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Computer-aided automated evaluation of *MYB* rearrangement in 10 cases of adenoid cystic carcinoma using fluorescent in situ hybridization: a single-institution study

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Abstract :
Objective: The *MYB-NFIB* fusion gene has recently been detected in adenoid cystic carcinomas (ACCs) of the salivary gland and other organs. Detection of *MYB* rearrangement appears to be diagnostically valuable for ACCs, particularly in high-grade transformed ACCs (HGT-ACCs). However, a consensus cutoff value defining *MYB* rearrangement using fluorescence in situ hybridization (FISH) has not been established. The aim of this study was to investigate an appropriate evaluation of *MYB* rearrangement in ACCs using FISH.
Study Design: We retrospectively selected 10 ACC cases diagnosed between 2000 and 2015. FISH analysis was performed using formalin-fixed paraffin-embedded sections and a MetaSystems image analysis system.
Results: The involved sites were the major salivary gland (n=4), oral cavity (n=2), lip (n=1), nasal cavity (n=1), external acoustic meatus (n=1), and trachea (n=1). One case was of HGT-ACC. Six cases (60%) including the HGT-ACC showed positive *MYB* rearrangement, exhibiting over 85% split *MYB* signals. The other cases were negative, demonstrating fewer than 10% split *MYB* signals, as observed in non-neoplastic cells.
Conclusion: The *MYB* rearrangement cutoff value in ACC cells was 10%. *MYB* rearrangement heterogeneity in ACC cells appeared to not be present. This genetic study supported the diagnosis of HGT-ACC.

Keywords : Adenoid cystic carcinoma, MYB rearrangement, fluorescent in situ hybridization

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1. Introduction

Adenoid cystic carcinoma (ACC) is the second most common malignant salivary gland tumor and accounts for <10% of all salivary gland neoplasms [1]. ACCs can arise in other sites, such as the ear canal, lacrimal glands, sinonasal tract, tracheobronchial tissue, breast, skin, and vulva [1, 2]. Conventional ACCs histologically consist of epithelial and myoepithelial tumor cells forming cribriform, tubular, and solid patterns. ACCs can undergo high-grade transformation characterized by cellular atypia beyond that of conventional ACC and loss of biphasic epithelial-myoepithelial architecture [3]. Histological diagnosis of high-grade transformed ACC (HGT-ACC) is difficult, if the typical histological features of ACC cannot be detected.

In 2009, Persson et al. identified a novel gene fusion in ACCs of the breast, head, and neck; recurrent translocation t(6;9)(q22-23;p23-24) involves the *MYB* oncogene and the transcription factor gene *NFIB* [4]. Such cytogenetic abnormality has also been detected in pulmonary ACCs [5] but has not been shown in other salivary gland tumors [4, 6-8], invasive ductal carcinoma of the breast [4], or other histological subtypes of lung cancer [5]. Thus, the *MYB-NFIB* fusion gene has been postulated to be a tumor-specific cytogenetic abnormality of ACCs. Two studies have demonstrated with fluorescence in situ hybridization (FISH) that some HGT-ACCs exhibit *MYB* rearrangement [9, 10], suggesting that detection of this genetic event supports diagnosis of HGT-ACC.

The *MYB-NFIB* gene fusion is evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) or FISH. *MYB* FISH in ACCs detects an unusual translocation of *MYB*, in addition to the one involving NFIB [11]; thus, *MYB* FISH may detect a broader range of *MYB* translocations than RT-PCR. However, *MYB* FISH studies on ACC have been limited [4-6, 9-14], and the rate of split *MYB* signals has not been determined in positive *MYB* rearrangement cases. Furthermore, no consensus cutoff value defining *MYB* rearrangement in ACCs has been established using FISH, because cutoff values have differed in each study, ranging from 10% to 30% (and incidentally, the reasoning for selecting these values was not described) [9, 11, 12]. Thus, evaluation of ACC with *MYB* FISH may be problematic.

In the present study, we performed *MYB* FISH using formalin-fixed paraffin-embedded (FFPE) tissues from ACC cases diagnosed in our hospital and evaluated the cutoff value of split *MYB* signals defining positive *MYB* gene rearrangement. Split *MYB* signals were automatically interpreted using a MetaSystems image analysis system.

2. Materials and Methods

2.1 Consent

Comprehensive agreement for this study was obtained from all patients, and anonymized clinical information and pathological diagnosis were used for analyses. This study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board of the Japanese Red Cross Kochi Hospital (No. 218).

2.2 ACC cases and classification

We retrospectively analyzed 10 cases of patients with ACC treated or diagnosed in our hospital between 2000 and 2015. Clinical information was obtained from medical records and included age, sex, tumor location, UICC stage at diagnosis, treatment, disease-free survival (DFS), and overall survival (OS). Surgically resected tumors were pathologically evaluated in 9 of the cases, and the other case (Case 8) was diagnosed by biopsy. Follow-up was performed from the date of surgery or biopsy to September 2017. Pathological data included maximum size (invasive depth), conventional ACC component grade, presence of HGT component, and perineural invasion. The growth patterns of ACCs are classified as tubular, cribriform, and solid [1]. Conventional ACC component grade was assessed according to the

criteria developed by Szanto et al [15]: grade I was mostly tubular with some cribriform component, grade II was either entirely cribriform or tubular/cribriform with a <30% solid component, and grade III had any growth pattern with a >30% solid component. The HGT component was assessed according to the description by Seethala et al [3].

2.3 FISH for MYB rearrangement and automated analysis of the FISH signals

FFPE tumor blocks used for pathological diagnosis were selected from each tumor, as the blocks included the largest cross-sectional slice of each tumor. Four-μm-thick sections were prepared from the FFPE tumor blocks for FISH analysis. ZytoLight SPEC *MYB* Dual Color Break Apart Probes (ZytoVision, Bremerhaven, Germany) were used. Pretreatment, hybridization, and posthybridization washes were performed according to the manufacturer's protocols. Pretreatment and hybridization were performed with a VP-2000 Processor (Abbott Molecular, Tokyo, Japan) and a ThermoBrite system (Abbott Molecular), respectively. Fluorescence signals were automatically analyzed using the Metafer 4 version 3.10.4 imaging system (MetaSystems GmbH, Altlussheim, Germany). At least 100 nuclei of tumor cells and 50 nuclei of background non-tumor cells, such as endothelial cells, inflammatory cells, or fibroblasts, were scored in each case. Cells with two fusion signals (one orange and one green fluorochrome) were defined as normal. Cells harboring *MYB* rearrangements had one normal fusion signal, one orange signal, and one green signal.

2.4 Statistical analysis

The relationship between *MYB* rearrangement and clinical factors was evaluated by an unpaired t-test. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was defined at $p < 0.05$.

3. Results

Clinicopathological and genetic data from

Table 1. Clinicopathological and genetic data for the 10 cases of adenoid cystic carcinoma.

Case	Age/ Sex	Tumor location	Conventional ACC grade	High-grade transformed component	Maximal size (invasive depth)	PI	UICC stage	Treatment	DFS/OS (mo.)	*MYB* FISH (Split signal %)
1	45/M	Trachea	2	(-)	NA	(-)	NA	Re	43/43 (L)	96
2	67/F	Oral cavity	2	(-)	18 mm (8 mm)	(-)	T2N0M0	Re	92/92 (AWoD)	98
3	70/M	Oral cavity	1	(-)	22 mm (11 mm)	(+)	NA	Re	118/126 (L)	99
4	73/M	Lip	1	(-)	20 mm (11 mm)	(+)	T3N0M0	Re	43/43 (L)	86
5	46/F	Parotid gland	Not found	(+)	30 mm	(+)	T3N0M0	Re + Ra	29/29 (AWoD)	92
6	74/F	Parotid gland	2	(-)	15 mm	(-)	T3N0M0	Re + Ra	18/51 (AWoD)	99
7	35/F	Parotid gland	3	(-)	10 mm	(-)	T1N0M0	Re	NA/NA	8
8	87/F	Submandibular gland	2	(-)	38 mm	(-)	T3N0M1 (lung)	P	None/28 (AWD)	9
9	74/F	Nasal cavity	2	(-)	27 mm	(-)	T3N0M0	Re	36/95 (AWD)	7
10	56/F	External acoustic meatus	2	(-)	7 mm	(-)	T1N0M1 (thigh)	Re	136/211 (AWD)	5

Abbreviations: M, male; F, female; NA, not available; PI, perineural invasion; Re, resection; Ra, radiotherapy; P, palliative care; DFS, disease-free survival; L, lost to follow-up; OS, overall survival; AWOD, alive without disease; AWD, alive with disease; FISH, fluorescent in situ hybridization.

our cases are summarized in Table 1. Patient age ranged from 35 to 87 years, with a median (interquartile range, IQR) of 68 (46-74) years. Patients were predominately female, with a male to female ratio of 3:7. Tumor locations were as follows: major salivary gland (n=4), oral cavity (n=2), lip (n=1), nasal cavity (n=1), external acoustic meatus (n=1), and trachea (n=1). Conventional ACCs of grade I (n=2), grade II (n=6, Figure 1A–C), grade III (n=1) were found in nine of the cases. The other case (Case 5, Figure 1D–F) was an HGT-ACC initially diagnosed as basal cell adenocarcinoma by histological and immunohistochemical studies. Perineural invasion was seen in three of the 10 cases, and distant metastases were observed in two of the 10 cases at diagnosis. Follow-up data were available for nine cases, and three of the nine cases were lost to follow-up. The median overall survival was 51 months (IQR: 39-102 months), and no tumor-related death was observed.

In FISH analysis, six of the 10 cases (60%) including the HGT-ACC case showed a high rate (in >85% of cells) of split *MYB* signals (Figure 1C and F), which was considered to indicate positive *MYB* rearrangement. In contrast, four of the 10 cases (40%) showed spilt *MYB* signals in fewer than 10% of cells, similarly to the background non-tumor cells. Thus, the *MYB* rearrangement cutoff value was postulated to be 10%. No equivocal result was obtained. Neither the presence nor the absence of MYB rearrangement was significantly correlated

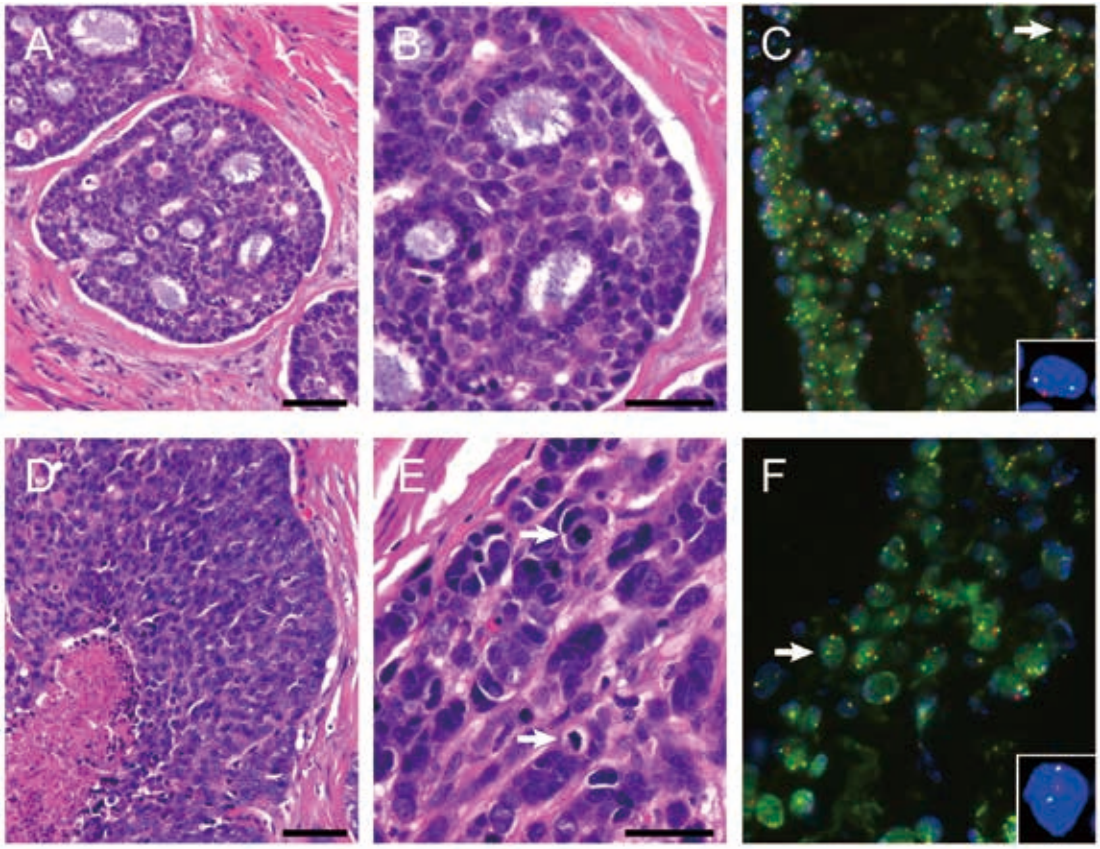

Figure 1. Histological and fluorescence in situ hybridization findings for adenoid cystic carcinomas.
A–C) Conventional adenoid cystic carcinoma (ACC). The tumor shows a cribriform growth of monomorphic tumor cells (A–B). Fluorescence in situ hybridization (FISH) of the MYB gene shows that most tumor cells have split signals (C). The inset in C is a magnified photo of the typical split signal indicated by an arrow. D–F) High-grade transformed ACC. The tumor shows solid (D) or trabecular (E) growth of atypical cells showing frequent mitosis (arrows in E). Central necrosis is seen in the solid nest (D). FISH demonstrates positive MYB gene rearrangement (F). The inset in F is a magnified photo of the typical split signal indicated by an arrow. A, B, D, and E are taken from sections stained with hematoxylin and eosin. Bars represent 50 μm.

with age, sex, maximum tumor size, perineural invasion, DFS, or OS.

4. Discussion

Our study demonstrated FISH to be a useful tool for evaluating *MYB* rearrangement using FFPE ACC sections, with the ability to make such evaluations in FFPE blocks prepared more than 17 years prior to analysis. Six of the 10 cases (60%) in our study were considered positive for *MYB* rearrangement, which is a rate consistent with that reported in previous studies (77/127 cases, 61%) [11, 13, 14]. No equivocal cases were found using FISH; therefore, an additional molecular assay such as RT-PCR, was not required. As shown in our study, *MYB-NFIB* tumor status was not significantly associated with OS or DFS [16].

Our study is the first to show the rate of split *MYB* signals in positive *MYB* rearrangement cases. Our cases with positive *MYB* rearrangement, i.e., five conventional ACCs and one HGT-ACC, suggested that heterogeneity in *MYB* rearrangement was not present. Thus, FISH analysis using a biopsy sample can estimate *MYB* rearrangement of the whole tumor. However, Costa et al. reported that in one case of ACC with high-grade transformation, *MYB-NFIB* translocation only occurred in areas with the HGT component and not in the areas with only conventional components [9]. Thus, further genetic studies on heterogeneity in *MYB* rearrangements in ACCs are warranted.

A high rate of split *MYB* signals in ACCs was obtained using the automated imaging analyzer. The automatically evaluated FISH data were preserved and can be reviewed retrospectively. Automatically analyzed data for the cancer tissues included that of tumor and non-tumor cells, such as endothelial cells, inflammatory cells, and fibroblasts. Non-tumor cell data could be retrospectively removed from the analyzed data, which may increase the rate of split *MYB* signals in the tumor cells. In contrast, the computer-aided analysis of the data may have reduced the cutoff value of split *MYB* signals compared to the 10 to 30% reported in previous studies [9, 11, 12]. In our study, counting of split *MYB* signals in non-tumor cells supported the postulated cutoff value defining positive *MYB* rearrangement. However, further additional study for optimal cutoff value of split *MYB* signals in ACCs will be needed because the present study dealed with only 10 cases.

One high-grade tumor (Case 5) was determined to be an HGT-ACC by positive *MYB* rearrangement, as basal cell adenocarcinoma has not been associated with positive *MYB* rearrangement [7]. Genetic studies of salivary gland HGT-ACCs have been limited [9], and the rate of *MYB* rearrangement in HGT-ACCs may be low, considering that breast HGT-ACCs have shown low rates of *MYB* rearrangement (2/16 cases, 12.5%) [10]. However, *MYB* FISH analysis of HGT-ACCs appears to be a viable option when conclusive diagnosis of such tumors cannot be obtained histologically or immunohistochemically.

5. Conclusions

Our automated analysis of *MYB* rearrangement in ACC cells using FFPE sections showed positive results in 60% of cases, and the cutoff value defining *MYB* rearrangement was postulated to be 10%. No heterogeneity of *MYB* rearrangement in ACC cells was present in our study. This genetic study supports the conclusive diagnosis of high-grade tumors.

Declarations

Conflicts of Interest: The authors declare that they have no competing interests.

Funding: This study was supported by Grant-in-Aid from the Life Fund of Kochi Shimbun and Kochi Broadcast in 2015.

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