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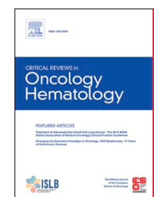

MYB-NFIB fusion transcript in adenoid cystic carcinoma: Current state of knowledge and future directions

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ABSTRACT

Adenoid cystic carcinoma (ACC) is the most common type of salivary gland cancer that can also arise in other primary sites. Regardless of the site, most ACC cases carry a recurrent chromosomal translocation - t(6;9)(q22–23;p23–24) - involving the *MYB* oncogene and the *NFIB* transcription factor. Generally, a long sequence of *MYB* is fused to the terminal exons of *NFIB*, yet the break can occur in different exons for both genes, resulting in multiple chimeric variants. The fusion status can be determined by a number of methods, each of them with particular advantages. *In vitro* and *in vivo* studies have been conducted to understand the biological consequences of *MYB-NFIB* translocation, and such findings could contribute to improving the current inefficient therapeutic options for disseminated ACC. This review provides a discussion on relevant evidence in the context of ACC *MYB-NFIB* translocations to determine the current state of knowledge and discuss future directions.

1. Introduction

Several malignancies carry specific chromosomal translocations as their hallmark. Resulting gene rearrangements can generate oncogenic fusion proteins when the original translocation site involves proto-oncogenes (Zheng, 2013). Since the early 1990s, karyotyping studies have shown that a translocation involving chromosomes 6 and 9 represents a non-random and common event in adenoid cystic carcinoma (ACC) (Nordkvist et al., 1994). A report by Presson and colleagues thoroughly investigated the t(6;9) in 11 cases of ACC from the salivary glands and breast and concluded it was indeed a recurrent event - present in 100 % of cases examined - leading to the fusion of the *MYB* oncogene, located at chromosomal band 6q23, with the *NFIB* transcription factor, located at chromosomal band 9p22–23 (Persson et al., 2009). Later, Mitani et al. (2016) reported that some t(6;9)-negative ACC cases carried gene rearrangements in the *MYB-like 1* (*MYBL1*) gene.

Overall, more than 70 % of ACC cases present aberrations involving *MYB* or *MYBL1* genes (Mitani et al., 2016; Fujii et al., 2017). *MYB* rearrangements are more common, usually found in up to 60 % of cases (Fujii et al., 2017), while *MYBL1* rearrangements occur in approximately 35 % of MYB-NFIB negative cases (Mitani et al., 2016). Interestingly, this recurrent event is not restricted to salivary gland and breast ACC (Persson et al., 2009) - *MYB* rearrangements have been detected in ACC from lacrimal gland (von Holstein et al., 2013), lung (Roden et al., 2015), vulva (Xing et al., 2017) and skin (North et al., 2015). Several studies have investigated different aspects of these fusions ranging from evaluating their diagnostic or prognostic value (Fujii et al., 2017; de Almeida-Pinto et al., 2019) to the biological implications of the chromosomal rearrangements (Gao et al., 2014). Our aim is to provide a comprehensive review of the most relevant evidence concerning the role of *MYB-NFIB* translocations in ACC, focusing on the investigative methods, biological consequences, and therapeutic repercussions.

2. *MYB-NFIB* translocation in ACC

2.1. *MYB-NFIB transcript variants*

A relevant aspect of the t(6;9)(*MYB-NFIB*) found in ACC is that the breakpoint sites are highly variable leading to multiple *MYB-NFIB* transcript variants. MYB gene is located at chromosomal band 6q23 (Fig. 1A). More than 41 different transcripts have been documented

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(Gene: MYB ENSG00000118513 – Ensembl.org). MYB is composed of 15 or 16 exons (depending on the variant) (Gene: MYB ENSG00000118513 – Ensembl.org) and encodes a protein with three helix-turn-helix DNA-binding domains that functions as a transcription regulator (MYB - Alliancegenome.org) (Fig. 1B). The NFIB transcription factor is located at chromosomal band 9p22–23 (Fig. 1C), has 22 transcript variants and is usually composed of 11–12 exons (Gene: NFIB ENSG00000147862 - Ensembl.org) (Fig. 1D). The locations of the DNA binding domain (DBD), transactivation domain (TAD) and negative regulatory domain (NRD) for both genes are shown according to Togashi et al. (2018).

In the original report by Persson et al. (2009), a total of 24 translocations were identified in the 11 cases analyzed, with some cases presenting more than one translocation, and at least 11 *MYB-NFIB* different transcript variants were identified due to alternative splicing and variable breakpoints in both *MYB* and *NFIB* genes (Persson et al., 2009); the fusion of *MYB* at exon 14 with *NFIB* at exon 9 was the most common rearrangement being found in 7/11 of cases. Mitani et al. (2011) observed a different trend, with the fusion of exon 8 of *MYB* and exon 12 of *NFIB* being the most frequent fusion in the cases they reported. The authors also reported recurrent translocations occurring at exon 15 of the *MYB* gene. Interestingly, a more thorough analysis reveals that the sequence "CCCCTTGCAG", reported by Persson et al. (2009) for the exon 14 fusion breakpoint, is the same sequence as that reported by Mitani et al. (2011) and McIntyre et al. (2019) for the exon 15 fusion breakpoint. *MYB* is a complex oncogene with frequent alternative RNA splicing, which can cause confusion in exon numbering and notation. Supplementary Table 1 provides information on the breakpoints reported in the analysis of 61t(6;9) translocations, extracted from 5 studies (Persson et al., 2009; Mitani et al., 2011; Panaccione et al., 2017; Togashi et al., 2018; Chahal et al., 2018). Overall, the key observation is that there are multiple variable breakpoints that usually occur in the *MYB* gene between exons 8 and 15 and in the NFIB gene between exons 8 and 12, or at the 3' untranslated region (UTR). This results in the fusion of a long portion of *MYB*, which contains the DBD and the TAD, to a small part of the *NFIB* gene (Fig. 1 E).

The biological consequences of these fusions will be addressed later, but the variability in breakpoint sites is reflected in the variability in success of the different investigative methods used to identify t(6;9) (*MYB-NFIB*)-positive ACC cases. For example, there is a significant risk of false negatives resulting from RT-PCR analysis in which an insufficient number of primer sets are used. Ono and Okada (2018) demonstrated that the translocation status was mainly detected by a set of primers that investigated exon 14 of the *MYB* gene, supporting the observations that this is the most common breakpoint site for the *MYB* gene. However, one case initially identified as negative in this analysis, was found to be positive when a new set of primers, comprising exon 9 of the *MYB* gene, was used (Ono and Okada, 2018). Researchers planning to investigate the fusion status of a tumour need to consider their final aims prior to selecting the most appropriate investigative method. A range of methods including karyotyping, fluorescent *in situ* hybridization (FISH), Southern blot, PCR, next-generation sequencing (NGS), nanopore sequencing, *etc.* have previously been studied (Mitani et al., 2011; Pasquier et al., 2016; Abel et al., 2014; Au et al., 2019; Hu et al., 2020). In this review we will focus our discussion on the more commonly used methods (FISH *vs.* PCR) and also discuss promising, emerging methods.

If the main goal is to determine the fusion status, for example to investigate clinical-molecular correlations, FISH provides a preferable method to PCR with fewer false-negative results (Mitani et al., 2011). Break-apart FISH probes have advantages over PCR analysis, in the context of chromosomal translocations with diverse chimeric variants, because the probes can be designed for large regions (up to 500 kb) (Burman et al., 2015). Mitani et al. (2011) reported that, in a sample comprising 30 ACC, none of the FISH negative cases had fusion transcripts detected by RT-PCR, which was performed using a wide-range of primers. Interestingly, some of the FISH positive cases lacked transcript products. Additional analysis, performed using 3′RACE amplification, revealed that some of these cases actually presented breakpoints that occurred distal to the 3′ end of the NFIB gene, in the 3′UTR (Mitani et al., 2011).

A further advantage of FISH is the ability to reliably carry out the analysis using formalin-fixed paraffin-embedded (FFPE) samples, which are the most commonly available samples. PCR analysis can also be performed on FFPE samples, with RNA extraction kits designed for this

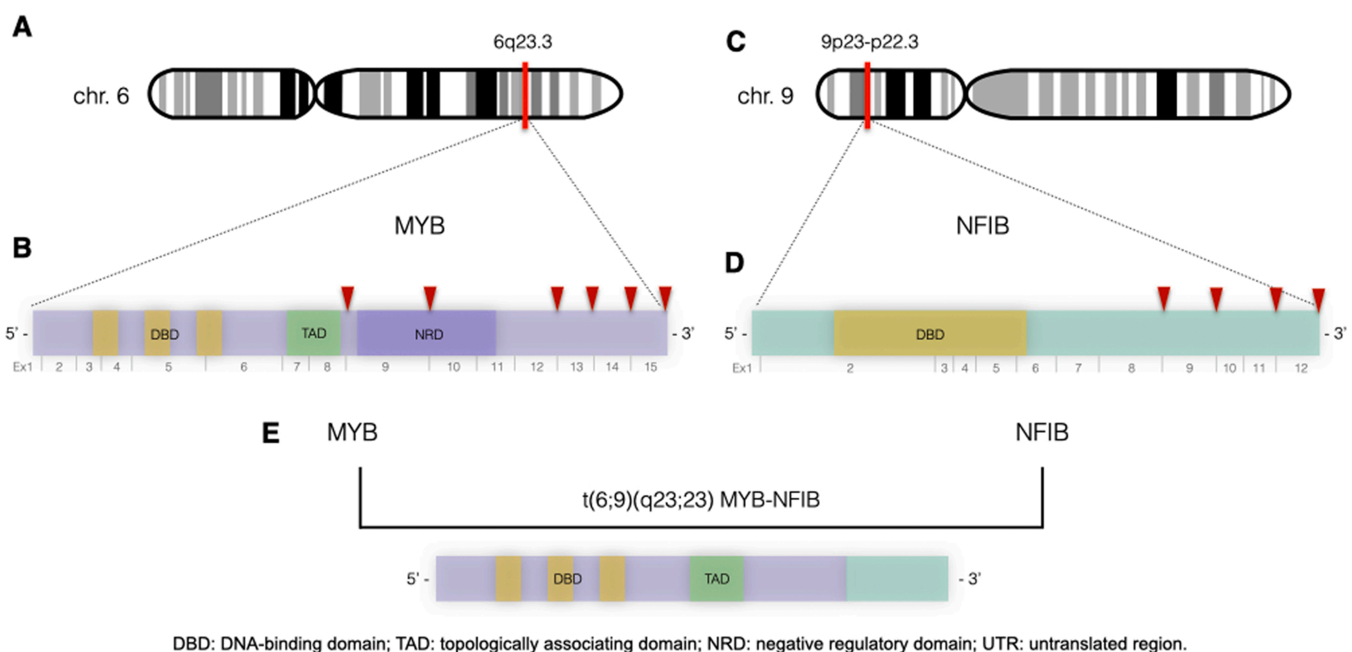

Fig. 1. Schematic representation of *MYB-NFIB* translocation in adenoid cystic carcinoma. (A) *MYB* oncogene located at chromosomal band 6q23. (B) Red arrowheads demonstrate the reported breakpoint sites for *MYB*, which occur between exons 8 and 15. (C) *NFIB* transcription factor, located at chromosomal band 9p22–23. (D) Red arrowheads demonstrate the reported breakpoint sites for *NFIB*, which occur between exons 8 and 12 or at the 3' untranslated region (UTR). (E) The fusion results in a significant part of the *MYB* gene being fused to a small part of the *NFIB* gene.

being continuously improved, however, to date, the results using frozen or fresh tissue are more reliable. Brill et al. (2011) investigated the *MYB-NFIB* transcript by RT-PCR using primers located in *MYB* exons 5, 6 and 12, and in NFIB exon 9, and demonstrated significant differences in the percentage of positive cases when comparing frozen tissue and FFPE samples: 86 % *vs.* 44 % respectively.

Despite these advantages, FISH also has some limitations, particularly in relation to analysis and interpretation. A large number of cells need to be visually inspected to detect a sufficient number of translocation events. Also, defining a split signal can be biased by evaluator subjectivity (Burman et al., 2015). Digital methods of evaluation such as high throughput imaging (HTI) and automated image analysis may help to eliminate such biases. Images can be acquired in 3D through confocal high-throughput microscopy and digitally analyzed to determine the spatial positioning and distance between FISH signals. This method has been previously used with success for the determination of NPM1-ALK chromosome breaks in anaplastic large cell lymphoma (Burman et al., 2015). To date there are no published reports of similar analysis being used for studies of ACC.

FISH is imprecise in identifying the precise chromosomal breakpoint, which can be viewed as an advantage as it gives the method a broader sensitivity but also represents a limitation. More comprehensive methods, such as NGS and nanopore sequencing, bring important advantages in this regard (Hu et al., 2020). NGS can accurately determine DNA-level breakpoints with single-nucleotide precision, and also offers the opportunity to identify previously unknown partner genes or breakpoint sites (Abel et al., 2014). This method has previously been validated to have similar sensitivity and specificity to FISH in detecting ALK and KMT2A rearrangements in malignant tumors (Abel et al., 2014). Nanopore long-read sequencing has also been validated as an effective method to determine fusion status and precise breakpoint sites in acute myeloid leukemia (Au et al., 2019). This method has some advantages over NGS such as lower cost and complexity. If cost and complexity are major barriers to analysis, RNA-based RT-PCR represents an appropriate alternative. Due to the diverse *MYB-NFIB* chimeric variants, it is important, as mentioned previously, to include a wide variety of primer sets in the analysis.

None of the methods described so far can establish precise correlations between the presence of fusion transcripts and key histological features such as cell type, perineural or perivascular invasion, and architectural pattern. ACC is a biphasic tumor composed of relatively uniform ductal and modified myoepithelial cells with a marked tendency for perineural invasion (PNI) that can be classified in three histological patterns: cribriform, tubular and solid, with the solid pattern being the most aggressive (Bell et al., 2011; Frerich et al., 2019). Immunohistochemistry (IHC) studies have demonstrated, through the use of two separate antibodies, that between 50 % and 65 % of ACC cases demonstrate MYB protein overexpression (Bell et al., 2011; Fujii et al., 2017; West et al., 2011). Interestingly, most studies have shown that MYB overexpression occurs mainly in myoepithelial cells (West et al., 2011; Bell et al., 2011; Fujii et al., 2017; Costa et al., 2014). However, a recent study by Goto et al. (2021), using a combination of FISH and IHC, reported that both p63-positive myoepithelial and p63-negative epithelial cells demonstrated *MYB* break-apart signals in cutaneous ACC (Goto et al., 2021). New methods, such as RNA *in situ* hybridization (ISH) (Rooper et al., 2021), have emerged and represent a reliable quantitative technique to determine the presence of RNA transcripts in routinely processed tissues. Rooper et al. (2021) evaluated *MYB* expression by RNA ISH, using a probe targeting bases 204-1289 of the *MYB* human gene. These bases are located in close proximity to the common *MYB* fusion breakpoints and thus would be expected to be conserved regardless of the fusion status. The investigation was performed on ACC and other salivary gland tumor samples, aiming to determine the diagnostic sensitivity and specificity of RNA ISH for ACC in comparison to FISH and MYB IHC. The authors determined that *MYB* RNA ISH was more sensitive than FISH and more specific than IHC, proving the technique to be an accurate tool for the diagnosis of ACC (Rooper et al., 2021). Interestingly, *MYB* RNA ISH was positive in both *MYB-NFIB* positive and negative tumors (as assessed by FISH), suggesting that the *MYB* transcript is present in ACC independent of the fusion status. This technique brings the advantage of enabling a direct visualization of the RNA transcripts by conventional optical microscopy in counterstained tissue and the authors were able to demonstrate that *MYB* RNA overexpression occurred mainly in the myoepithelial cells in tubular or cribriform ACC cases. In solid cases the pattern of expression was more diffuse (Rooper et al., 2021). It would be interesting to further evaluate a specifically designed probe targeting *MYB-NFIB* fusion transcripts through this same technique. The fact that MYB protein overexpression is not uniquely correlated to the MYB-NFIB fusion is also supported by studies that assessed both MYB IHC expression and FISH (West et al., 2011). For example, a study by West et al. (2011) demonstrated that 46 % of MYB-NFIB-negative ACC cases showed MYB IHC overexpression (defined as strong staining in more than 50 % of the neoplastic cells) compared to 88 % of MYB-NFIB-positive cases (West et al., 2011) while Fujii et al. (2017) observed MYB IHC overexpression (also defined as more than 50 % of neoplastic cells showing strong positivity) in only 9 out of 16 (56 %) MYB-NFIB-positive cases (Fujii et al., 2017).

To advance our understanding of the role of the *MYB-NFIB* fusions in ACC development, routine fusion assessment could become part of the standard pathologic evaluation of ACC cases. Accurate, technically feasible and cost effective methods are necessary for this to become a reality. IHC is consistently used for diagnostic purposes in pathology services as it is a relatively straightforward and inexpensive method. Recently, antibodies identifying specific chromosomal translocations have been successfully developed. Azorsa et al. (2020) developed specific monoclonal antibodies that target the junction region of the *PAX3-FOXO1* fusion protein, a recurrent event observed in alveolar rhabdomyosarcoma. The authors demonstrated that IHC analysis achieved a specificity of 100 % and a sensitivity of 91 % (Azorsa et al., 2020). Baranov et al. (2020) found similar specificity and sensitivity values for a fusion-specific antibody designed to identify the breakpoint of *SS18-SSX*, a recurrent event of synovial sarcoma (Baranov et al., 2020). In the salivary gland cancer context, a polyclonal antibody to detect the *MECT1–MAML2* translocation, a recurrent event in mucoepidermoid carcinoma, has been developed in rabbits by immunizing them with a peptide corresponding to sequences that span the fusion point at the N-terminal part of the fusion protein (Behboudi et al., 2006). The antibody was assessed on two fusion-positive MEC cell lines using western blot, confirming the presence of the fusion protein. In addition, immunostaining using the antibody in three fusion positive cases revealed the presence of nuclear staining in the majority of tumor cells, including mucous, epidermoid and intermediate cells, while stromal cells were negative (Behboudi et al., 2006). Due to the highly variable breakpoint sites of *MYB-NFIB* fusion the development of a specific antibody in this context will be more challenging, however, attempts should be made as IHC would positively impact the correct identification of the fusion status of ACC tumors, especially in services with no access to more robust techniques.

2.2. *MYB-NFIB: biological consequences*

Following the discovery of *MYB-NFIB* as a recurrent event in ACC, several studies were conducted aimed at understanding the consequences of the fusion at the molecular level and how this is associated with tumor development or progression. Most studies suggest that, regardless of the chimeric variants and breakpoints observed, the overall *MYB-NFIB* translocation outcome is *MYB* overactivation leading to an increase in Myb protein levels (Persson et al., 2009; Gao et al., 2014; Rettin et al., 2018). It has been demonstrated that *MYB-NFIB* positive cases have increased mRNA levels of *MYB* compared to fusion negative cases (Rettig et al., 2018) and that the Myb protein half-life in cells

expressing the *MYB-NFIB* fusion construct is significantly prolonged (Gao et al., 2014). Thus, to fully understand the consequences of de novo expression of the translocation in ACC, it is important to reflect on known information about the oncogene, *MYB*.

The history behind the discovery of *MYB* as a human oncogene dates back to the beginning of the 20th century. In the 1930s, the Avian Myeloblastosis Virus (AMV), a virus responsible for a form of avian leukemia, was identified (Hall et al., 1941). Some decades later, it was discovered that almost all of the retroviral envelope genes had been substituted by a sequence of cellular origin: *MYB* (Duesberg et al., 1980; Souza et al., 1980). Subsequent studies demonstrated that *MYB* induced not just avian and murine leukaemogenesis, but was also involved in the development of human hematological malignancies and colon cancer (reviewed in Ramsay and Gonda, 2008). *MYB* acts mostly as a transcriptional activator, which means that the great majority of *MYB* targets are positively regulated, while relatively few targets are *MYB*-repressed. It plays important roles in embryogenesis and homeostasis, such as haematopoietic lineage determination (Ramsay and Gonda, 2008). As an example, *MYB* gene knockout is lethal at embryonic day 15, with animals lacking any differentiated blood cell lineages (Mucenski et al., 1991). However, as noted above, this gene can also act as an oncogene, and many genes regulated by *MYB* are linked to aspects of oncogenicity, such as *MYC, CCNA1, CCNB1, CCNE1* and *KIT* (cell proliferation); *BCL2, HSPA5* and *HSP70* (cell survival); and *GATA3* (cell differentiation) (Ramsay and Gonda, 2008). Interestingly, organs and tissues in which *MYB* has a normal physiological role appear to be particularly susceptible to *MYB*-dependent oncogenesis, such as bone marrow (acute myeloid leukaemia and chronic myeloid leukaemia), colon (colorectal cancer) and salivary gland (adenoid cystic carcinoma) (Ramsay and Gonda, 2008). Interestingly, Matsumoto et al. (2016) demonstrated that *MYB* acts as a repressor of Kit expression during submandibular salivary gland development, and Kit downregulation inhibits end bud morphogenesis and cell differentiation into proacini. Thus *MYB* activation contributes to the correct timing of organogenesis by conserving cells in an undifferentiated state (Matsumoto et al., 2016).

MYB involvement in chromosomal translocations is not restricted to ACC as other malignancies present *MYB* rearrangements with different gene partners being involved such as *TCRB* in T-cell acute lymphoblastic leukemia (T-ALL) (Ramsay and Gonda, 2008), *GATA1* in acute basophilic leukemia (Quelen et al., 2011) and *QKI* in angiocentric glioma (Bandopadhayay et al., 2016). In ACC, *MYB-NFIB* fusion causes Myb overactivation, however, the exact mechanism of action remains to be elucidated. It is not known whether the increase in Myb expression is a result of loss in the *MYB* gene, or if the small region of *NFIB* present in the new fusion gene plays the more significant role. The part of the *MYB* gene that is lost depends on where the breakdown occurs and can comprise only the final exons and the 3' end, if the break occurs at exon 12 or after, or also include the NRD - fully or partially when the breakpoint occurs at exons 8 and 9, respectively (Fig. 1). Corradini et al. (2005) have previously demonstrated the importance of the segment of Myb corresponding to amino acids 358 – 452 (located in the NRD) in protein turnover. Hematopoietic cells with cDNA lacking the nucleotides corresponding to these amino acids had increased protein stability (Corradini et al., 2005), and thus the loss of this regulatory portion in the *MYB-NFIB* translocation could contribute to enhanced Myb activity in ACC. It has also previously been proposed that *MYB* overactivation could be a result of disruptions in microRNA (miRNA) binding sites located in the 3' UTR of *MYB* (Persson et al., 2009). miRNA-15a induces an active autoregulatory feedback of *MYB*, directly binding to the 3'-UTR of Myb mRNA (Zhao et al., 2009). miR-150 (Xiao et al., 2007) and miRNA-16 (Chung et al., 2008) have also been shown to act as negative regulators. Persson et al. (2009) demonstrated that miRNA15a/16 and miRNA-150 overexpression in MOLT-4 cells (T-cell acute lymphoblastic leukaemia cell line overexpressing *MYB*) resulted in a 30 % down-regulation of *MYB* mRNA. However, this significant decrease was not observed when primary fusion-positive ACC cells were assessed (Persson et al., 2009).

Gao et al. (2014) investigated the global miRNA signature in fusion positive and negative ACC cases and argued against miRNA binding sites as being a main target for the t(6;9) translocation. The authors observed no meaningful differences in the miRNA expression between *MYB* fusion positive ACC with deleted 3'UTR sequences and fusion-negative cases (Gao et al., 2014). Their analysis did show, however, that in both fusion positive and negative ACC cases, miR-150 was downregulated in the tumor area compared to matched adjacent normal tissue. This suggests that miR-150 plays a role in ACC tumorigenesis even if this is not associated with the fusion status of the tumour (Gao et al., 2014). The authors also generated normal salivary gland cells expressing full-length *MYB* with intact 5' and 3'UTR; *MYB* with no 3'UTR, truncated *MYB* with a stop codon inserted at exon 15 and *MYB-NFIB* fusion. Interestingly, a slight increase in Myb protein expression was noted in cells expressing *MYB* with no 3'UTR and truncated *MYB* but this change was not as significant as that seen in cells expressing the *MYB-NFIB* fusion. Furthermore, only cells expressing the *MYB-NFIB* fusion construct significantly prolonged Myb protein half-life, suggesting that the *NFIB* gene plays a significant role in increasing Myb protein activity.

Significant advances in our understanding as to how *MYB-NFIB* triggers Myb activation were made by Drier et al. (2016). The authors mapped the chromatin landscape and examined the genomic loci that were translocated to *MYB* in ACC cases and identified several super-enhancers in the rearranged portions of *NFIB*, which suggest that the *MYB-NFIB* rearrangement triggers the repositioning of potent regulatory elements in proximity to *MYB* (Drier et al., 2016). Chromosome Conformation Capture analysis confirmed that the super-enhancers interact with the *MYB* promoter, which can create a loop and sustain *MYB* high-level expression. Interestingly, these mechanisms don't seem to be *NFIB* dependent and occur with other *MYB* translocation partners such as *TGFBR3* and *RAD51B* (Drier et al., 2016). These data suggest that promoter–enhancer interactions are a key event in ACC tumorigenesis. A recent study by Frerich et al. (2019) found that ACC tumors make use of an alternative *MYB* promoter, TSS2, that produces a Myb protein with a N-terminal truncation, lacking the first 20 amino acids. This event is similar to that observed in the oncogenic v-Myb protein encoded by AMV, which lacks the first 72 amino acids. The ACC isoform has intact DNA-binding and regulatory domains and a distinct transcriptional activity compared to wild type Myb (Frerich et al., 2019).

The downstream consequences of the fusion protein or Myb overactivation in ACC have also been previously investigated. Two important functional *in vitro* studies were conducted by Gao et al. (2014) and Andersson et al. (2020) in which primary normal salivary gland cells and non-tumorigenic glandular epithelial cells (MCF10A), respectively, were transfected with *MYB-NFIB* or *MYB* wildtype constructs. Their findings demonstrated that the fusion results in changes in cell cycle progression, enhanced proliferative capacity, survival and organoid formation in three-dimensional cultures (Gao et al., 2014; Andersson et al., 2020). Normal salivary gland cells harbouring the *MYB-NFIB* have increased capacity for colony formation and organoid formation (Gao et al., 2014). Glandular epithelial cells (MCF10A) overexpressing wild type *MYB* and the *MYB-NFIB* were more abundant in the S-phase of the cell cycle and their increased proliferative capacity seems to be *MYB* dependent, as it can be inhibited by naphthol phosphate (NAS) treatment (an inhibitor of *MYB* and *CREB* interaction) (Andersson et al., 2020). Using the ACC cell line SACC-83, Xu et al. (2019) used a lentiviral vector to induce *MYB* overexpression and siRNA to knockdown *MYB* expression. Their studies suggested that *MYB* was directly associated with increased cell proliferation, migration and invasion (Xu et al., 2019), however, the results need to be interpreted with caution as SACC-83 cells have not been fully validated to present the most common molecular alterations of ACC and, when injected into mice, do not accurately replicate ACC morphology. *MYB* knockdown by lentiviral transfection has also been shown to inhibit the viability of UFBT and UFH2 cell lines generated from a murine ACC-like mammary tumor and an ACC patient derived xenograft

(PDX) model (Jiang et al., 2019).

Changes in cell behaviour are probably a consequence of *MYB* hyperfunction as a transcriptional activator. ACC fusion-positive cases exhibit upregulation of known direct targets of *MYB* involved with apoptosis (*API5, BCL2, BIRC3, HSPA8, SET*), cell growth or angiogenesis (*CD53, FGF2, KIT, MYC, VEGFA*), cell cycle control (*CCNB1, CDC2, MAD1L1*) and cell adhesion (*CD34*) (Presson et al., 2009). *MYB* overexpression in SACC-83 induced upregulation of genes involved with cell proliferation (*CCND1, MCL1*) and cell migration and invasion *(ICAM1, VEGFA, MMP7, MMP9*) (Xu et al., 2019). Gao et al. (2014) noted the upregulation of several genes in ACC harbouring high *MYB* expression, with significantly increased expression being seen in *ART3, EPHA7, SERPINE2, SCRG1* (Gao et al., 2014). Other known targets of *MYB*, such as *BIRC3, CDC2,* and *CXCR4*, have been shown to be upregulated in MCF10A cells harbouring wildtype *MYB* and *MYB-NFIB* (Andersson et al., 2020). Interestingly, many other genes not previously associated with *MYB* were also found to be upregulated, such as *CCNA2, CENPE, ESCO2, MYBL1, NDC80, PBK, TTK* (Andersson et al., 2020). Ontology analysis demonstrated that the biological processes impacted to a greater extent were cell cycle, cell division, DNA repair, mitosis, and DNA replication (Andersson et al., 2020). At the protein level, *MYB* overexpression in SACC-83 cells triggered β-catenin, N-cadherin, vimentin and α-SMA production and inhibited E-cadherin, suggesting a role in epithelial-mesenchymal transition, which could implicate an increased risk of metastasis (Xu et al., 2019).

Drier et al. (2016) investigated potential downstream effects of *MYB* by identifying MYB-bound enhancers associated with genes that are expressed in ACC and thus revealing putative MYB target genes. The authors identified genes associated with development, migration, cell signalling, cell cycle, transcription regulation and angiogenesis, such as *MYC, BCL2, AURKA, CCND1, MET, FGFR2, IGF1R, MALAT1, CASC4* and *NENF* (Drier et al., 2016). Notably, *NOTCH1, JAG1* and *JAG2* (Notch activators), and *SPEN* (a Notch transcriptional repressor) were also among the putative targets. There was an overlap between *MYB* target genes in ACC and normal salivary glands, but interestingly, 12 % of the genes identified were exclusive to ACC, among which cell cycle regulators stood out (*CDK6* and *GMNN*). The authors suggest that *MYB* triggers two distinct regulatory pathways in ACC, the first that reinforces the neurodevelopmental program already present in normal salivary gland cells and the second that triggers cell proliferation. In the search to identify pathways that cooperate with *MYB* in ACC, it was noted that 81 % of *TP63* binding sites in ACC were co-bound by *MYB*. The authors further identified that the cooperation between *MYB* with *NOTCH* and *TP63* were cell-type dependent, occurring mainly in luminal and myoepithelial cells respectively (Drier et al., 2016).

Global gene expression analysis after *MYB* siRNA knockdown in a *MYB-NFIB* positive ACC cell line demonstrated that *MYB* inhibition triggers the downregulation of genes involved with DNA replication/repair, cell cycle regulation, mRNA transport and RNA processing, including *CCNB1, FGF2, PPP3CA,* and *FABP5*. Ingenuity pathway analysis demonstrated that *MYC* was a top upstream transcriptional regulator associated with *MYB-NFIB* activity. Interestingly, *MYB* knockdown resulted in reduced MYC protein but not mRNA levels, suggesting an indirect regulation of *MYC* target genes by *MYB-NFIB* through alteration of protein levels (Andersson et al., 2017).

Frerich et al. (2019) investigated differences in the transcription activity of wild type Myb and a unique ACC isoform triggered by the TSS2 promoter. The authors identified an overlap in gene expression but also distinctive activity. Notably, the cell functions of genes differentially expressed included cell cycle regulation (*CDK3, COPS2, HSF4*), oncogenic activity (*MALAT1, GPC2, LINC-PINT*) and metastasis (*RAB40B, PRSS3, NME1*). Also, this Myb isoform activated *MYH9, PLXNB1, RAC2, ARHGEF11, RHOC,* and *LIMK2* - genes that belong to the *SEMA4D*-associated pathway, a signalling pathway previously linked to cell migration and perineural invasion. Interestingly, this Myb isoform silenced genes associated with immature or stem cell phenotype (Frerich et al., 2019).

Cancer stem cells (CSCs) have been increasingly associated with tumor progression, relapse, and therapy resistance (Adams et al., 2013). The *MYB-NFIB* fusion effect on CSCs has not been investigated in detail, however, Panaccione et al. (2017) demonstrated that CSCs (CD133+) isolated from a PDX ACC (Accx11) that harbours a *MYB-NFIB* fusion, expressed high levels of β-catenin and were enriched with *STAT3*. Among the different cell lines assessed by the authors Accx11 was the most robust cell line and spontaneously formed spheroids in 3-dimensional cultures, which are recognized to have an enriched CSC phenotype. The tumorigenicity of these spheroids was further confirmed by injection into immunodeficient mice, with 100 % tumor formation (Panaccione et al., 2017). Keysar et al. (2018) investigated the percentage of CSCs in 8 PDX models of salivary gland cancer, including 4 ACC cases but only one MYB-NFIB positive (CUSG005). Notably, the CSC fraction (ALDH+,C44+) within the tumor bulk of the CUSG005 model was 17 % whilst in all other cases of salivary gland cancers evaluated, the CSC fraction was between 0.15 % and 4.5 %. These studies did not investigate a direct association between CSC and MYB-NFIB fusion, however, the results suggest that fusion-positive tumors have an enriched CSCs population with highly tumorigenic features.

In vivo experimental studies have been conducted to explore the oncogenic potential of MYB in ACC. Andersson et al. (2020) could not detect tumor formation after a period of 5 months following the injection of *MYB* and *MYB-NFIB* overexpressing MCF10A cells into immunodeficient mice, concluding that stable transfection of *MYB* or *MYB-NFIB* is not sufficient to achieve tumorigenic potential of a normal mammary gland cell line *in vivo*. Xu et al. (2019) demonstrated that SACC-83 *MYB*-overexpressing cells injected into NOD/SCID mice formed bigger tumors than SACC-83 control cells and also induced more lung metastasis. The histological features of the primary tumor and the metastasis are not discussed fully in the study, thus it is not possible to determine if ACC morphology is replicated.

Mikse et al. (2016) were the first to use a genetically-engineered mouse model to investigate the role of *MYB-NFIB* in salivary and breast gland tumorigenesis. The authors generated tri-allelic mice by crossing bi-allelic mice (*MYB-NFIB/MMTV-Cre*) that expressed *MYB-NFIB* in salivary and breast tissues with p53fl/fl mices (p53 was used to accelerate tumor formation). Notably, no mice developed salivary gland cancer, yet poorly differentiated breast tumors were observed in *MYB-NFIB/MMTV-Cre/p53+/fl* mice. Jiang et al. (2019) studied genetically-engineered *MYB-NFIB/MMTV-Cre* mice and also observed no tumor formation in the salivary glands, however, most animals developed B-cell neoplasm which resulted in a shorter survival time. Interestingly, when these mice were further crossed with transgenic mice with Ink4a+/−/Arf+/−, one mouse developed a mammary tumor with cribriform ACC-like adenocarcinoma features that overexpressed Myb protein levels and was positive for the human *MYB-NFIB* transgene (Jiang et al., 2019). In the same study, the authors observed key *in vivo* findings that link *MYB* to ACC tumor growth in that the tumors generated by injection of control ACC cells (ACCX22 and UFH1PDX) into NOD/SCID mice were significantly bigger than those resulting from cells with *MYB* knockdown by shRNA lentivirus (Jiang et al., 2019).

2.3. Drug based therapy targeting MYB-NFIB-driven pathways

In the era of personalized and targeted medicine a recurrent molecular event involving an oncogene might be seen as a promising therapeutic target. The prognostic value of *MYB* is still arguable, with discrepant results being published. A recent report by Hanna et al. (2020) comprising a cohort of 123 cases of ACC with a long-term follow-up, identified that patients with *MYB* alterations had improved survival compared to those without this genetic change (Hanna et al., 2020). On the other hand, Mitani et al. (2016) demonstrated a direct correlation between *MYB* alterations and the presence of metastasis,

which was further supported by the *in vitro* and *in vivo* assays of Xu et al. (2019). Elevated *MYB* mRNA levels have also been identified in ACC cases with a tendency for worse survival compared to less aggressive cases (Ferraroto et al., 2021), and *MYB* rearranged cases were found to have lower overall survival (Han et al., 2019). Concerning the specific *MYB-NFIB* fusion event, most individual studies suggest no direct association with disease-free survival (Ferraroto et al., 2021; McIntyre et al., 2019; Ho et al., 2019; Togashi et al., 2018), highlighted by a recent meta analysis (Liu et al., 2019).

Independent of its prognostic value, the high proportion of ACC cases harbouring the *MYB-NFIB* translocation support the event as representing a promising therapeutic target. An important starting point in providing supportive evidence is screening for potential drugs that can inhibit *MYB* activity. A number of studies have used this rationale. Mandelbaum et al. (2018) performed a genetic screen of 3840 bioactive small molecules in a pluripotent zebrafish blastomere culture system involving a bacterial artificial chromosome transgenic reporter with GFP at the ATG of the *MYB* gene. The authors identified retinoic acid agonists as potent suppressors of *MYB* and further tested the *in vivo* effect of all-trans retinoic acid (ATRA) and isotretinoin in three ACC patient-derived xenografts (PDXs). Both drugs were capable of inhibiting tumor growth in all three PDXs evaluated with an average of 87 % inhibition. Interestingly, ChIP-seq analysis demonstrated that ATRA induced a modest decrease in *MYB* binding at the *MYB* promoter. In parallel, ATRA also triggered an increase in retinoic acid receptor (RAR) levels in the *MYB* promoter and enhancer, which have repressive functions. These outcomes could further act by weakening the mechanisms of the positive feedback loop of *MYB* (Mandelbaum et al., 2018). The use of ATRA represents a perfect example of a "bench to bedside" approach. It also represents an example of drug repurposing: a valid strategy in the search of new therapeutic options as it makes the process from bench to bedside more straightforward. ATRA, or Tretinoin, received its first FDA approval in 1995 and was initially used to manage acne. In 2004 the drug was further approved for oncologic treatment in patients with acute promyelocytic leukemia. In ACC, this drug has progressed from basic research to clinical trial analysis with the results from the phase II trial being published recently. Eighteen patients with recurrent, metastatic ACC were included and 11 (68 %) achieved disease stability; no case displayed partial or complete response. The authors concluded that despite not achieving the expected effect on reversing disease progression, ATRA was an effective and safe therapy for disease stabilization, as no important adverse effects were observed (Hanna et al., 2021). The use of retinoic acid inhibitors in ACC has also been evaluated in conjunction with a PI3K inhibitor, alpelisib, in an animal model. The rationale was that the PDX ACC tumor chosen for drug testing harboured the *MYB-NFIB* fusion alongside a PIK3CA^{R88Q} mutation. The combination of drugs induced an inhibition in tumor growth, decreased the number of proliferative cells (Ki67 positive) and also *MYB* levels (Sun et al., 2021). Recently, preliminary results investigating the use of ATRA in association with low-dose apatinib (VEGFR2 small-molecule inhibitor) for recurrent/metastatic ACC have been published. Sixteen patients were enrolled of which 3 (19 %) achieved a partial response and 13 (81 %) achieved stable disease (SD), for an overall disease control rate of 100 % (Ye et al., 2021).

Other drug screening strategies have searched for small molecule inhibitors capable of inhibiting *MYB* using a luciferase-based *MYB* reporter cell line (Yusenko et al., 2020). The search was performed using a database of more than 1200 off-patent approved drugs with monensin, a potent inhibitor, being identified as the most promising for further testing. The tests were initially performed in myeloid leukemia cells with promising results: monensin triggered *MYB* degradation and also inhibited its transcriptional activity on a transcriptome wide scale. In primary ACC cells, monensin impaired cell viability and sphere-forming capacity, and also inhibited mRNA and protein *MYB-NFIB* levels and *MYB* transcription activity (Yusenko et al., 2020).

Subsequently, this group expanded the search to cover more than 4000 biology-annotated compounds. The proteasome inhibitor oprozomib showed the highest *MYB*-inhibitory activity in HEK293T cells transiently transfected with a *MYB* construct and was selected for subsequent tests. The authors demonstrated that oprozomib successfully inhibited ACC primary cell growth at nanomolar concentrations, induced ACC cell apoptosis and also impaired sphere forming capacity (Yusenko et al., 2021).

A promising therapeutic role for the ATR/BRCA pathway was identified in a global gene expression and Gene Set Enrichment Analysis (GSEA) of cells overexpressing *MYB* and *MYB-NFIB* (Andresson et al., 2020). The authors tested the effects of an ATR inhibitor, (VX-970/berzosertib) *in vitro* and *in vivo* using ACC PDX derived cells and ACC PDX models. *In vitro* results showed a dose-dependent cell growth inhibition with induction of apoptosis. Corroborating evidence came through *in vivo* studies in which VX-970 treatment led to a significant inhibition of PDX tumor growth. The same group had previously studied the effect of IGF1R/AKT inhibition on *MYB* levels and ACC growth (Andresson et al., 2017). This pathway was chosen based on phospho-arrays analysis to identify top receptor tyrosine kinase (RTK) activation in ACC cells. Linsitinib, an oral small molecule inhibitor of IGF1R, was efficient in decreasing *MYB-NFIB* mRNA levels and inhibited ACC spherogenesis *in vitro*, however, there was no impairment of PDX tumor growth *in vivo* (Andresson et al., 2017).

Drug selection has also been made based on previous findings in the literature, with studies mostly being performed on acute myeloid cells that overexpress MYB, and further tested on ACC models. Drier et al. (2016), tested the BET bromodomain inhibitor, JQ1, as previous data had demonstrated *MYB* suppressive activity in acute myeloid leukemia (Roe et al., 2015). In ACC PDX, cribriform-type ACC (low grade tumors), comprising a biphasic cell population (epithelial and myoepithelial cells), tumor growth was significantly slowed through a modest decrease in MYB mRNA levels and in *MYB* target gene expression. Solid ACC (high grade tumors), composed mainly of epithelial cells, did not respond to JQ1 treatment which led to the hypothesis that the presence of more abundant myoepithelial cells in cribriform tumors make it more dependent on the *MYB* regulatory circuit (Drier et al., 2016) than the greater association with *NOTCH* activation in epithelial cells. Jiang et al. (2019) also based their drug choice on previous data from acute myeloid cells. Celastrol, a natural low-molecular-weight compound, inhibited the growth of *MYB-NFIB* positive cells (derived from a murine ACC-like mammary tumor and from a human ACC PDX), however, this result appeared to be more cytotoxic-related than as a consequence of *MYB* inhibition, as non *MYB* dependent cell lines were also sensitive to the drug. Based on this limitation, the authors tested a drug that was structure-guided and molecularly-designed to target the MYB:CBP/P300 co-transcriptional protein complex and interfere in the assembly of these proteins. The peptidomimetic inhibitor (MYBMIM) demonstrated a dose-dependent effect on cell viability of *MYB*-activated cells along with a limited effect on non-*MYB* dependent cells, suggesting an important *MYB*-specific mechanism of action (Jiang et al., 2019).

A phase I trial is currently underway for patients with metastatic ACC or colorectal cancer (which is also *MYB*-driven), that uses *MYB* inactivation as the therapeutic target (Pham et al., 2019). The trial is testing a vaccine, TetMYB, that targets *MYB*, in association with an anti-PD1 antibody, BGB-A317. This DNA vaccine has been formulated to generate a *MYB*-specific immune response, using a full-length *MYB* cDNA bound to two potent CD4-epitopes extracted from tetanus toxoid (Cross et al., 2015). Three disabling mutations (W115A, W134A and D382V) were introduced to guarantee inactivation of the oncogene transcriptional activation capacity (Cross et al., 2015). Preliminary results with an *in vivo* model, using nude mice, suggested that the vaccine alone was only effective if initiated when the tumor burden was low (2-days post tumor cell inoculation, with boosts on days 7 and 12), and had no positive effect if initiated afterwards. To overcome this, the authors combined the use of immunotherapy which restored the vaccine efficacy even if applied at a later time point after inoculation (Cross

et al., 2015). The ongoing clinical trial represents the first testing of this vaccine in humans.

3. Conclusions and perspectives

Significant advances have been made in our understanding of how the *MYB-NFIB* translocation impacts ACC tumor development since it's discovery in 2009. The *MYB-NFIB* fusion, however, brings significant challenges due to the multiple breakpoint sites. In spite of these different variants, it appears that the fusion of a long sequence of *MYB* with a small part of *NFIB* has a similar outcome in that the activity of the *MYB* oncogene is enhanced. The presence of super-enhancers in the rearranged portions of *NFIB* appears to be key in promoting a regulatory feedback mechanism that maintains *MYB* activity. The outcome is a direct impact on key tumorigenic events through the activation of *MYB*-regulated genes and pathways. Different methods have been used to determine which genes are involved with *MYB* in ACC. Some genes overlap between studies while others do not, but overall it is possible to state that *MYB* overexpression in ACC triggers genes involved with cell proliferation, survival and cell cycle progression.

This review has identified that *MYB* inhibition represents a promising pathway for targeted systemic therapy for ACC, with several preclinical studies using this rationale already achieving promising results. Currently, there are no FDA approved systemic drugs to treat ACC, however, *MYB* is being investigated as a therapeutic target in one completed and one ongoing clinical trial for ACC. The process of new drug discovery, testing and approval poses major challenges in terms of cost and time. Drug repurposing or re-tasking brings significant advantages in this context, as the active compound has already been proven to be safe in humans and the formulation development has already been achieved (Pushpakom et al., 2019). Drug screening strategies have shown that oprozomib and monensin are potent *MYB* inhibitors and other studies have identified berzosertib and linsitinib as putative inhibitors. These drugs have been successfully developed by pharmaceutical companies, but they are not yet fully approved by the FDA for human use. We believe that two pathways should be followed: testing these drugs in further *in vivo* models with clinical trials running alongside new drug screening studies to identify other potent *MYB* inhibitors; special attention should be paid to FDA-approved drugs.

CRediT authorship contribution statement

Vivian P. Wagner and Lynne Bingle contributed to the conception and design of the study. Vivian P. Wagner searched literature and wrote the initial draft. All authors critically revised the data retrieved, revised and gave inputs into the manuscript and approved the final version.

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Conflict of interest statement

The authors certify that they have no commercial or associative interest with that represents a conflict of interest in connection with the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2022.103745.

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