBL1* altered in ACC cells.

There are few studies regarding to the difference in treatment response between ACCs with *MYB* and *MYBL1* alterations. This is mainly because that the molecular aberrations in ACC are only recently identified, and clinical research is yet limited due to the rarity and slow growth kinetics of the tumors.[24] However, since previous studies have shown consistently low response rates to chemotherapy for metastatic ACCs,[24] it is unlikely that tumors with *MYB* and *MYBL1* alterations respond differently to cytotoxic chemotherapies. On the other hand, recent and ongoing clinical trials evaluating efficacy of molecularly targeted therapies and cancer immunotherapies may reveal different response rates

among different genetic groups of ACCs. For example, in a recent report of clinical study of axitinib (a receptor tyrosine kinase inhibitor) for patients with incurable ACC, Ho et al. noted that a trend (not significant) toward superior progression-free survival was observed with the *MYB*/*NFIB* rearrangement.[25] Future research may delineate the impact of the type of genetic aberrations on medical therapies for patients with incurable ACC.

In this study, there were 13 ACCs that showed a strong MYB expression in immunohistochemistry despite no *MYB–NFIB* fusions detected by RT-PCR. In these cases, molecular aberrations different from the *MYB–NFIB* fusion formation are considered to have resulted in MYB overexpression. For example, previous studies have demonstrated that i): fusions between *MYB* and genes other than *NFIB* (such as *PDCD1LG2*, *EFR3A*, *TGFBR3* and *RAD51B*),[4, 7] ii): fusions between *MYB* and intergenic sequences downstream of the 3′ untranslated region of *NFIB*,[4, 10] and iii): fusions between the 5′ end of *NFIB* and genes around the *MYB* locus (such as *NKAIN2* and *AIG1*) [4, 9] were associated with MYB overexpression in ACCs. As the unifying features of these various genetic alterations, Drier et al. have recently advocated a model that chromosomal rearrangements that relocate super-enhancers within the loci of *NFIB* or other genes to the vicinity of *MYB* results in the formation of a positive feedback loop that sustains MYB expression.[7] It is highly likely that such rearrangements have also occurred in the 13 cases of our cohort.

There are several limitations to the present study. First, the size of the study group is relatively small, as only 36 patients were included in the study. Second, gene alterations involving *MYB* and *MYBL1* were not completely investigated. Although *MYB*/*MYBL1–NFIB* fusion is the dominant pattern of gene alterations involving *MYB*/*MYBL1* in ACCs, since other alterations noted in some ACCs are diverse, we were unable to detect such alterations in the present study using FFPE specimens. Moreover, we performed *MYBL1* break-apart FISH to identify additional tumors with *MYBL1* alterations, but the results were interpretable only in 11 of the 19 tumors. Accordingly, the importance of appropriate fixation and storage of FFPE tissues for molecular analysis was reaffirmed through the present study.

In conclusion, we conducted a clinicopathological and molecular analysis using 36 cases of ACC and noted a potential relationship between *MYBL1* alterations and the mandibular regions. On survival analysis, we observed a trend toward a longer disease-specific survival in patients with *MYBL1* alterations as compared with that in patients with *MYB–NFIB* fusions and/or strong MYB expression. The shortcomings of the study included the small sample size, which may have resulted in the low statistical power to detect some of the differences between tumors with *MYB* and *MYBL1* alterations. Further research with larger cohorts is thus required to verify the results in the present study.

Acknowledgments: This work was supported by the research fund granted by director of Tottori University Hospital.

The authors declare no conflict of interest.

REFERENCES

1 Stenman G, Licitra L, Said-Al-Naief N, van Zante A, Yarbrough WG. Adenoid cystic carcinoma. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ, editors. (WHO classification of head and neck tumours, 4nd ed. Lyon: IARC Press; 2017. p. 164-5.)

2 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. PMID: 19841262; PMCID: PMC2773970.

3 Mitani Y, Li J, Rao PH, Zhao YJ, Bell D, Lippman SM, et al. 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. PMID: 20702610; PMCID: PMC5862426.

4 Mitani Y, Rao PH, Futreal PA, Roberts DB, Stephens PJ, Zhao YJ, et al. Novel chromosomal rearrangements and break points 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. PMID: 21976542; PMCID: PMC3225955.

5 West RB, Kong C, Clarke N, Gilks T, Lipsick JS, Cao H, et al. MYB expression and translocation in adenoid cystic carcinomas and other salivary gland tumors with clinicopathologic correlation. Am J Surg Pathol. 2011;35:92-9. PMID: 21164292; PMCID: PMC3127258.

6 Brill LB 2nd, Kanner WA, Fehr A, Andrén Y, Moskaluk CA, Löning T, et al. Analysis of MYB expression and MYB-NFIB gene fusions in adenoid cystic carcinoma and other salivary neoplasms. Mod Pathol. 2011;24:1169-76. PMID: 21572406.

7 Drier Y, Cotton MJ, Williamson KE, Gillespie SM, Ryan RJ, Kluk MJ, et al. An oncogenic MYB feedback loop drives alternate cell fates in adenoid cystic carcinoma. Nat Genet. 2016;48:265-72. PMID: 26829750; PMCID: PMC4767593.

8 Brayer KJ, Frerich CA, Kang H, Ness SA. Recurrent Fusions in MYB and MYBL1 Define a Common, Transcription Factor-Driven Oncogenic Pathway in Salivary Gland Adenoid Cystic Carcinoma. Cancer Discov. 2016;6:176-87. PMID: 26631070; PMCID: PMC4744535.

9 Mitani Y, Liu B, Rao PH, Borra VJ, Zafereo M, Weber RS, et al. Novel MYBL1 Gene Rearrangements with Recurrent MYBL1-NFIB Fusions in Salivary Adenoid Cystic Carcinomas Lacking t(6;9) Translocations. Clin Cancer Res. 2016;22:725-33. PMID: 26631609; PMCID: PMC4807116.

10 Togashi Y, Dobashi A, Sakata S, Sato Y, Baba S, Seto A, et al. MYB and MYBL1 in adenoid cystic carcinoma: diversity in the mode of genomic rearrangement and transcripts. Mod

Pathol. 2018;31:934-46. PMID: 29410490.
11 Rettig EM, Tan M, Ling S, Yonescu R, Bishop JA, Fakhry C, et al. MYB rearrangement and clinicopathologic characteristics in head and neck adenoid cystic carcinoma. Laryngoscope. 2015;12:E292-9. PMID: 25963073; PMCID: PMC4546892.
12 Wysocki PT, Izumchenko E, Meir J, Ha PK, Sidransky D, Brait M. Adenoid cystic carcinoma: emerging role of translocations and gene fusions. Oncotarget. 2016;7:66239-54. PMID: 27533466; PMCID: PMC5323230.
13 Kugoh H, Shigenami K, Funaki K, Barrett JC, Oshimura M. Human chromosome 5 carries a putative telomerase repressor gene. Genes Chromosomes Cancer. 2003;36:37-47. PMID: 12461748.
14 Fujii K, Murase T, Beppu S, Saida K, Takino H, Masaki A, et al. MYB, MYBL1, MYBL2 and NFIB gene alterations and MYC overexpression in salivary gland adenoid cystic carcinoma. Histopathology. 2017;71:823-34. PMID: 28594149.
15 Kim J, Geyer FC, Martelotto LG, Ng CK, Lim RS, Selenica P, et al. MYBL1 rearrangements and MYB amplification in breast adenoid cystic carcinomas lacking the MYB–NFIB fusion gene. J Pathol. 2018;244:143-50. PMID: 29149504; PMID: 29149504.
16 Rettig EM, Talbot CC Jr, Sausen M, Jones S, Bishop JA, Wood LD, et al. Whole-Genome Sequencing of Salivary Gland Adenoid Cystic Carcinoma. Cancer Prev Res (Phila). 2016;9:265-74. PMID: 26862087; PMCID: PMC4818686.
17 Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res. 2008;14:6618-24. PMID: 18927303.
18 Lin E, Li L, Guan Y, Soriano R, Rivers CS, Mohan S, et al. Exon array profiling detects EML4-ALK fusion in breast, colorectal, and non-small cell lung cancers. Mol Cancer Res. 2009;7:1466-76. PMID: 19737969.
19 Yu W, Wang Y, Jiang Y, Zhang W, Li Y. Genetic analysis and clinicopathological features of ALK-rearranged renal cell carcinoma in a large series of resected Chinese renal cell carcinoma patients and literature review. Histopathology. 2017;71:53-62. PMID: 28199742.
20 Lovly CM, Gupta A, Lipson D, Otto G, Brennan T, Chung CT, et al. Inflammatory myofibroblastic tumors harbor multiple potentially actionable kinase fusions. Cancer Discov. 2014;4:889-95. PMID: 24875859; PMCID: PMC4125481.
21 Antonescu CR, Suurmeijer AJ, Zhang L, Sung YS, Jungbluth AA, Travis WD, et al. Molecular characterization of inflammatory myofibroblastic tumors with frequent ALK and ROS1 gene fusions and rare novel RET rearrangement. Am J Surg Pathol. 2015;39:957-67. PMID: 25723109; PMCID: PMC4465992.
22 Foo WC, Jo VY, Krane JF. Usefulness of translocation-associated immunohistochemical stains in the fine-needle aspiration diagnosis of salivary gland neoplasms. Cancer Cytopathol. 2016;124:397-405. PMID: 26882287.
23 Byrne M, Wray J, Reinert B, Wu Y, Nickoloff J, Lee SH, et al. Mechanisms of oncogenic chromosomal translocations. Ann N Y Acad Sci. 2014;1310:89-97. PMID: 24528169.
24 Dillon PM, Chakraborty S, Moskaluk CA, Joshi PJ, Thomas CY. Adenoid cystic carcinoma: a review of recent advances, molecular targets, and clinical trials. Head Neck. 2016;38:620-7. PMID: 25487882; PMCID: PMC6166139.
25 Ho AL, Dunn L, Sherman EJ, Fury MG, Baxi SS, Chandramohan R, et al. A phase II study of axitinib (AG-013736) in patients with incurable adenoid cystic carcinoma. Ann Oncol. 2016;27:1902-8. PMID: 27566443; PMCID: PMC5035791.