Fusion Oncogenes in Salivary Gland Tumors: Molecular and Clinical Consequences

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Abstract Salivary gland tumors constitute a heterogeneous group of uncommon diseases that pose significant diagnostic and therapeutic challenges. However, the recent discovery of a translocation-generated gene fusion network in salivary gland carcinomas as well in benign salivary gland tumors opens up new avenues for improved diagnosis, prognostication, and development of specific targeted therapies. The gene fusions encode novel fusion oncoproteins or ectopically expressed normal or truncated oncoproteins. The major targets of the translocations are transcriptional coactivators, tyrosine kinase receptors, and transcription factors involved in growth factor signaling and cell cycle regulation. Notably, several of these targets or pathways activated by these targets are druggable. Examples of clinically significant gene fusions in salivary gland cancers are the *MYB–NFIB* fusion specific for adenoid cystic carcinoma, the *CRTC1–MAML2* fusion typical of low/intermediate-grade mucoepidermoid carcinoma, and the recently identified *ETV6–NTRK3* fusion in mammary analogue secretory carcinoma. Similarly, gene fusions involving the *PLAG1* and *HMGA2* oncogenes are specific for benign pleomorphic adenomas. Continued studies of the molecular consequences of these fusion oncoproteins and their down-stream targets will ultimately lead to the identification of novel driver genes in salivary gland neoplasms and will also form the basis for the development of new therapeutic strategies for salivary gland cancers and, perhaps, other neoplasms.

Keywords Fusion oncogenes · Salivary gland neoplasms · Adenoid cystic carcinoma · Mucoepidermoid carcinoma · Biomarker · Targeted therapy · *MYB–NFIB* · *CRTC1–MAML2* · *ETV6–NTRK3*

Introduction

Chromosome rearrangements, in particular translocations, may result in fusion oncogenes encoding oncoproteins with transforming properties. More than 800 fusion oncogenes have thus far been described in a variety of human neoplasms and many of these are now being recognized as important diagnostic and prognostic biomarkers and as novel targets for therapy [1, 2]. Recent studies have shown that gene fusions may account for at least 20 % of human cancer morbidity. The majority of gene fusions are found in leukemias and sarcomas and comparatively few in carcinomas [1]. The reason for this difference is not fully known but may at least partly be due to the fact that recurrent balanced chromosome rearrangements are rare in carcinomas whereas they are common in leukemias and sarcomas [2]. In contrast, several carcinomas have recently been shown to express fusion oncogenes resulting from submicroscopic, often intrachromosomal, rearrangements [3–7]. The most prominent of these are the *ETS* (*ERG* or *ETV1*) and *TMPRSS2* fusions found in a high frequency of prostate cancer [4]. Several intrachromosomally generated gene fusions have also been identified in salivary gland neoplasms [3, 6–8]. Future studies using next generation sequencing strategies are expected to lead to the identification of new fusions oncogene in epithelial malignancies, including those derived from salivary glands [9].

Fusion genes are clinically important, potent oncogenes as demonstrated by their ability to induce tumors in various

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transgenic mouse tumor models. For example, the *ETV6–NTRK3* fusion, typical of secretory carcinoma of the breast and mammary analogue secretory carcinoma of the salivary glands, can induce breast cancer in mice through transformation of committed alveolar bipotent or CD61+ luminal progenitor cells [10]. Many fusion oncogenes have also been shown to be tumor-type specific and are therefore useful as diagnostic biomarkers [8, 11, 12]. The majority of fusions identified in solid tumors encode aberrant transcription factors while a minority express chimeric proteins that deregulate growth factor signaling [13]. Taken together, these and other studies (references in Stenman et al. [8]) clearly demonstrate that fusion genes and their downstream targets are pathogenetically and clinically significant oncogenes and as such also key targets for the development of new cancer therapies. The perhaps most well-known example of this is the tyrosine kinase inhibitor Imatinib mesylate that effectively inhibits the *BCR–ABL* fusion oncoprotein in chronic myeloid leukemia [14].

Several subtypes of salivary gland tumors are characterized by recurrent chromosome translocations which recently have been shown to result in a network of oncogenic gene fusions (Fig. 1) [8, 11]. The fusions encode novel fusion proteins as well as ectopically expressed normal or truncated proteins, and are found in both benign and malignant tumors. The major targets of the translocations are transcriptional coactivators, tyrosine kinase receptors, and transcription factors involved in growth factor signaling and cell cycle regulation. The aim of this paper is to review the current literature on fusion oncogenes in benign and malignant salivary gland tumors and discuss their molecular, clinical, and therapeutic consequences.

***MYB–NFIB* Gene Fusion in Adenoid Cystic Carcinoma**

Adenoid cystic carcinoma (ACC) is the second most common salivary gland malignancy but may also occur in exocrine glands in several other anatomical locations, including breast, sinonasal tract, tracheobronchial tree, cervix, and vulva [15, references in 8, 9]. It is an aggressive, but slowly growing cancer with an often fatal outcome. More than 80 % of patients with head and neck ACC die in 10–15 years after diagnosis. Until recently, little was known about the molecular pathogenesis of ACC. However, we recently showed that a recurrent t(6;9)(q22–23;p23–24) translocation in ACC [16] consistently results in a fusion of the *MYB* oncogene to the transcription factor gene *NFIB* (Fig. 1) [17]. *MYB* belongs to a family of proteins that functions as transcriptional regulators. The *MYB* protein contains three functional key domains, an N-terminal DNA-binding domain, a centrally located transcription activation domain, and a C-terminal negative regulatory domain involved in transcriptional repression [reviewed in 18, 19]. *MYB* plays an important role in the control of cell proliferation, apoptosis, and differentiation and is highly expressed in immature, proliferating cells and is down-regulated as cells become differentiated [19].

In the *MYB–NFIB* fusion oncogene, which is highly overexpressed in ACC, the 3'-part of *MYB*, including target sites for negatively regulating microRNAs, is replaced by one or more of the last coding exons of *NFIB* (Fig. 2a). The predicted *MYB–NFIB* fusion proteins retain the DNA-binding and transactivation domains of *MYB*, and is therefore expected to activate *MYB* target genes. Indeed, several *MYB* targets, including *BCL2*, *KIT*, *CD34*, *BIRC3*, *MYC*, and *MAD1L1*, were shown to be overexpressed in

Fig. 1 A translocation-generated network of oncogenic gene fusions in salivary gland tumors. The multiple translocation target genes *MYB*, *HMGA2*, and *PLAG1* are indicated in red. ACC adenoid cystic carcinoma, MEC mucoepidermoid carcinoma, HCCC hyalinizing clear cell carcinoma, MASC mammary analogue secretory carcinoma, Pl. Ad. pleomorphic adenoma

Fig. 2 a Schematic illustration of the *MYB* and *NFIB* genes as well as the *MYB-NFIB* fusion oncogene (coding exons are shown in darker red and blue colors) and the resulting fusion oncoprotein. Translocation breakpoints are shown by vertical arrows and binding sites for negatively regulating miRNAs in the 3'-UTR of *MYB* are indicated. *DBD* DNA binding domain, *TAD* transactivation domain, *NRD* negative regulatory domain. **b** and **c** FISH analysis of *MYB* rearrangements in adenoid cystic carcinoma using a dual-color *MYB*

break-apart probe consisting of a centromeric (green) and a telomeric (red) probe covering the *MYB* locus and its flanking sequences. Interphase nuclei from a *MYB-NFIB* fusion-positive tumor (**b**), showing an intact signal (fused red/green signals) and a split signal (separated red and green signals), indicating a breakpoint within the *MYB* gene. Panel **c** shows interphase nuclei from a tumor with 1–2 intact red/green signals as well as 1–2 green signals indicating a selective gain of the *MYB* gene

ACC compared to normal salivary gland and breast tissue [8, 17].

Previous studies have identified a subset of ACC that are *MYB-NFIB* fusion-negative but still overexpress *MYB* mRNA and protein. Detailed genomic characterization (including next generation sequencing) of several such cases have revealed insertions of a segment from 9p23–p22.3, including the 3'-part of *NFIB*, immediately centromeric to the *MYB* locus (Fig. 3) [9, 20, 21]. In these cases we can only speculate that enhancer elements upstream of *MYB* perhaps in combination with other regulatory elements in the 3'-part of *NFIB* and its flanking sequences may contribute to the activation of *MYB*. There are also cases reported with *MYB* activation and breakpoints distal to *MYB* (Fig. 3) [9, 21]. These cases may be similar to the t(6;7) translocations found in a subset of T-ALL in which the *TCRB* C β enhancer is juxtaposed 10–100 kb distal to *MYB*, leading to transcriptional deregulation of *MYB* [22]. Taken together, available data indicate that at least 80–90 % of ACC have *MYB* activation by gene fusion or other mechanisms leading to overexpression of *MYB-NFIB* fusion proteins or an apparently normal *MYB* oncoprotein. In contrast, the *MYB-NFIB* fusion has not been found in any non-ACC carcinomas of the head and neck, confirming

the high specificity of the *MYB-NFIB* fusion for ACC. The fact that *MYB* activation is found in such a high frequency of ACCs regardless of the site of tumor origin, indicate that the *MYB-NFIB* fusion is a key oncogenic event and hallmark of ACC [9, 17, 21, 23, 24]. From a diagnostic point of view, the *MYB-NFIB* fusion and/or *MYB* activation may be detected by RT-PCR analysis of fusion transcripts, FISH analysis using probes for *MYB*, and/or *NFIB* (Fig. 2b, c), or by immunohistochemical staining of *MYB*-proteins. In addition to being a diagnostic biomarker for ACC, *MYB* and its downstream targets are also potential therapeutic targets. Continued studies aiming at identifying the transcriptional targets of the fusion will therefore be crucial to develop new therapies that may improve the survival of patients affected by this aggressive and often fatal disease.

Similar to the *CRTC1-MAML2* and *EWSR1-POU5F1*-fusions (see below), the *MYB-NFIB* fusion has also been encountered in sporadic dermal cylindromas [25]. These are benign tumors often located in the head and neck region with certain histopathologic similarities to ACC. Two-thirds (67 %) of cylindromas either express *MYB-NFIB* fusion transcripts and/or stain positive for *MYB* proteins. These results together with our previous observations [26–29] further strengthens the evidence for common molecular

Fig. 3 Schematic illustration of two alternative mechanisms of activation of *MYB* in fusion-negative adenoid cystic carcinomas, that is insertions of a segment from 9p23–p22. 3, including the 3'-part of *NFIB*, immediately centromeric to the *MYB* locus or t(6;9)

translocations with breakpoints 10–100 kb distal to *MYB*. In both cases, the proximity of the 3'-part of *NFIB* and its flanking sequences to *MYB* leads to transcriptional activation of an apparently intact *MYB* gene

pathways of importance for the development of both benign and malignant, breast, salivary, and skin adnexal tumors.

***CRTC1–MAML2* Gene Fusion in Mucoepidermoid Carcinoma**

Mucoepidermoid carcinoma (MEC), the most common salivary gland carcinoma, is characterized by a unique and recurrent t(11;19)(q21–22;p13) translocation that occur in a high frequency of MECs [8, 11, 30]. The translocation results in a fusion of the two transcriptional coactivators *MAML2* and *CRTC1* (also known as *MECT1*, *TORC1* or *WAMTP1*) and is a characteristic feature of MECs of salivary, bronchial, and thyroid glands [31–33]. The *CRTC1–MAML2* fusion gene is composed of exon 1 of *CRTC1* linked to exons 2–5 of *MAML2*. *CRTC1* belongs to a family of highly conserved CREB (cAMP response element-binding protein) coactivators [34, 35] whereas *MAML2* belongs to a family of Mastermind-like, nuclear proteins that functions as coactivators for Notch receptors [36, 37]. The fusion encodes a chimeric protein in which the Notch-binding domain of *MAML2* is replaced by the CREB-binding, coiled-coil domain of *CRTC1* fused to the transactivation domain of *MAML2* [31, 32]. The molecular consequences of the fusion is not yet fully understood. However, functional studies have shown that the N-terminal part of the fusion protein, including the CREB-binding domain, is crucial for transforming activity [38] and that the fusion protein activates transcription of cAMP/CREB target genes, including *PEPCK1*, *AREG*, *MMP10*, *IL6*, *NR4A2*, and *NR4A3* [39, Enlund et al. unpublished data]. Preliminary studies using different small molecule inhibitors of the *EGFR* (AREG-amphiregulin) or PKA (cAMP-dependent kinase) pathways have shown that they can inhibit the proliferation of MEC-derived cell lines in vitro, suggesting that targeting these pathways may offer a new

approach to systemic treatment of *CRTC1–MAML2* positive MECs [39].

Several independent and well-documented studies have shown that the *CRTC1–MAML2* fusion preferentially occurs in low/intermediate-grade MECs with favorable prognosis [29, 40 and refs. therein]. Based on a recent arrayCGH study of genomic imbalances in fusion-positive and fusion-negative MECs, we proposed that MEC may be subdivided in (a) low-grade, fusion-positive tumors with no or few genomic imbalances and favorable prognosis, (b) high-grade, fusion-positive tumors with multiple genomic imbalances (including deletions of the tumor suppressor gene *CDKN2A*) and unfavorable prognosis, and (c) a heterogeneous group of high-grade, fusion-negative non-MEC adenocarcinomas with multiple genomic imbalances and unfavorable outcome [40]. There is sufficient evidence at hand indicating that the *CRTC1–MAML2* fusion is a clinically useful biomarker that distinguishes true MECs, most of which have an excellent prognosis, from fusion-negative MEC-like tumors with a more unfavorable prognosis.

Is the *CRTC1–MAML2* Gene Fusion a Recurrent Feature in Warthin Tumor?

Previous cytogenetic studies have shown that a subset of Warthin tumors, the second most common subtype of benign salivary gland tumor, have an apparently identical t(11;19)(q21–22;p13) translocation as the one found in MEC [32 and refs. therein]. RT-PCR analysis of a few such cases have shown that this translocation also results in a *CRTC1–MAML2* gene fusion [32, 33]. Subsequent studies have suggested that these tumors may be described as metaplastic variants of Warthin tumors, representing early signs of MEC developing within Warthin tumors or misdiagnosed mucoepidermoid carcinomas [41, 42]. Interestingly, an identical *CRTC1–MAML2* fusion has also been identified in clear cell hidradenoma of the skin and breast

[26, 28]. This is a benign skin adnexal tumor with certain morphologic similarities to MEC. Taken together, these observations indicate that the *CRTC1–MAML2* fusion is etiologically linked to benign and low-grade malignant, histogenetically similar tumor types originating from exocrine glands in different anatomical locations.

***EWSR1–POU5F1* Gene Fusion in High-Grade *MAML2*-Negative Mucoepidermoid Carcinoma**

As discussed above, there is a subgroup of high-grade *CRTC1–MAML2* fusion-negative tumors with a partly MEC-like morphology. In an effort to further characterize these tumors we have identified cases with a t(6;22)(p21;q12) translocation resulting in an *EWSR1–POU5F1* gene fusion (Fig. 1) [27]. Moreover, we also identified an identical gene fusion in a subset of less well-differentiated cutaneous hidradenomas (in contrast to the clear cell variants) [27]. The chimeric *EWSR1–POU5F1* protein consists of the N-terminal domain of *EWSR1* linked to the DNA-binding domain of the transcription factor *POU5F1*. *POU5F1* is important during the early stages of development to maintain the pluripotent status of embryonic stem cells. Notably, the morphology of the *EWSR1–POU5F1* positive tumors were more immature compared to the *CRTC1–MAML2* positive tumors, in line with the known consequences of overexpression of *POU5F1*.

A similar *EWSR1–POU5F1* fusion has also been found in a case of an undifferentiated bone tumor of the pelvis [43] as well as in a subset of deep-seated benign and malignant soft tissue myoepithelial tumors of children and young adults [44]. In contrast, none of five salivary myoepithelial carcinoma ex pleomorphic adenomas analyzed in that study had rearrangements of *EWSR1*, suggesting that at least a subset of myoepithelial tumors of the salivary glands may not be related to their soft tissue counterparts.

***EWSR1–ATF1* Gene Fusion in Low-Grade Hyalinizing Clear Cell Carcinoma**

Recently, a rare salivary gland carcinoma, hyalinizing clear cell carcinoma (HCCC), was shown to have t(12;22)(q13;q12) translocations resulting in *EWSR1–ATF1* gene fusions [45, Fehr et al. unpublished data]. HCCC is a low-grade carcinoma with distinctive clear-cell morphology and pattern of hyalinization often in combination with focal mucinous differentiation. The *EWSR1–ATF1* fusion has been found in >80 % of HNCCC. By contrast, the fusion is not detected in any of the morphological mimics: epithelial-myoepithelial carcinoma, myoepithelial carcinoma or MEC, demonstrating its usefulness as a diagnostic biomarker for HCCC.

Interestingly, *EWSR1–ATF1* fusions were originally described in conventional clear cell sarcomas (of tendons and aponeurosis) [46] and have recently also been encountered in angiomatoid fibrous histiocytomas [47] as well as in a few cases of soft tissue myoepithelial tumors [48]. Taken together, these observations provide evidence for a unifying concept of salivary gland and soft tissue tumors with clear cell morphology.

***ETV6–NTRK3* Gene Fusion in Mammary Analogue Secretory Carcinoma of Salivary Glands (MASC)**

Mammary analogue secretory carcinomas of the salivary glands (MASC) is a recently described subtype of salivary gland carcinoma, with strong histologic and immunohistochemical resemblance to secretory carcinoma (SC) of breast [49, 50]. In addition to the morphologic similarities, MASC and SC of the breast also have important genetic similarities since they both share a t(12;15)(p13;q25) chromosomal translocation [51], resulting in an identical *ETV6–NTRK3* gene fusion (Fig. 1). The fusion is found in the majority of MASCs (>90 %) and is an important biomarker that may help distinguish MASC from acinic cell carcinoma and low-grade cystadenocarcinoma. The *ETV6–NTRK3* fusion gene, encodes a chimeric tyrosine kinase that activates two major effector pathways, i.e., the Ras-MAP kinase (MAPK) mitogenic pathway and the phosphatidylinositol-3-kinase (PI3K)-AKT pathway both of which seem to be required for *ETV6–NTRK3* transformation [51–53]. It should be noted that *ETV6–NTRK3* gene fusions are found in several other tumor types, including congenital mesoblastic nephroma, congenital fibrosarcoma, and acute myeloid leukemia [52], indicating that the fusion oncoprotein has transforming activity in a variety of cell types. Interestingly, a recent study of mammary-type secretory carcinoma of the skin suggests these tumors are negative for the *ETV6–NTRK3* gene fusions [54]. The finding of the *ETV6–NTRK3* fusion in SC of the breast and salivary glands is of particular interest because of the recently described *MYB–NFIB* fusion in ACCs of both glands [17]. These observations further strengthen the evidence for common molecular pathways of importance for development of breast and salivary gland neoplasms.

***PLAG1* and *HMGA2* Gene Fusions in Pleomorphic Adenoma**

Pleomorphic adenoma is the most common histologic subtype of salivary gland tumor. It is a benign tumor with a highly variable morphology that sometimes may cause

diagnostic problems. Extensive cytogenetic studies of pleomorphic adenomas have shown that they are characterized by recurrent translocations or intrachromosomal rearrangements with breakpoints preferentially affecting 8q12 (>50 % of the cases) and 12q14–15 (10–15 % of the cases) [reviewed in 8, 11]. The translocations/rearrangements invariably result in gene fusions involving the transcription factor genes *PLAG1* and *HMGA2* [8, 11, 55, 56]. *PLAG1* encodes a developmentally regulated DNA-binding zinc finger protein that is part of a family of cell cycle progression-related proteins. Ectopic overexpression of *PLAG1* due to promoter swapping with at least one of five other genes (*CTNBN1*, *FGFR1*, *LIFR*, *CHCHD7*, *TCEA1*) (Fig. 1), cause deregulation of *PLAG1* target genes and activation of the IGF-II signaling pathway [57, reviewed in 8, 11]. Previous studies have demonstrated that *PLAG1* is also involved in gene fusions with two other genes (*HAS2* and *COL1A2*) in benign lipoblastomas [reviewed in 8, 11].

HMGA2 belongs to the high mobility group (HMG) protein gene family which encodes proteins that are heterogeneous, nonhistone components of chromatin [reviewed in 8, 11]. *HMGA2* functions as an architectural transcription factor which regulates transcription through its binding to the minor groove of AT-rich DNA and is also involved in the regulation of recombination and chromatin structure. The protein contains three DNA-binding domains, a spacer domain, and a highly acidic C-terminal domain. Genomic rearrangements of the 3'-part of *HMGA2* due to fusions with the 3'-parts of *NFIB*, *WIF1* or *FHIT* (Fig. 1) result in activation of the expression of *HMGA2* and its target genes, including the cell cycle regulators *CCNA1* and *CCNB2* [58, 59]. The molecular mechanism leading to activation of *HMGA2* is still partly unknown. Recent studies have indicated that a major mechanism may include loss of target-sites for negatively regulating Let-7 microRNAs in the 3'-UTR as a consequence of gene fusion [60]. Interestingly, *HMGA2* is also involved in gene fusion in a variety of benign mesenchymal tumors with 12q14–15 rearrangements, including lipoma, uterine leiomyoma, hamartomas of the breast and lung, fibroadenoma of the breast, angio-myxoma, endometrial polyps, and bone and soft tissue chondroma [reviewed in 8, 11].

The *PLAG1* and *HMGA2* fusions in pleomorphic adenoma have not been encountered in any other histopathologic subtypes of salivary gland neoplasms and may therefore be useful as biomarkers in diagnostically challenging cases with morphologies partly overlapping with adenoid cystic carcinoma, polymorphous low-grade adenocarcinoma and other salivary gland carcinomas.

Gene Fusion in Carcinoma-Ex-Pleomorphic Adenoma

Our knowledge about the molecular abnormalities involved in the transformation of a benign pleomorphic adenoma into a carcinoma-ex-pleomorphic adenoma (Ca-ex-PA) is still limited. The malignant component is frequently a poorly differentiated adenocarcinoma or undifferentiated carcinoma but may also be virtually any other subtype of salivary gland carcinoma, such as MEC, salivary duct carcinoma or ACC [15]. Molecular studies of small series and single cases of Ca-ex-PA have shown that they express pleomorphic adenoma specific gene fusions involving *PLAG1* and *HMGA2* [7, 61]. In addition, amplification of multiple genes within 12q13–15 (in particular *MDM2* and *HMGA2-WIF1* gene fusions, *TP53* mutation, deletions of 5q23.2–q31.2, gains of 8q12.1 (*PLAG1*) and 8q22.1–q24.1 (*MYC*), and amplification of *HER2* have been identified as genetic events of importance for malignant transformation [7].

Conclusions

Salivary gland tumors constitute a heterogeneous group of uncommon diseases that pose significant diagnostic and therapeutic challenges. The primary treatment of salivary gland neoplasms is surgical resection with or without post-operative radiotherapy. For patients presenting with locally advanced, recurrent or metastatic disease the treatment options are currently limited and mainly palliative. However, the recent discovery of a comprehensive translocation-generated gene fusion network in salivary gland carcinomas and benign salivary gland neoplasms opens up new avenues for improved diagnosis and development of specific targeted therapies. The fusions encode novel fusion proteins or ectopically expressed normal or truncated proteins. The major targets of the translocations are transcriptional coactivators, tyrosine kinase receptors, and transcription factors involved in growth factor signaling and cell cycle regulation. Continued molecular characterization of these fusion oncoproteins and their downstream targets will ultimately lead to the identification of novel driver genes in salivary gland neoplasms and will also form the basis for the development of new therapeutic strategies for salivary gland cancers and, perhaps, other neoplasms.

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