Novel MYBL1 Gene Rearrangements with Recurrent MYBL1–NFIB Fusions in Salivary Adenoid Cystic Carcinomas Lacking t(6;9) Translocations

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Abstract

Purpose: Adenoid cystic carcinoma (ACC) is an indolent salivary gland malignancy, characterized by t(6;9) translocations and MYB–NFIB gene fusions in approximately 50% of the tumors. The genetic alterations underlying t(6;9)-negative and t(6;9)-positive/MYB–NFIB fusion–negative ACC remain unknown. To uncover the genetic alterations in ACC lacking the canonical translocation and fusion transcript and identify new abnormalities in translocation positive tumors.

Experimental Design: We performed whole-genome sequencing in 21 salivary ACCs and conducted targeted molecular analyses in a validation set (81 patients). Microarray gene-expression data were also analyzed to explore the biologic differences between fusion positive and negative tumors.

Results: We identified a novel MYBL1–NFIB gene fusion as a result of t(8;9) translocation and multiple rearrangements in the MYBL1 gene in 35% of the t(6;9)-negative ACCs. All MYBL1 alterations involved deletion of the C-terminal negative regulatory domain and were associated with high MYBL1 expression. Reciprocal MYB and MYBL1 expression was consistently found in ACCs. In addition, 5’-NFIB fusions that did not involve MYB/MYBL1 genes were identified in a subset of t(6;9)-positive/fusion-negative tumors. We also delineated distinct gene-expression profiles in ACCs associated with the length of the MYB or MYBL1 fusions, suggesting a biologic importance of the C-terminal part of these fusions.

Conclusions: Our study defines new molecular subclasses of ACC characterized by MYBL1 rearrangements and 5’-NFIB gene fusions.

Introduction

Adenoid cystic carcinoma (ACC), the second most common salivary gland malignancy, is characterized by a remarkable morphologic heterogeneity and protracted clinical course (1–3). Approximately 50% of ACC patients experience recurrence and metastasis in 5 to 10 years, to which no effective therapy is available (4–6). Despite mounting efforts aimed to improve the management of ACC patients, no significant progress has been made.

Recent molecular genetic studies of ACC have demonstrated low mutation frequency (7, 8), with tumor-specific t(6;9) translocations resulting in MYB-NFIB gene fusions and high MYB expression in over 50% of tumors (9–11). The genetic alterations associated with ACCs lacking t(6;9) remain unknown (11, 12). We posit that in depth genomic analysis of this subset of tumors will uncover potential driver events.

To achieve this goal, we investigated a large cohort of ACC patients with and without t(6;9) and the MYB–NFIB fusion using whole-genome sequencing (WGS) and multiple conventional molecular techniques for in depth characterization of their genomic alterations.

Materials and Methods

Patient samples

Tumor specimens were harvested from primary tumors of patients who underwent surgical resection at The University of Texas MD Anderson Cancer Center (Houston, TX) between 1981 and 2011, reviewed by a head and neck pathologist, immediately frozen and stored at –80°C until used (Supplementary Table S1). The study was approved by the MD Anderson Cancer Center Institutional Review Board.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance
We report novel recurrent MYBL1–NFIB gene fusions, MYBL1 gene rearrangements, and MYBL1 overexpression in t(6;9)-negative ACCs, and 5′-NFIB gene fusions in a subset of t(6;9)-positive/MYB-NFIB-negative ACCs. These findings provide new insights into the complex genetics of ACC and expand the role of the MYB gene family members in ACC tumorigenesis, for future targeted therapies of ACC patients.

Whole-genome sequencing
Genomic DNA was extracted using the Gentra Puregene Tissue Kit (QIAGEN) according to the manufacturer's protocols. WGS was performed by Complete Genomics (CG) using ACC tumors and matching normal samples. This platform is based on the unchained combinatorial probe anchor ligation (cPAL) chemistry on arrays of self-assembling DNA nanoballs (DNB; ref. 13). Raw data were processed by CG using Cancer Pipeline 2.4. Reads were aligned to the reference genome [National Center for Biotechnology Information (NCBI) Build 37] and variants were called and scored using a local de novo assembly approach. The Complete Genomics Cancer Pipeline 2.4 generated the somatic mutation call with the somatic score ≥ 10 (Supplementary Table S2). The visualization of circos plots was also generated on the basis of the highConfidenceJunctions files by the pipeline.

Identification of gene fusions
The Ingenuity Variant Analysis (IVA) software (QIAGEN) was used to retrieve the in-frame fusions with genomic annotations. On the basis of the ‘highConfidenceJunctions.tsv’ files and MasterVar files from CG, the IVA system aligns sequences of left and right junctions to the reference genome (Human NCBI Build 37). Strand sequences consistently matched and located on separate genes were recorded as gene fusion (Supplementary Table S3).

Validation of the MYBL1–NFIB fusion by RT-PCR
Total RNA was extracted with the RNasy Universal Kit (QIAGEN) and the first-strand cDNA was synthesized using 2 μg of total RNA by oligo(dT) primer and the SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Then, the cDNA was used to detect MYBL1–NFIB fusion transcripts by PCR, using specific primers (Supplementary Table S4) and KAPA2G Fast PCR Kits (KAPA Biosystems). PCR products were gel-purified and either sequenced directly or cloned into the pCR2.1 vector (Invitrogen). Sanger sequencing was performed using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) at the DNA sequencing core facility of MD Anderson Cancer Center.

Fluorescence in situ hybridization
FISH was performed on touch preparations of fresh ACCs tissues to screen for the t(8;9) translocation. MYBL1 gene BAC clone (RP11-27101) and the NFIB gene clones (RP11-54D21 and RP11-79B9) were labeled with spectrum red and spectrum green, respectively (Abbott Laboratories). Probes hybridization was performed according to previously published procedure (10). For scoring, 200 individual nuclei were counted in each case and positive fusion was scored when >3% of cells had yellow signal. Individual cells were captured and processed using the Quantitative Image Processing System (Applied Imaging).

Quantitative RT-PCR for MYBL1 expression
Quantitative RT-PCR using duplicate samples was performed using the Applied Biosystems 7900HT Real-Time PCR Systems (Applied Biosystems) with the KAPA SYBR FAST Kit (KAPA Biosystems). Primers corresponding to the N-terminus of MYBL1 were used for amplification of MYBL1 transcripts, and ACTB gene was used as internal control (Supplementary Table S4). MYBL1 expression was determined by the ΔΔCt method, and relative expression was calculated relative to MYBL1 expression of pooled normal salivary gland tissue (Clontech).

Western blotting
Protein lysates from fresh tumor tissues was extracted using RIPA buffer. Aliquots of 20 μg of protein were loaded on SDSPAGE gels, transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-MYBL1 (HPA008791; Sigma-Aldrich), MYB (EP769Y; Abcam) and anti-ACTB (Sigma-Aldrich) antibodies.

Immunohistochemistry
IHC was performed using the MYBL1 antibody (HPA008791; Sigma-Aldrich). Freshly processed 4-μm-thick unstained sections of MYBL1 fusion positive and negative ACCs underwent incubation with primary antibody on Autostainer Link 48 (Dako) according to the manufacturer's instructions. Nuclear staining in tumor cells was scored as positive.

3′Rapid amplification of cDNA ends
Two μg of total RNA was used to synthesize the first-strand cDNA using the 3′Rapid amplification of cDNA ends (3′RACE) adapter primer (Invitrogen) and the SuperScript III reverse transcriptase (Invitrogen), using MYBL1-specific primers (GSP), and nested PCR was performed using KAPA HiFi PCR Kits (KAPA Biosystems) with touch-down protocol and 3′RACE universal primer (AUAP).

Expression array analysis
Illumina HumanHT-12 V4 BeadChips was used for the gene-expression microarrays that have been deposited in Array Express (E-MTAB-1397; ref. 8). The expression array data were processed by linear normalization and further subjected to the differential analysis with the ‘limma’ Bioconductor package (14). Gene Set Enrichment Analysis (GSEA) was downloaded from the website (http://www.broadinstitute.org/gsea/index.jsp; ref. 15). MYB and MYBL1 fusion description is mentioned in Supplementary Table S5.

Results
Whole-genome sequencing analysis of ACC
To identify genomic alterations in ACCs lacking the t(6;9) translocation and in t(6;9)-positive/fusion-negative tumors, we performed WGS on a set of 21 fresh-frozen tumors and matched normal tissue specimens (Supplementary Table S1). This test set comprised of nine t(6;9)-positive tumors, six of which had MYB–NFIB fusion transcripts, and 12 t(6;9)-negative tumors. WGS
MYBL1-NFIB Fusions in Adenoid Cystic Carcinoma

Figure 1.
MYBL1–NFIB gene fusion identified in ACC lacking t(6;9) translocations. A, schematic outline of a representative example of MYBL1–NFIB gene fusion in t(6;9)-negative ACCs (left). In this case, exon 8 of MYBL1 gene on chromosome 8 fused with exon 11 of the NFIB gene on chromosome 9. A circos plot illustrates global genomic rearrangements of the tumor containing this MYBL1–NFIB fusion (right); the chromosomal translocations identified by WGS are shown with orange lines. B, incidence of t(6;9) and t(8;9) translocations and associated MYB–NFIB (red) and MYBL1–NFIB (blue) fusions, in the 21 ACCs analyzed by WGS. The presence of the translocations was assessed by FISH. The MYBL1–YTHDF3 fusion (green) is also indicated. 5′ NFIB fusions (orange) represent tumors in which the 5′ end of NFIB was found fused to genes other than MYBL1 or MYB (see Supplementary Table S3). C, RT-PCR analysis for the detection of the MYBL1–NFIB and MYBL1–YTHDF3 transcripts in t(6;9)-negative ACCs (left). Sanger sequencing confirmed the MYBL1–NFIB gene fusions (right). Note that two different fusion transcripts were identified in tumor AC11: MYBL1 exon 8 linked to NFIB exon 11 (fusion 1) and exon 12 (fusion 2). (‘) MYBL1–YTHDF3 fusion was not identified by WGS in AC15. D, FISH analysis using BAC clones containing MYBL1 (red) and NFIB (green) genes. The yellow signals pointed by white arrow represent the MYBL1 and NFIB gene fusion. E, schematic representation of the exon structure of the MYBL1 and MYB genes showing the breakpoint locations in these genes in their fusions to NFIB. The number of cases with MYBL1–NFIB or MYB–NFIB fusions is listed above each gene and exon numbers are shown below the gene. (‘) two tumors had two different MYBL1 fusions each (see Table 1, tumors AC13 and AC73).
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A

B

AC79

Chr. 8

FBXO32

MYBL1

Exon 10

124,590,836

67,488,502

C

MYBL1

MYB

ACTB

D

RNA_PROCESSING

E

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confirmed the presence of MYB–NFIB fusions in all six previously reported tumors (10, 11). In addition, this analysis revealed the presence of numerous gene fusions in 20 of the 21 tumors sequenced (Supplementary Table S3).

Remarkably, rearrangements of the MYBL1 gene were found in 5 of the 12 t(6;9) negative tumors. These comprised of 4 tumors with MYBL1–NFIB fusions and one tumor with an intra-chromosomal rearrangement resulting in the fusion of the MYBL1 and YTHDF3 genes (Fig. 1A and B; Supplementary Fig. S1 and Supplementary Table S3). The breakpoints in the four tumors with MYBL1–NFIB fusions were located in introns 8 and 14 of the MYBL1 gene and in intron 10 of the NFIB gene. Accordingly, only the last two exons of NFIB were part of the gene fusions.

In addition, three of the t(6;9)-positive/MYB–NFIB transcript-negative tumors were found to have the 5’ end of the NFIB gene fused to different gene partners (non-MYB/MYBL1 related), including X RCC4, NRRN2, PTTR9D, and AIC1 (Fig. 1B; Supplementary Table S3; see detailed information below).

Analysis of MYBL1–NFIB and MYBL1–YTHDF3 gene fusions in ACCs

The finding of MYBL1 rearrangements in five of 12 t(6;9)-negative tumors and its structural similarity to the MYB gene (16, 17) led us to conduct in-depth analyses of MYBL1–NFIB and MYBL1–YTHDF3 fusions. To confirm MYBL1 rearrangements identified by WGS, we used a panel of primers designed to detect these alternations by RT-PCR (Supplementary Fig. S3A and Supplementary Table S4). The presence of MYBL1–NFIB fusion transcripts was confirmed in all four tumors identified by WGS (Fig. 1C and Supplementary Fig. S3B). In addition, Sanger sequencing of the MYBL1–NFIB transcripts showed that exon 8 of MYBL1 to be fused with either exon 11 or exon 12 of the NFIB gene in the two tumors in which the MYBL1 breakpoint had been localized to intron 8 by WGS (AC10 and AC11, Fig. 1C and Supplementary Fig. S4). In the other two tumors (AC12 and AC13), where WGS identified the breakpoints in MYBL1 intron 14, RT-PCR and Sanger sequencing showed MYBL1 exons 14 or 15 to be fused with exon 11 and/or 12 of the NFIB gene (Fig. 1C and Supplementary Fig. S4).

Notably, we found that part of NFIB exon 12 was transcribed in alternative reading frame in the presence of NEIB exon 11, resulting in distinct amino acid sequences (Supplementary Fig. S4). The expression of multiple fusion transcripts in the same tumor with different exons of MYBL1 or NFIB supports alternative splicing mechanism. FISH analysis using MYBL1 and NFIB BAC clones confirmed the presence of t(8;9) translocations in all four tumors in which MYBL1–NFIB fusions were initially identified by WGS (Fig. 1D). We also confirmed the MYBL1–YTHDF3 fusion in AC14 and detected this fusion in another tumor (AC15, Fig. 1C and Supplementary Fig. S5).

Table 1. MYBL1 gene rearrangements in (t(6;9))-negative ACCs

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>t(6;9)*</th>
<th>MYBL1 rearrangement</th>
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<tbody>
<tr>
<td>AC01</td>
<td>Positive</td>
<td>MYBL1 exon8–NFIB exon11</td>
</tr>
<tr>
<td>AC02</td>
<td>Positive</td>
<td>MYBL1 exon8–NFIB exon11</td>
</tr>
<tr>
<td>AC04</td>
<td>Negative</td>
<td>MYBL1 exon9–YTHDF3 exon4</td>
</tr>
<tr>
<td>AC05</td>
<td>Negative</td>
<td>MYBL1 exon8–YTHDF3 exon4</td>
</tr>
<tr>
<td>AC06</td>
<td>Negative</td>
<td>MYBL1 exon14–NFIB exon12</td>
</tr>
<tr>
<td>AC09</td>
<td>Negative</td>
<td>MYBL1 exon14–NFIB exon12</td>
</tr>
<tr>
<td>AC10</td>
<td>Negative</td>
<td>MYBL1 exon14–NFIB exon12</td>
</tr>
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<td>Negative</td>
<td>MYBL1 exon14–NFIB exon12</td>
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<tr>
<td>AC12</td>
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<tr>
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<td>Negative</td>
<td>MYBL1 exon14–NFIB exon12</td>
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*Confirmed by RT-PCR, Sanger sequencing, and 3’ RACE.

Figure 2.

Mutually exclusive overexpression of MYBL1 and MYB in ACCs. A, quantitative RT-PCR analysis for MYB and MYBL1 mRNA expression performed on 102 ACCs. Tumors with t(6;9) translocation and MYB–NFIB fusion (red), t(6;9) without MYB–NFIB (orange), 1(t(8;9) translocation (blue), and tumors lacking both t(6;9) and t(8;9) (green). Tumor samples in the top and bottom were aligned to visualize the expression of MYBL1 (top) and MYB (bottom) in the same tumors. All of t(6;9) tumors but two had MYBL1–NFIB fusions; these two tumors had high MYB expression. Note that MYBL1 alterations not involving NFIB (MYBL1 alt) were found in a subset of tumors lacking t(6;9) and t(8;9). Specifically, two tumors had an MYBL1–YTHDF3 fusion and three tumors had MYBL1 truncations, as indicated. MYBL1 overexpression was found in all tumors with MYBL1 rearrangements. Gene fusions involving the 5’ end of NFIB were identified by WGS in three cases, indicated with (*): 1 (AC07), 2 (AC08), and 3 (AC09). B, schematic representation of an MYBL1 truncation at exon 10 as a result of an intrachromosomal translocation of chromosome 8. The sequencing profile for this truncation is shown on the right. C, Western blotting analysis for MYBL1 expression in ACCs. Samples labeled in red or blue indicate MYB–NFIB or MYBL1–NFIB positive cases, respectively. Normal samples represent matched normal specimens for cases AC10 and AC11. Note that the MYBL1 antibody was raised against the C-terminal end of MYBL1, and only MYBL1–NFIB fusions with exon 14/15 of MYBL1 could be identified. Note that MYB was not detected in tumors carrying MYBL1–NFIB fusions. D, IHC for MYBL1 showing nuclear staining in an ACC with MYBL1–NFIB fusion (left). MYBL1 was not detected in ACC tumor without MYBL1 alterations (right). E, unsupervised analysis of expression profiles of ACCs with different MYB and MYBL1 alterations (top). The numbers indicate the breakpoint exon for MYB or MYBL1 in MYB–NFIB or MYBL1–NFIB fusions, respectively. Note that tumors with proximal and distal fusions tend to cluster together, respectively. The GSEA analysis indicates enrichment of RNA processing in "long fusion," which were fusions that occurred after exon 11 of MYB or MYBL1. "Short fusion" comprised tumors with fusions at exons 8 or 9.
Validation analysis of the MYBL1 rearrangement (81 tumors)

To assess the prevalence of MYBL1 alterations, we analyzed a validation set of 81 ACCs comprised of 45 positive tumors for the t(6;9) translocation and 36 (6;9)-negative tumors (10, 11). FISH analysis identified the t(8;9) translocation in 10 (27.8%) of the 36 (6;9)-negative tumors; eight of these ACCs also had MYBL1–NFIB fusions (Table 1), where MYBL1 exons 8, 9, 14, or 15 were found fused to exon 11 or 12 of the NFIB gene. Thus, all the fusions involving the MYBL1 gene had intact DNA binding and transactivation domains and a disrupted C-terminal negative regulatory domain (Fig. 1E). No MYBL1–YTHDF3 fusion was identified in any of the tumors in the validation set.

Expression of MYBL1 transcript in ACCs with MYBL1 rearrangements

To evaluate the mRNA expression of MYBL1 in ACCs, we performed quantitative RT-PCR on all 102 ACCs (21 in the test set and 81 in the validation set). Remarkably, we observed high levels of MYBL1 mRNA in all 12 tumors with MYBL1-NFIB fusions and in the two tumors with MYBL1–YTHDF3 fusions (Fig. 2A). Surprisingly, we noted high MYBL1 expression in three tumors lacking both MYBL1 fusions and t(6;9)/(8;9) translocations. To explore additional genomic events associated with MYBL1 overexpression in these three tumors, we performed 3’RACE using specific primers for MYBL1 (Supplementary Figs. S3A and S6A) and found MYBL1 gene truncation at exons 9 or 10 in all three tumors (Table 1). In one tumor (AC79), a MYBL1 transcript with nine additional coding nucleotides (Fig. 2B) was identified in an interchromosomal translocation involving exons 9 of the second tumor (AC78), an MYBL1 truncation occurred as a result of an interchromosomal translocation between exon 9 of MYBL1 and intron 5 of the RAD51B gene (chromosome 14; Supplementary Fig. S6B). In the third tumor (AC77), an MYBL1 variant containing 15bp of intron 9 resulted in the generation of a premature termination codon (Supplementary Fig. S6B). A similar finding has previously been observed in pediatric low-grade gliomas (18).

Interestingly, we observed an inverse relationship between MYB and MYBL1 expression in ACCs. The majority of the t(6;9)-positive tumors, expressed high MYB and low MYBL1 mRNA levels (Fig. 2A). Conversely, tumors with MYBL1 gene rearrangements expressed high MYBL1 and low MYB mRNA levels. The small subset of ACCs that lacked both of the t(6;9) and t(8;9) translocations and MYB or MYBL1 rearrangements expressed variable levels of MYB mRNA.

MYBL1 protein expression was assessed by Western blotting using a specific antibody against the C-terminal domain of MYBL1. Because the antibody recognized only MYBL1 variants with intact exon 10, this analysis is relevant to tumors with MYBL1 exon 14/15 fusions. We observed high MYBL1 protein expression in the 3 ACCs with MYBL1 exon14/15–NFIB exon11 gene fusions (Fig. 2C), but not in tumors with other MYBL1 rearrangements. On the other hand, MYB protein was not detected in tumors with MYBL1–NFIB fusions. To assess the cellular localization of MYBL1 protein, we performed immunohistochemical staining using the same MYBL1 antibody. This analysis revealed robust nuclear staining for MYBL1 in myoepithelial cells in all ACCs with MYBL1 alterations at exons 14 or 15, but not in tumors lacking MYBL1 rearrangements (Fig. 2D). Normal salivary gland, including the ductal and acinar elements, was uniformly negative.

Expression profiling of MYB–NFIB and MYBL1–NFIB in ACCs

To explore global gene expression associated with MYB–NFIB and MYBL1–NFIB fusions in ACCs, we analyzed microarray data previously generated in this study (Supplementary Table S5; ref. 8). Differential expression analysis comparing 13 tumors with MYB or MYBL1 fusions to six tumors lacking MYB or MYBL1 fusions showed no significant changes in gene expression. Further GSEA analysis of the MYB–MYBL1 fusion and non-fusion tumors showed that 126 genes sets enriched in the non-fusion group (Supplementary Table S6, listed top highest 37 gene sets), whereas no differential gene set was enriched in the MYB–MYBL1 fusion group. Of note the 6 tumors lacking MYB or MYBL1 fusions used in this analysis expressed high levels of MYB.

Interestingly, unsupervised clustering analysis of the 13 tumors with MYB or MYBL1 fusions revealed that tumors with fusions that occurred after exon 11 of MYB or MYBL1 ("long fusions") clustered together and away from tumors with fusions at exons 8 or 9 ("short fusions"; Fig. 2E, top). Although supervised analysis found no significant expression differences between ‘long’ and ‘short’ fusions, GSEA analysis identified 19 gene sets significantly enriched in “long fusion,” and five gene sets enriched in the “short fusion” group (Supplementary Table S6). It is interesting to note that most of the gene sets enriched in the ‘long fusion’ samples are related to RNA processing and regulation of translation (Fig. 2E, bottom), whereas gene sets related to tissue development were enriched in short fusions (Supplementary Table S6). These results suggest that the MYB and MYBL1 fusions may share common biologic functions based the length of the fusion products.

Gene fusions involving the 5’ end of the NFIB gene

As noted above, WGS identified fusions of the 5’NFIB region including exons 1 and 2, with no-MYB or MYBL1 gene partners in three of the t(6;9)-positive tumors (Fig. 3A). In one tumor (AC09), we confirmed the fusion of NFIB exon 2 to XRCC4 exon 4 as a result of t(5;9) (Fig. 3B). This tumor also had a translocation that fused NFIB intron 3 to an intergenic region located approximately 1Mb upstream of MYB (position 134,485,793 in chromosome 6). A second tumor (AC08) contained complex rearrangements of chromosomes 6 and 9 that resulted in fusion of NFIB exon 2 to PTPRD and NKAIN2 genes (Supplementary Fig. S7A). However, we were unable to detect fusion transcripts by RT-PCR in this tumor. Of note, the NKAIN2 gene was translocated to approximately 10Mb upstream of the MYB gene. The third tumor (AC07), with NFIB–AIG1 gene fusion (Supplementary Fig. S7B), has previously been confirmed (11). This tumor also had translocation of NFIB to a genomic region located approximately 0.1Mb upstream of the MYB gene.

In summary, we identified 12 tumors with MYBL1–NFIB fusions in a total of 102 ACCs (12%), representing 25% (12/48) of all t(6;9)-negative cases. MYBL1 truncations were found in three cases and MYBL1–YTHDF3 fusion was only detected in two cases in the entire cohort. Three tumors were found to have fusions of the 5’end of the NFIB gene with XRCC4, NKAIN2, PTPRD, and AIG1 genes (Fig. 3C).
Clinicopathologic correlations with 
MYB and MYBL1 alterations

Clinicopathologic correlations with the molecular findings of the entire cohort showed only significant association between MYB alterations and recurrence and metastasis (\(P = 0.042\), Supplementary Table S7). Kaplan–Meier analysis also showed a shorter survival for patients with MYB alterations compared with that of patients with MYBL1 alterations (\(P = 0.010\), log-rank test, Supplementary Fig. S8).

Figure 3.
Analysis of NFIB gene fusions. A, location of NFIB gene breakpoints in MYBL1–NFIB and MYB–NFIB fusions and those in which the 5' end of NFIB is fused to XRCC4, PTPRD, NKAIN2, and AIG1. Note that the breakpoints of NFIB in the MYBL1–NFIB fusions are clustered in intron 10, whereas those in MYB–NFIB fusions tend to be scattered across introns 6–10. All the breakpoints in the NFIB gene in 5' NFIB fusions were found in intron 2. Adjacent table lists all three cases with 5' NFIB fusions and the location of the breakpoints in fusion partners. B, schematic representation of the NFIB–XRCC4 fusion depicting the 5'end of the NFIB gene in fusion with the 3' end of XRCC4 (left). The corresponding circos plot for the tumor with this alteration is shown on the right; the chromosomal translocations identified by WGS are shown with orange lines. The NFIB–XRCC4 fusion transcript in AC09 was confirmed by RT-PCR. Tumors AC07 and AC08 were used a negative controls. C, summary of the frequency of t(6;9) and t(8;9) translocations and the associated MYB–NFIB and MYBL1–NFIB fusions and other MYBL1 rearrangements (MYBL1 alt) identified in the 102 ACCs analyzed in this study. The tumors comprised 53% with t(6;9), 14% with t(8;9), and 33% of tumors lacking both t(6;9) and t(8;9). The incidence of MYB–NFIB and MYBL1–NFIB fusions is indicated. Overall, MYBL1 alterations were found in 17% of the tumors, 12% of them containing MYBL1–NFIB fusions and 5% of them (MYBL1 alt) with MYBL1 truncations or MYBL1–YTHDF3 fusions.
Discussion

We identified novel inter- and intrachromosomal rearrangements of the MYB/L1 gene in 35% of t(6;9)-negative ACCs. These alterations comprised MYB/L1–NFIB fusions as a result of chromosomal translocations of 8q13 and 9p23 regions, intrachromosomal rearrangements leading to MYB/L1–YTHDF3 fusions and MYB/L1 truncations. These findings, together with our previously reported MYB gene rearrangements, highlight the unprecedented occurrence of two distinct chromosomal translocations involving two of the MYB gene family members in a mutually exclusive and tumor specific manner. We contend that the lack of these gene rearrangements in other salivary gland malignancies studied (data not shown) suggests that t(6;9) and t(8;9) translocations occur at spatially adjacent domains in ACC progenitor cell facilitating the homologous recombination during cell division in ACC (19).

Interestingly, all MYB/MYBL1 fusions and truncations retained the DNA binding and the transactivation domains, suggesting an important role for these MYB/MYBL1 variants in regulating gene expression and biologic functions in ACC tumorigenesis. In addition, we observed that the breakpoints in these fusions resulted in the truncation of the C-terminal negative regulatory domain, a mechanism that has been linked to the activation of MYB family members (17, 20–22). The presence of MYB/L1–NFIB fusions in ACC has been confirmed in an independent study (23). However, the finding of MYB full-length expression in some tumor suggests that loss of the C-terminal domain may not be an absolute requirement in the regulation of these genes in ACC. Our MYB/L1 gene rearrangements findings, however, are at variance with studies of pediatric gliomas where duplications and/or amplification of MYB/L1 are the main findings, and suggest that tissue specificity play a role (18, 24).

Our gene expression results show a mutually exclusive relationship between MYB and MYBL1 alterations in most tumors in our cohort. Only two tumors with t(8:9) translocation but negative MYB/L1–NFIB fusion had low MYB/L1 and high MYB mRNA expression. The underlying event associated with the expressions of these genes is currently unknown. The results, however, suggest that both genes coordinately involved in ACC tumorigenesis. This is supported by evidence that MYB and MYBL1 regulate the expression of common and specific set of genes (16), and by our current expression analysis and those of others (7, 25, 26). Interestingly, the finding of distinct differential gene profiles between ‘long’ and ‘short’ fusions of both genes and their association to RNA processing and translation highlights a potential biological importance of the C-terminal part of these fusions.

Further analysis of somatic mutations of the WGS data identified limited number of genes with alterations in ACC. Comparative mutational analysis of tumors based on the structural abnormalities of each group showed mutations in MAP10 and OTOF in the MYBL1 fusion group and mutations of MUC12 and TAF4 genes in the MYB alterations group. The oncogenic role and the clinical significance of these mutations will be investigated upon the validation if these mutations in larger cohort. Interestingly, we found that tumors lacking MYB–MYBL1 alterations had mutations in NOTCH1, SPEN, and MUC16, genes that have been previously reported to be mutated in ACC (7, 8), and lend further support to a potential role in ACC development. These findings suggest that different genetic events may underlie non-fusion derived ACC. In this context, alterations of the NOTCH pathway would characterize preferentially non-MYB–MYBL1 tumors, whereas different mutations are linked to fusion-positive tumors. These findings, if validated, may allow for the stratification of patients for targeted therapy, especially with small molecule therapy for NOTCH1.

We also underscore a potential driver role of NFIB gene in a subset of ACC. In three t(6:9) positive/MYB fusion negative tumors, the 5’ end of NFIB was fused to non-MYB/MYBL1-related genes. Interestingly, all tumors with 5’NFIB fusions had genomic segments translocated to upstream of the MYB gene and expressed high MYB, suggesting that placement of genomic segment in proximity to the MYB gene play a functional role in enhancing activation. Interestingly, all three tumors with these alterations occurred in male patients in their early forties and had distant metastatic disease.

Our clinicopathologic correlations showed an association between MYB alterations and poor patients’ outcome in contrast with those with MYBL1 alterations. Although these results may appear to conflict with the concept of coordinate functional relationship between MYB and MYBL1, the aggressive nature of MYB associated tumors could be attributed to other factors, including enhanced genomic instability and/or epigenetic alterations with MYB associated tumors.

In summary, this study identifies distinct molecular subclasses of ACC, characterized by translocation of 8q and 9p and alterations and high expression of the MYB homologue, MYBL1, in tumors negative for t(6;9) that also lack MYB expression. These findings advance efforts to identify common biologic pathways in ACC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.K. El-Naggar
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