Comprehensive Analysis of the *MYB-NFIB* Gene Fusion in Salivary Adenoid Cystic Carcinoma: Incidence, Variability and Clinicopathological Significance

Yoshitsugu Mitani¹, Jie Li¹, Pulivarthi H. Rao², Yi-Jue Zhao², Diana Bell¹, Scott M. Lippman³, Randal S. Weber⁴, Carlos Caulin⁴ and Adel K. El-Naggar¹

Departments of ¹Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; ²Pediatrics, Baylor College of Medicine, Houston, Texas; ³Thoracic/Head and Neck Medical Oncology, ⁴Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

**Corresponding Authors:**

Adel K El-Naggar, MD, PhD, Department of Pathology, Unit 85, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX, 77030-4009, USA.

Phone number: 713-792-3109, Fax number: 713-792-5532

Email: anaggar@mdanderson.org

Carlos Caulin, PhD, Department of Head and Neck Surgery-Research, Unit 123, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX, 77030-4009, USA.

Phone number: 713-794-5603, Fax number: 713-745-2234

Email: ccaulin@mdanderson.org
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State of Translational Relevance

Adenoid cystic carcinoma (ACC) is one of the most aggressive cancers that develop in the salivary gland. Here we report the first comprehensive analysis to determine the incidence and the potential clinical significance of the MYB-NFIB gene fusion in salivary ACCs. The MYB-NFIB fusion was identified in approximately one-third of the ACCs, but not in any other salivary tumor type. In addition, we found that most of the ACCs with MYB-NFIB fusions and over half of the fusion-negative ACCs overexpress MYB. Collectively, these findings indicate that MYB overexpression, as a result of the MYB-NFIB fusion or through alternative mechanisms, is a significant feature of most salivary ACCs, suggesting that MYB may be involved in ACC development and could be a potential target to develop novel therapeutic strategies for ACC treatment.

Abstract

Purpose: The objective of this study was to determine the incidence of the MYB-MFIB fusion in salivary adenoid cystic carcinoma (ACC), to establish the clinicopathological significance of the fusion and to analyze the expression of MYB in ACCs in the context of the MYB-NFIB fusion.

Experimental Design: We performed an extensive analysis involving 123 cancers of the salivary gland, including primary and metastatic ACCs, and non-ACC salivary carcinomas. MYB-NFIB fusions were identified by reverse transcription-PCR (RT-PCR) and sequencing of the RT-PCR products, and confirmed by fluorescence in situ hybridization. MYB RNA expression was determined by quantitative RT-PCR and protein expression was analyzed by immunohistochemistry.
Results: The *MYB-NFIB* fusion was detected in 28% primary and 35% metastatic ACCs, but not in any of the non-ACC salivary carcinomas analyzed. Different exons in both *MYB* and *NFIB* genes were involved in the fusions, resulting in expression of multiple chimeric variants. Notably, *MYB* was overexpressed in the vast majority of the ACCs, although *MYB* expression was significantly higher in tumors carrying the *MYB-NFIB* fusion. The presence of the *MYB-NFIB* fusion was significantly associated (p = 0.03) with patients older than 50 years of age. No correlation with other clinicopathological markers, factors and survival was found.

Conclusions: We conclude that the *MYB-NFIB* fusion characterizes a subset of ACCs and contributes to *MYB* overexpression. Additional mechanisms may be involved in *MYB* overexpression in ACCs lacking the *MYB-NFIB* fusion. These findings suggest that *MYB* may be a specific novel target for tumor intervention in patients with ACC.
Introduction

Adenoid cystic carcinoma (ACC), the second most common malignancy of the salivary glands, is characterized by heterogeneous morphology, propensity for perineural permeation and variable progressive clinical course (1-6). In contrast, similar tumors at other non-head and neck sites including those of mammary and pulmonary origins were reported to pursue less aggressive behavior (7-9). The underlying factors for these distinctive biological and clinical differences remain poorly understood. To unravel the molecular events associated with the development and biological progression of ACC and to identify targets for their management, numerous molecular genetic studies have been conducted. While no universally accepted clinically or biologically relevant marker/alteration has been identified, the existing data indicate that deletions and/or translocations of the terminal regions of the long arm of chromosome 6 are consistent findings in salivary ACC (10-13).

Cytogenetic studies of ACCs have reported the involvement of chromosome 6q terminal loci in reciprocal translocations with different chromosomal partners and/or loss of 6q in a subset of these tumors (14-24). In these studies, the short arm of chromosome 9 was identified as the partner of chromosome 6q in 42.8% of the ACCs that contained 6q translocations, and t(6;9) translocations were identified in 14% of the ACCs analyzed (12, 14, 15, 17, 20, 21, 25-27). Moreover, t(6;9) was found to be the sole cytogenetic alteration in some of these tumors, suggesting a primary role and/or an early involvement of the 6q region in the development of a subset of ACCs (14, 21, 22, 27). Subsequent molecular and genome-wide analyses have also shown frequent loss at the terminal loci of chromosome 6q in ACCs (28-35). Together, these data suggest that two different genetic events may underlie the pathogenesis of ACC: one event involves the generation of a fusion gene resulting from reciprocal translocation of 6q terminal...
region with chromosome 9p or other partners, and the other event constitutes a loss of genetic materials at the same location, denoting the presence of a tumor suppressor gene(s). Efforts to identify a tumor suppressor gene at these loci in ACC, however, have been unsuccessful (30).

Recently, the MYB-NFIB fusion gene, resulting from a reciprocal translocation between the terminal region of the long arm of chromosome 6 and the short arm of chromosome 9, was detected in all 11 ACCs analyzed, 5 mammary and 6 from head and neck origins (36). This translocation results in the formation of a fusion transcript that lacks the MYB 3′-UTR, containing target sequences for certain miRNAs (miR-15a, miR-16 and miR-150) (36-39). The putative function of this fusion gene, as in many reciprocal translocations in solid tumors, lies in the elevated expression of the MYB oncogene in ACCs that carry the fusion (38-40). If validated in larger cohorts, these findings may provide strong support for a primary/early involvement of the MYB-NFIB fusion in the development of ACCs. The ubiquitous identification of the fusion transcript in all of salivary ACCs tested (36), however, is at variance with the low incidence of t(6;9) in karyotypically published ACCs (14%) and with multiple molecular studies of these tumors (14-24, 28-35). These observations and the small number of salivary ACCs analyzed in the recent study (36) warrant a comprehensive analysis to determine the incidence of MYB-NFIB fusions in ACCs of the salivary gland.

The central issue, therefore, is whether the MYB-NFIB fusion gene represents a universal event necessary for the development of salivary ACC or one of the genetic alterations that define a subset of this entity. To address this question, we undertook an extensive analysis involving a total of 123 salivary carcinomas, including primary ACCs of the salivary gland, metastatic ACCs, non-ACC salivary carcinomas and normal salivary gland tissue. Our objectives were to account for 1) incidence and specificity of MYB-NFIB fusion in ACCs of the salivary gland, 2) to analyze
the nature of the transcript variants involved in these fusions, 3) to determine the expression of \textit{MYB} in ACCs in the context of the \textit{MYB-NFIB} fusion status and salivary tumor type and 4) to explore the clinicopathologic significance of this alteration.
Materials and Methods

Tissue samples

Tissues used in this study comprised of 72 primary salivary gland ACCs, 5 corresponding normal salivary gland tissues, 17 ACCs metastatic to lung and lymph nodes, 5 each of myoepithelial, acinic cell, mucoepidermoid, salivary duct carcinomas, 11 polymorphous low grade adenocarcinomas, and 3 epithelial-myop epithelial carcinomas. Patients were treated at The University of Texas M.D. Anderson Cancer Center where fresh tissue samples were immediately taken from surgical specimens and frozen in liquid nitrogen and stored at -80°C until used. Tumors were classified according to the histological classification of salivary gland tumors by WHO. All tissue was obtained according to an IRB approved protocol for non-mucosal head and neck cancer by specialized head and neck pathologist. For immunohistochemical analysis, tissue microarray constructed from formalin-fixed, paraffin-embedded tissues of 300 ACCs, including most tumors used in this study and 79 salivary adenocarcinomas were used.

Detection of MYB-NFIB transcripts

Total RNA was extracted with the TRIzol reagent (Invitrogen) and treated with recombinant DNase I, RNase-free (Roche) prior to RT-PCR and converted subsequently to cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo(dt) primers according to the manufacturer’s instructions. For amplification of the MYB-NFIB fusion transcripts, the primers and annealing temperatures used were described previously (36). The MYB-NFIB fusion transcripts were detected by PCR analysis with Platinum Taq DNA polymerase (Invitrogen). ACTB was used as internal control using the following primers: ACTB, 5’-CTGTCTGGCGGCACCACCAT-3’ and 5’-GCAACTAAGTCATAGTCCGC-3’.
**MYB-NFIB fusion gene sequencing**

RT-PCR products were purified and sequenced directly or cloned into the pCR2.1 vector (Invitrogen). The PCR fragments were sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) at the DNA sequencing core facility of M.D. Anderson Cancer Center. To characterize the **MYB-NFIB** variants, the nucleotide sequences and genomic organization of **MYB** (accession number NM_001130173) and **NFIB** (ENSG00000147862) were obtained from NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org), respectively.

**Quantitative RT-PCR**

Quantitative RT-PCR was performed using the Applied Biosystems 7900HT Real-time PCR Systems (Applied Biosystems) with Power SYBR® Green PCR Master Mix (Applied Biosystems). The following primers were used for amplification of **MYB** transcripts: **MYB-E2** (forward in exon 2): 5’-GACTATGATGGGCTGCTTCC-3’, **MYB-E3** (reverse in exon 3): 5’-TGTTCCATTCTGTCCGACCA-3’, **MYB-E15** (forward in exon 15): 5’-GCATTTCAGATACCTAAAACAGGTC-3’, **MYB-E16** (reverse in exon 16): 5’-CCCAGTCAACTTGAGTGTTCC-3’. The **ACTB** gene was used as internal control using primers 5’-TCACCGAGCGGCTGCTC-3’ and 5’-TAATGTCACGCAGATTCCC-3’. Duplicate samples for each tumor or tissue were analyzed. The expression of **MYB** transcripts was determined by the ΔCT method (Average CT-**MYB** - Average CT-**ACTB**), and relative **MYB** expression in each tumor was calculated relative to **MYB** expression in pooled normal salivary gland tissue (Clontech).

**Immunohistochemistry**
We used the MYB rabbit monoclonal antibody clone (EP769Y) raised against an aminoterminal peptide of MYB (Abcam) for immunohistochemical evaluation of MYB protein expression. Tissue sections were pretreated in 10mM sodium citrate buffer in a pressure cooker and stained with DAKO Envision+™ system (DakoCytomation). The antibody optimum concentration was attained at 1:25 in dilution experiments using colon carcinoma control tissue. Tissue arrays of 300 ACCs, including 61 primary adenoid cystic carcinomas used in this study were used along with a tissue array of 75 salivary adenocarcinomas for comparison. The MYB expression scoring was performed based on the visualization of nuclear staining in tumor cells as compared to those in the built-in control core and the absence of any staining in negative control. Tumors were scored as negative if nuclear staining or faint staining was observed in less than 10% of the tumor cells.

**Fluorescent in-situ hybridization (FISH):**

FISH was performed on touch preparations of ACCs to identify MYB/NFIB rearrangements. BAC clones containing MYB gene (RP11-104D9) and NFIB gene (RP11-54D21 and RP11-79B9) were labeled with Spectrum Green and Spectrum Red, respectively (Abbott Laboratories, Abbott Park, IL). Hybridization and detection were performed according to the manufacturer's protocols. To determine the gene fusion status, 200 individual nuclei were analyzed for each case. The interphase nuclei were captured and processed using the Quantitative Image Processing System (Applied Imaging, Santa Clara, CA). In nuclei containing the MYB-NFIB fusion, green and red signals from the MYB and NFIB genes overlap in a red/green (yellow) signal.

**Statistical analysis**
Association between clinicopathological parameters and the MYB-NFIB fusion status was performed by a paired univariate statistical analysis using Fisher’s exact test. Differences in the relative expression level of MYB between the MYB-NFIB fusion-positive, fusion-negative ACCs and non-ACCs were analyzed by the nonparametric Mann-Whitney U test. p values lower than 0.05 were considered statistically significant.
Results

Incidence of *MYB-NFIB* fusions in salivary ACCs.

To determine the frequency and specificity of *MYB-NFIB* fusions in salivary gland carcinomas we carried out a comprehensive analysis involving 72 primary salivary ACCs, 17 metastatic ACCs and 34 non-ACCs that included myoepithelial, acinic cell, mucoepidermoid, salivary duct carcinomas, polymorphous low grade adenocarcinomas, and epithelial-myoeplithelial carcinomas. Two sets of primers designed to detect transcripts resulting from the fusion between the *MYB* and *NFIB* genes (36) were used for the RT-PCR analysis (Fig. 1A). As summarized in Table 1, this analysis revealed the presence of *MYB-NFIB* fusion transcripts in 20 (28%) primary ACCs, 6 (36%) metastatic ACCs and none of the 34 non-ACC salivary carcinomas, including the 14 polymorphous and epi-myoeplithelial carcinoma subtypes analyzed. Overall, *MYB-NFIB* fusions were identified in 20 tumors using primers *MYB1-NFIB1* and in 10 tumors with primers *MYB2-NFIB1* (Fig. 1B), with fusions in four tumors detected with both primer sets. All 5 matching normal salivary tissues used as controls were negative for the fusion gene, as expected (Supplementary Fig. S1). The identity of the fusion transcripts was confirmed by sequence analysis of the RT-PCR products. Nested PCR using internal primers did not result in increased detection of fusion products, but generated false positives in four tumors, as sequencing of discrete bands obtained by nested PCR in these tumors did not show *MYB* or *NFIB* sequences.

Multiple *MYB-NFIB* fusion variants expressed in ACCs.

All of the *MYB-NFIB* fusion transcripts identified in this analysis were characterized relative to the exons involved in the fusion, in both the *MYB* and *NFIB* genes. As indicated in Table 2, 14 different fusion transcripts involving different exons of *MYB* and *NFIB* were found.
in the ACCs analyzed, some of which were present in different tumors. In this analysis, exons were enumerated according to the genomic organization described in the NCBI database for *MYB* (accession number NM_001130173) and Ensembl for *NFIB* (ENSG00000147862). For the *MYB* gene, the most frequent fusion variants found in the salivary ACCs involved exons 13 and 8b (each found in 7 tumors and 2 metastases). In addition, other fusions were found in exon 11 (3 tumors and 2 metastases), exon 15 (5 tumors), exon 9b (2 tumors) and exons 8a, 14 and 16 (one tumor each). In the *NFIB* gene, the majority of the variants involved exon 12 (18 tumors and 3 metastasis) and exon 11 (6 tumors and 2 metastasis), while exons 9 and 9’ were found in one tumor each. We also identified five tumors with more than one *NFIB* variant (Supplementary Table S1), most likely resulting from alternative splicing in the last exons of *NFIB* (Supplementary Fig. S2). A representative tumor with 2 *MYB-NFIB* transcripts is shown in Fig. 1C. Nucleotide sequencing of the PCR products (Fig. 1D) revealed that the smaller product (fusion 1) corresponded to a fusion transcript linking exon 15 of *MYB* to exon 12 of *NFIB*, whereas the larger product (fusion 2) contained a chimeric variant fusing exon 15 of *MYB* to exon 11 of *NFIB*. Note that both variants contain the “tctgtaacctgggctag” sequence in the *NFIB* part of the fusion in alternative reading frames, resulting in distinct amino acid sequences, according to the predicted genomic structure of the *NFIB* gene (Supplementary Fig. S2). To verify that ACCs that were negative for the *MYB-NFIB* transcript lacked genomic translocations involving these two genes we performed FISH analyses using BAC clones containing the *MYB* gene (labeled with spectrum green) and *NFIB* gene (labeled with spectrum red), in three randomly selected fusion-negative ACCs. Three fusion-positive ACCs were used as positive controls for the *MYB-NFIB* fusion. This analysis revealed lack of translocations involving the
MYB and NFIB genes in fusion-negative tumors (Fig. 1E), thus confirming that the RT-PCR analysis was extremely robust in identifying MYB-NFIB fusions.

**MYB overexpression in salivary ACCs.**

To determine the impact of the MYB-NFIB fusion in MYB expression, we analyzed the expression levels of MYB transcripts in the salivary tumors by quantitative RT-PCR using primers for exons 2-3 of MYB. We detected elevated MYB expression in 17 of 20 fusion-positive tumors, 14 of 20 of fusion-negative tumors and 2 of 34 of the non-ACCs tumors (Fig. 2A). Overall, MYB expression in the fusion-positive tumors was over two-fold higher than in fusion-negative ACCs and 100-fold higher than in non-ACCs (Fig. 2C). As these primers cannot discriminate MYB-NFIB fusion transcripts from non-fused MYB, an additional set of primers was designed to detect transcripts containing exon 16 of MYB, the last MYB exon, which is lost in all MYB-NFIB fusions. Therefore, these primers only amplify non-fused MYB. Remarkably, these primers detected elevated MYB expression in 60% of the fusion-negative ACCs, but not in the fusion-positive ACCs (Fig. 2B), indicating that non-fused MYB is not expressed in ACCs carrying the MYB-NFIB fusion, whereas fusion-negative tumors overexpress MYB through mechanisms independent of the MYB-NFIB fusion. Overall, the expression of non-fused MYB in ACCs lacking the MYB-NFIB fusion was approximately 20-fold higher than in fusion-positive tumors and 30-fold higher than in non-ACCs salivary carcinomas (Fig. 2D). It is worth noting that we also found very low expression of MYB RNA in 3 of the fusion positive tumors, which were also negative for the protein expression (Fig. 3C, see below). In addition, we noted that among the 5 fusion-positive tumors with the highest level of MYB expression (cases # 4, 5, 7, 15 and 21) 4 had fusions linking exon 8b of MYB to exon 12 of NFIB, suggesting that fusions involving these specific exons may be functionally relevant.
Expression of *MYB* protein in salivary ACCs.

The expression of *MYB* protein in salivary tumors was evaluated by immunohistochemistry analysis using a monoclonal antibody raised against the N-terminal domain of human *MYB*, on tissue microarrays containing the 20 fusion-positive tumors and 41 of the fusion-negative ACCs. This analysis revealed strong nuclear staining for *MYB* in 17 (85%) of the 20 fusion-positive tumors (Fig. 3A-B) and 25 (61%) of the 41 fusion-negative tumors (Fig. 3D). Only the 3 fusion-positive tumors that expressed the lowest levels of *MYB* RNA also lacked *MYB* protein (Fig. 3C). We also noted that in tubular and cribriform ACCs with dual epithelial and myoepithelial cells composition, *MYB* expression was limited to the myoepithelial cells (Fig. 3A). Interestingly, the ACCs with translocations involving t(6q;14p) and t(6q;15p), which were negative for the *MYB-NFIB* fusion were also negative for *MYB* protein expression (Fig. 3E-F). *MYB* expression was not detected in either the ductal or acinar structures of normal parotid salivary glands (not shown). In fusion-negative ACCs, tumors with high *MYB* transcripts were also positive for the *MYB* protein by immunohistochemistry, while tumors with low level transcript showed negative nuclear *MYB* staining.

Clinicopathological correlations

Of the 72 patients with primary ACCs analyzed for the *MYB-NFIB* fusion, 58 patients were followed up for at least three years and were included in the clinicopathological analysis. In this group, 16 patients (27.5%) had fusion-positive ACCs and 42 (72.5%) were fusion-negative. The clinicopathological analysis of this cohort is represented in Table 3. Statistically significant correlation was found between age (p=0.03) and fusion status, as patients older than 50 years of age had significantly higher fusion-positive tumors. No significant association between fusion status and other clinicopathological factors was found.
Discussion

Our comprehensive analysis identified MYB-NFIB chimeric transcripts resulting from the balanced t(6;9) (q^{22-23}; p^{23-24}) in 28% of primary and 35% of metastatic salivary ACCs. The frequency of this alteration was considerably lower than that reported by Persson et al, in which all 11 head and neck and mammary ACCs analyzed, including six of salivary gland origin, carried this translocation (36). This difference can be attributed, at least in part, to either the selection of tumors used in the earlier study or the size of both cohorts. Despite the difference, our results confirmed the presence of this fusion gene in approximately one-third of the salivary ACCs and support a role for the MYB-NFIB fusion in the development of a subset of ACCs. Accordingly, ACC joins an increasing list of epithelial malignancies with specific gene fusions that may be targeted for biological investigation and therapeutic intervention (40-43). Our findings are compatible with the incidence of t(6;9) reported in cytogenetic studies (14-27) and with genomic studies (28-35) of these tumors by our group (14, 27, 35) and others (14-28, 33, 34, 44). In these studies, only a subset (14%) of the ACCs had the t(6;9)(q^{22-ter}; p^{23-24}), while approximately 60% of this entity manifested genomic loss at the same 6q terminal region, using different molecular and genomic assays (17, 19, 20, 25, 26). These data suggest that multiple genetic events, including translocation and loss at chromosome 6q, may characterize the underlying events associated with the pathogenesis of the majority of ACCs.

The sequencing analysis of all 26 fusion transcripts identified in this study revealed multiple MYB-NFIB fusion variants involving different exons of the MYB and NFIB genes at the fusion point. These variants may arise from distinct breakpoints involved in the t(6;9) and/or alternative splicing for the MYB and NFIB genes. Our data suggests that multiple breakpoints may occur within the MYB gene, whereas the different variants at the NFIB gene are consistent
with alternative splicing in the last exons of *NFIB* (Supplementary Fig. S2). One of the most common fusions found in our tumors involved exon 8 of *MYB* and exon 12 of *NFIB*, which was identified in tumors with the highest *MYB* expression, suggesting a significant functional association. In addition, we identified multiple previously unreported variants of the *MYB* fusion gene involving exons 9, 11, 13 and 15. Despite the multitude of variants in both *MYB* and *NFIB* genes, the minimally critical region common to all fusions was restricted to the DNA binding domain and the Wos2 domain of the MYB protein. A major consequence arising from the fusion of *MYB* to *NFIB* is the dramatic increase in *MYB* expression that may be attributed to the loss of the *MYB* sequences containing the regulatory miRNA binding sites in *MYB-NFIB* transcripts. Indeed, we found high expression of *MYB* RNA in fusion-positive ACCs, in agreement with the Persson et al study (36).

We also found that *MYB* expression in fusion-negative ACCs was over 40-fold higher than in non-ACCs and only about 2-fold lower than in fusion-positive ACCs (Fig. 2). Since fusion-positive tumors only overexpress the chimeric, but not the non-fused *MYB* transcript, we contend that the *MYB* overexpression in these tumors occurs as a consequence of the *MYB-NFIB* fusion. On the other hand, alternative mechanisms may be responsible for *MYB* overexpression in fusion-negative ACCs. Consistently, *MYB* protein expression was found in the majority of fusion-positive (85%) and approximately 60% of fusion-negative ACCs. The high expression of *MYB* RNA and protein in a subset of fusion-negative tumors may raise the possibility that chimeric transcripts were missed in the analysis of these tumors. However, the elevated expression of full-length *MYB* transcripts in fusion-negative, but not in fusion-positive ACCs, the absence of *MYB* translocations by FISH in fusion-negative tumors and the lack of *MYB* expression in subsets of both *MYB-NFIB* fusion-positive and negative ACCs, suggest that this
specific fusion did not take place in these tumors. This is further supported by the lack of MYB expression in the two previously reported ACCs with the translocations t(6q;14p) and t(6q;15p), suggesting that other genes exclusive of MYB may be involved in these translocations (27). Interestingly, we report, for the first time, the restricted expression of MYB protein to myoepithelial cells of tubular and cribriform ACCs, suggesting the preferential occurrence of MYB-NFIB fusion and/or the activation of the MYB expression in myoepithelial cells in these tumors.

The clinicopathological correlation of this large cohort, based on a single parameter statistical analysis, showed a lack of correlation between the fusion status of the ACCs and traditional factors and outcome, except for a significant association between the presence of MYB-NFIB fusions and tumors that developed in older patients. Several lines of evidence support these results: 1) the equal incidence of fusion-positive primary and metastatic lesions, 2) the absence of fusion genes in two matched primary and metastatic lesions, 3) the presence of MYB-NFIB fusions in bronchial and breast ACCs (36), tumors known to pursue a better clinical course, and 4) the lack of association between fusion status and patients’ outcome. Further studies of a large cohort of ACC patients with long-term follow-up information using multi-parameter statistical models, however, are needed to definitively address this issue. Our findings are in favor of a specific association between the MYB-NFIB fusion gene and the ACC phenotype. This is supported by the lack of MYB-NFIB fusions in all non-ACCs tumors, including those with dual cell composition and overlapping morphologic features. Similar results have been reported recently in mucoepidermoid carcinomas, where the MEC1/MAML2 gene fusion was restricted to this phenotype in salivary and non-salivary origins (45-48).
In summary, our results indicate that the \textit{MYB-NFIB} fusion defines a subset of salivary ACCs and suggest that \textit{MYB} overexpression, resulting from \textit{MYB-NIFB} fusion or through alternative mechanisms, is a key alteration during ACC development and a potential target for therapeutic intervention in most of these tumors. The functional consequences of \textit{MYB} overexpression in ACCs await further investigations, following the lead of studies in other tumor types that overexpress \textit{MYB}, including mammary carcinoma (38, 49, 50).
References


Acknowledgements

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<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Positive*</th>
<th>Negative†</th>
<th>Total</th>
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<tr>
<td>ACC</td>
<td>20 (27.7)</td>
<td>52 (72.3)</td>
<td>72</td>
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<tr>
<td>M-ACC</td>
<td>6 (35.2)</td>
<td>11 (64.8)</td>
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</tr>
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<td>Non-ACC</td>
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<td>34</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>26</strong></td>
<td><strong>97</strong></td>
<td><strong>123</strong></td>
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</table>

ACC, primary adenoid cystic carcinoma; M-ACC, metastatic adenoid cystic carcinoma; Non-ACC, non-adenoid cystic carcinomas.

* n (%) of tumors expressing MYB-NFIB fusion transcripts
† n (%) of tumors lacking MYB-NFIB fusion transcripts
**Table 2.** Frequency of MYB-NFIB fusion variants identified in primary and metastatic ACCs

<table>
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<th>MYB-NFIB fusion variants</th>
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<th>Metastasis*</th>
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<tr>
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<tr>
<td>MYB exon 16 - NFIB exon 12</td>
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* number of tumors or metastases expressing the indicated fusion variant
Table 3. Clinicopathological correlation and *MYB-NFIB* fusion status in patients with salivary ACC

<table>
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<th>Factor</th>
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<td><strong>PNI†</strong></td>
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<tr>
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<td>DOD</td>
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A, alive; AWD, alive with disease; DOD, died of disease; Exp, expression; S, solid pattern; PNI, perineural invasion; T/C, tubular/cribriform.

* Fisher's exact test.
† PNI was determined for 18 fusion-positive tumors.
‡ All 20 fusion-positive tumors and 41 fusion-negative were stained for MYB
Figure Legends

Figure 1. Detection of MYB-NFIB fusion transcripts in ACCs. A, schematic representation of the MYB and NFIB genes. Arrows indicate primers used to identify MYB-NFIB fusion transcripts by RT-PCR. B, RT-PCR analysis of MYB-NFIB fusion transcripts using the indicated primers in ACCs and non-ACCs, including a myoepithelial carcinoma (#44), acinic cell carcinoma (#49), mucoepidermoid carcinoma (#50), salivary duct carcinoma (#53), Polymorphous low grade adenocarcinoma (#58), and epithelial-myoepithelial carcinoma (#61). Samples 62(M) and 63(M) represent metastatic ACCs. ACTB (beta actin) was used as internal control. C, identification of tumors expressing multiple MYB-NFIB fusion variants. RT-PCR using RNA from tumor #8, amplified with primers MYB1-NFIB1, showing two PCR products. D, sequencing profile generated from fusion transcripts shown in C, confirming MYB exon 15 linked to NFIB exon 12 (fusion 1) and exon 11 (fusion 2). MYB-NFIB fusion cDNA and predicted protein sequences are indicated. The nucleotide and protein sequence of MYB are based on NCBI database (accession number NM_001130173). NFIB sequences and exon numbers were obtained from accession number ENSG0000147862 for NFIB in the Ensembl database. E, FISH analysis using BAC clones containing MYB (green) and NFIB (red) genes in fusion-negative ACCs (top panels, samples #29, #33, #43) and fusion-positive ACCs (bottom panels, samples #6, #15, #22). Note overlap of the NFIB and MYB probes in the fusion-positive tumors but not in fusion-negative ACCs.

Figure 2. Expression of MYB transcripts in salivary gland tumors and normal salivary glands. A and B, relative expression of MYB using primers that amplify MYB transcripts containing coding sequences of exons 2-3 (A) or exons 15-16 (B). Relative expression of MYB represents fold
expression compared to normal salivary gland. Red bars: fusion-positive ACCs; Blue bars: fusion-negative ACCs; Yellow bars: non-ACCs; N: normal salivary gland; ACC: Adenoid cystic carcinoma; MYC: Myoepithelial carcinoma; Aci: Acinic cell carcinoma; MEC: mucoepidermoid carcinoma; SDC: salivary duct carcinoma; PLGA: Polymorphous low grade adenocarcinoma; EMC: Epithelial-myoepithelial carcinoma. The asterisks in B denote tumors with MYB-NFIB fusions that occurred after exon 15 of MYB. C and D, cumulative expression of MYB exon 2-3 (C) or MYB exon 14-15 (D) in salivary gland tumors. Relative MYB expression for each tumor is represented with circles. White circles: normal salivary gland (N); Red circles: fusion-positive ACCs; Blue circles: fusion-negative ACCs; Yellow circles: non-ACCs. Horizontal bars represent the mean for each group.

**Figure 3.** Expression of MYB protein in salivary ACCs. A-C, immunostaining for MYB in MYB-NFIB fusion-positive ACCs. Each panel depicts a different fusion-positive ACC, showing strong staining in myoepithelial cells in the tumor shown in panel (A), ubiquitous expression in panel (B) and lack of staining in the ACC shown in (C). D-E, immunostaining for MYB in ACCs negative for the MYB-NFIB fusion, showing a representative tumor with high MYB expression (D) and another tumor that lacks MYB expression (E).
Mitani et al. Figure 3
Comprehensive Analysis of the MYB-NFIB Gene Fusion in Salivary Adenoid Cystic Carcinoma: Incidence, Variability and Clinicopathological Significance

Yoshitsugu Mitani, Jie Li, Pulivarthi H Rao, et al.

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