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REVIEW ARTICLE

The evolving role of molecular pathology in the diagnosis of salivary gland tumours with potential pitfalls

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Abstract

Salivary gland tumors are diagnostically challenging owing to the morphological diversity within any tumor type and overlapping histomorphology and immunohistochemistry amongst different tumours. In past two decades, rapid progress has been made in the field of understanding the pathogenesis of these tumours with the discovery of many tumour specific translocations and rearrangements. This includes *CRTC1-MAML2* and *CRTC-MAML2* in mucoepidermoid carcinoma, *MYBNFIB* and *MYBL1-NFIB* fusions in adenoid cystic carcinoma, *PLAG1* and *HMGA2* in pleomorphic adenoma, *ETV6-NTRK3* in secretory carcinoma, *NR4A3* rearrangements in acinic cell carcinoma, *PRKD1* mutations in polymorphous adenocarcinoma and *EWSR1-ATF1* in clear cell carcinoma. This review is a lens for progress made till date in the molecular pathology of salivary gland tumours with a special focus on their role as diagnostic tools and implications on clinical management of the patient as prognostic and predictive markers.

Keywords Salivary gland tumours · Mutational analysis · Diagnostic and prognostic role

Abbreviations

MEC:	Mucoepidermoid Carcinoma
AdCC:	Adenoid Cystic Carcinoma
AciCC:	Acinic Cell Carcinoma
PA:	Pleomorphic Adenoma
BCA:	Basal Cell Adenoma
PAC:	Polymorphous Adenocarcinoma
SDC:	Salivary Duct Carcinoma
SC:	Secretory Carcinoma
IC:	Intraductal Carcinoma
CCC:	Clear Cell Carcinoma
EMC:	Epithelial-Myopeithelial Carcinoma
CCMC:	Clear Cell Myopeithelial Carcinoma
CA Ex-PA:	Carcinoma Ex Pleomorphic
CAMSG:	Cribriform Adenocarcinoma Of Minor Salivary Glands
BCAC:	Basal cell adenocarcinoma

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Introduction

Salivary gland tumors are heterogeneous group of neoplasms which constantly pose a diagnostic challenge owing to the vast morphological diversity within any tumor type and overlapping histomorphology and immunohistochemistry (IHC) across different tumors. With rapid expansion and understanding of the genomic landscape and the pathogenesis, a large proportion of salivary gland tumours are now found to have fusion-driven tumour specific rearrangements. This has resulted in reclassification of salivary tumours with deletion of some and recognition of new entities. Currently 4th Edition of the World Health Organization (WHO) Classification of Head and Neck Tumours, 2017 has identified over 30 distinct types of salivary gland neoplasms [1].

Molecular diagnostic techniques including Fluorescence in situ hybridization (FISH), Reverse transcriptase polymerase chain reaction (RT-PCR), and next-generation sequencing (NGS) are now being utilized with increasing frequency for the exact characterization of difficult salivary gland tumors. Even the role of IHC has expanded as surrogate markers for mutations with new antibodies targeting molecular alterations. This has not only diagnostic and prognostic implications but has led to the identification of many targetable mutations. Latest NCCN guidelines identify the use of targeted therapy as an option for advanced salivary gland tumors with distant metastasis. Androgen receptor positive (AR), NTRK and Her2neu are currently validated predictive markers [2]. Future integration of genetic profiles with histopathological parameters for therapeutic stratification of certain salivary gland neoplasms is inevitable.

This review elaborates the panorama of molecular genetics of salivary gland tumors and focuses on its diagnostic potential with potential pitfalls. Details of all chief mutations have been described in Table 1.

Mucoepidermoid carcinoma (MEC)

MEC is the most common salivary gland malignancy involving both the major and minor salivary glands. It is composed of mucinous, intermediate and squamoid tumour cells arranged in cystic and solid patterns. It is a heterogenous category and includes a broad spectrum from low grade to aggressive high-grade tumours with variable clinical course [1].

In nearly 55–88% of MEC, a recurrent (11;19) (q21; p13) translocation, resulting in fusion of exon 1 of the *CRTC1* gene at 19p13 and exon 2 to 5 of the Mastermind-like gene family (*MAML2* gene) at 11q21 has been reported [3–5].

In normal cell, *CRTC1* gene encodes a protein called CREB-regulated transcription coactivator 1 (CRTC1). CREB is an acronym for cAMP response element binding protein and regulates genes involved in cell proliferation and differentiation. CRTC1 protein binds to CREB and increases its transcription. CRTC1 also modulates cellular pathways mediated by activating protein 1 (AP-1). Mastermind-like proteins act as critical transcriptional co-activators for Notch signaling. Amino-terminal (N-terminal) domain of MAML2 protein is responsible for its interaction with ankyrin repeat domain of Notch receptor in the nucleus while carboxyl terminal (C-terminal) contains a transcriptional activation domain (Fig. 1a) [3–5].

In MEC, the CRTC1-MAML2 fusion onco-protein consists of the 42-amino acid CREB binding domain (CBD) of CRTC1at N-terminal fused with the 981-amino acid transcriptional activation domain (TAD) of MAML2 at C-terminal. So, N terminal of MAML2 protein which is required for binding to intracellular Notch is now replaced by CREB binding domain of CRTC1. CRTC1-MAML2 fusion now interacts with the transcription factor CREB through the CRTC1 CBD domain and through the MAML2 TAD domain constitutively activates CREB-mediated transcription of many genes such as *PEPCK1*, *AREG*, *MMP10*, *IL6*, *NR4A2*, and *NR4A3* directly without requirement of cAMP (Fig. 1b) [3–5].

A small subset of MECs (nearly 5%) show different but similar translocation t(11;15)(q21;q26) generating *CRTC3-MAML2* fusions. *CRTC1-MAML2* and *CRTC3-MAML2* mutations are reported to be mutually exclusive [3].

Another rare translocation is t(6;22)(p21;q12) where *EWSR1* exon 6 is fused to *POU5F1* exon 2 resulting in fusion of the N-terminal transactivation domain of EWSR1 to the POU and the homeobox (DNA binding domain) of POU5F1 [3, 6].

Genome-wide, array-based comparative genomic hybridization (arrayCGH) study revealed that fusion positive tumours are mostly low grade and have few or no genomic imbalances while high-grade tumors have numerous genomic imbalances and are often fusion-negative [7].

Diagnostic role

MEC can be diagnosed on histomorphology alone in most of the cases and genetic testing is not always needed. However, molecular tests are especially helpful to diagnose the rare variants of MEC such as oncocytic (Fig. 2a), warthin like or clear cell type particularly in small biopsies and cytology materials. *CRTC1/3-MAML2* fusions can be detected by RT-PCR or by FISH using dual-color break-apart probes for *MAML2* [3, 4]. Amphiregulin (AREG) is an epidermal growth factor receptor (EGFR) ligand and its transcription is aberrantly upregulated by CRTC1-MAML2 fusion protein.

Table 1 Key mutations in salivary gland tumours

Tumour	Gene	Sequencing based molecular tests	PCR	Fish	Surrogate IHC markers
Mucopeidermoid carcinoma	CRTC1-MAML2 CRTC3-MAML2 EWSR1-POU5F1	(a) SalvGlandDx (b) NGS	(a) CRTC1-MAML2 (b) CRTC3-MAML2 Fusions by RT-PCR	MAML2 (Breakapart probes)	AREG
Adenoid cystic carcinoma	MYB-NFIB; MYBL1-NFIB	(a) Amplicon sequencing (b) SalvGlandDx (c) NGS	MYB-NFIB RTPCR	MYB and MYBL1 (Breakapart probes) MYB-NFIB (fusion probe)	MyB
Acinic cell carcinoma	SCPP-NR4A3 MSANTD3-HTN3	SalvGland Dx (b) NGS	SCPP-NR4A3 RT-PCR	NR4A3	NR4A3
Secretory carcinoma	ETV6-NTRK3 ETV6-RET ETV6-MET	SalvGlandDx (b) NGS	ETV6-NTRK3 Fusion by RT-PCR	ETV6 NTRK3 (Breakapart probes)	Pan-Trk
Polymporphous adenocarcinoma	PRKD1 E710D	(1) Sanger sequencing (2)SalvGlandDx (b) NGS	–	–	–
Cribriform adenocarcinoma of minor salivary gland	ARID1A-PRKD1, PRKD1-DDX3X, PRKD2 and PRKD3 fusions	SalvGlandDx (b) NGS	–	FISH	–
Clear cell carcinoma	EWSR1-ATF1 EWSR1-CREM Fusion	NGS SalvGlandDx	RT-PCR	EWSR1 ATF1 breakapart probes	–
Salivary duct carcinoma	AR gene alterations ERBB2 amplification TP53, PIK3CA, H-RAS, KIT, EGFR, BRAF, N-RAS, AKT1, FBXW7, ATM, NF1 mutations	Sequencing NGS	–	PLAG1 HMAG2	PLAG1 HMAG2 AR Her2
Pleomorphic adenoma, CAexPA	PLAG1 rearrangements (50–60%) HMAG2 rearrangements (10–20%)	(1) Amplicon sequencing (2) SalvGlandDx (b) NGS	RT PCR	PLAG1 HMAG2	PLAG1 HMAG2
Epithelial myoepithelial carcinoma	HRAS p.Q61R	(1) Hotspot mutation analysis by direct DNA sequencing (2) Amplicon sequencing (3) SalvGlandDx b) NGS	–	–	RAS Q61R
Basal cell adenoma	CTNNB1	(1) Hotspot mutation analysis by direct DNA sequencing (2) Amplicon sequencing (3) SalvGlandDx (b) NGS	PCR	–	β-Catenin, LEF-1

Table 1 (continued)

Tumour	Gene	Sequencing based molecular tests	PCR	Fish	Surrogate IHC markers
Basal cell adenoma	CYLD	(1) hotspot mutation analysis by direct DNA sequencing (2) NGS	PCR	–	CYLD LEF-1 But negative for β-Catenin
Intraductal carcinoma: intercalated duct type	NCOA4-RET	SalvGlandDx (b) NGS	–	–	–
Intraductal carcinoma: hybrid type	TRIM27-RET fusion	SalvGlandDx (b) NGS	–	–	–
Intraductal carcinoma: Apocrine type	PIK3CA HRAS	SalvGlandDx (b) NGS	–	–	–
Microsecretory adenocarcinoma	MEF2C-SS18 SS18-ZBTB7A	SalvGlandDx (b) NGS	–	SS18 breakapart probe	–
Sialadenoma papilliferum	BRAF V600E	(1) Amplicon sequencing (2) SalvGlandDx (b) NGS	–	–	–
Intraductal papillary mucinous neoplasm	AKT1 E17K	SalvGlandDx (b) NGS	–	–	

Its expression by IHC is used as surrogate marker for detection of fusion positive MEC [3, 8].

Hence, these markers can be incorporated in routine diagnostics for distinguishing low-grade MEC with oncocytic epithelium and prominent lymphoid stroma also called as Warthin like MEC from benign Warthin tumour (WT) and salivary lymphadenoma since the former needs closer follow up and clear resection margins. In such cases, the detection of *MAML2* rearrangement confirms the diagnosis of MEC. Although some authors have reported *MAML2* translocations in WT but others argue these cases may represent low-grade MEC mimicking WT [9–11]. Most consider the finding *MAML2* rearrangement strongly favor a diagnosis of MEC over WT [10, 11]. *MAML2* rearrangement should always be investigated before rendering a diagnosis of WT in a young, non-smoking, female patient [8]. Since these fusions are highly specific to MEC they are a useful diagnostic marker in problematic cases but their absence does not rule out MEC.

Another morphological variant of MEC which needs molecular evaluation is oncocytic MEC which needs to be distinguished from an oncocytic carcinoma and acinic cell carcinoma (AciCC). In such cases, FISH evaluation for *MAML2* is indispensable (Fig. 2a, b). Recently a purely oncocytic MEC with complete absence of mucocytes was diagnosed on NGS which detected *CRTC3-MAML2* fusion [12].

Molecular methods can also be used in differentiating high grade MEC from salivary duct carcinoma (SDC), adenosquamous carcinoma and squamous cell carcinoma; all of which, in general, have poor prognosis than high-grade MEC. SDC is frequently immunopositive for androgen receptor (AR) and negative for p63, which contrasts with MEC, which is usually AR-negative and shows reactivity for p63 (Fig. 2c, d) [1]. Adenosquamous and squamous cell carcinoma are generally considered to arise from surface epithelium, in contrast to MEC, which does not have a surface in situ component. Presence of focal keratinization favors the diagnosis of squamous cell carcinoma over MEC. Identification of a *MAML2* rearrangement in the parotid is diagnostic for MEC, because it excludes the possibility of metastasis. But high-grade MECs are less likely to harbor *MAML2* rearrangements, hence the absence of a rearrangement does not rule out diagnosis of MEC [7, 13].

Recently two cases of adenocarcinoma of minor salivary glands have been described showing concurrent rearrangement of both *MAML2* and *EWSR1* genes. These tumors showed unique histomorphology comprising of corded and nested growth pattern with focal glandular differentiation and prominent vascularization. They had bland, round nuclei with inconspicuous nucleoli, and eosinophilic or clear cytoplasm. No epidermoid or intermediate cells were reported. MEC never been reported to show dual gene rearrangement. Since they did not fit into any known type of salivary gland neoplasm, hence these tumors were diagnosed as adenocarcinoma, not otherwise specified. But more studies are needed for the validation of this entity [14].

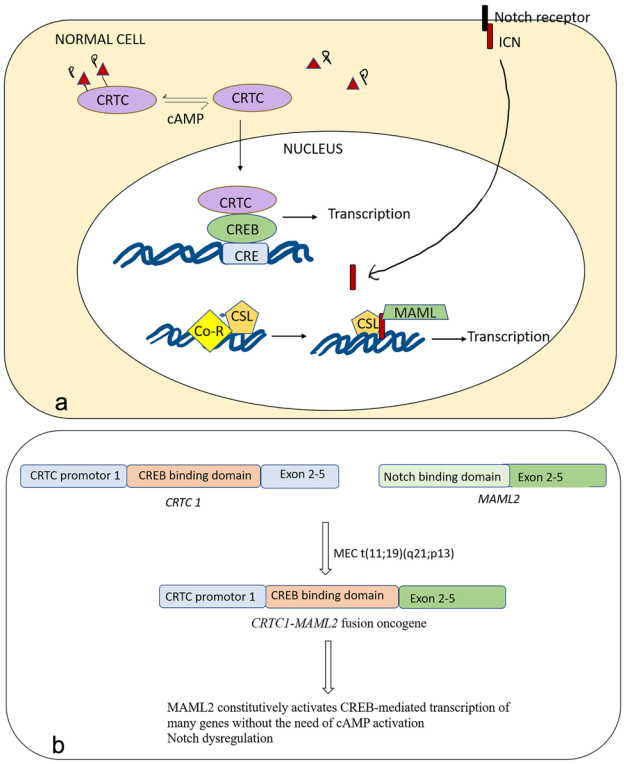

Fig. 1 Simplified illustration of pathogenesis of MEC. **a** In a normal cell, cyclic AMP and calcium signals regulate cAMP-responsive element (CRE)-binding protein (CREB) target genes by stimulating the nuclear translocation of cAMP-regulated transcriptional co-activators (CRTCs). cAMP and calcium signals promote CRTC dephosphorylation, dephosphorylated CRTC is translocated to the nucleus where it binds to CREB and stimulates its activity. Ligand binding of Notch receptor induces proteolytic cleavage producing free intracellular domain of Notch (ICN). ICN translocates to the nucleus and binds to a transcription factor, CSL. An active Notch transcriptional complex, consisting of the ICN/MAML/CSL core components, is formed through displacement of the transcriptional co-repressor complex (Co-R). **b** In MEC, t(11;19) forms CRTC1-MAML2 fusion oncogene where the Notch-binding domain of MAML2 is replaced by the CREB binding domain of CRTC1 and is fused to exons 2–5 of the MAML2 gene. This oncogene remains under the control of the CRTC1 promoter

Prognostic role

Studies on prognostic impact of translocation have not produced uniformly reproducible results. Some have found that overall survival was better for fusion positive tumors while in other studies fusion positive tumors failed to show any prognostic significance. A molecular classification for MEC is proposed:

(i) low-grade, fusion-positive mucoepidermoid carcinomas with no or few genomic imbalances and favorable prognosis,
(ii) high-grade, fusion-positive mucoepidermoid carcinomas with multiple genomic imbalances and unfavorable prognosis,
(iii) heterogeneous group of high-grade, fusion-negative adenocarcinomas with multiple genomic imbalances and unfavorable outcome [7, 13].

Adenoid Cystic Carcinoma (AdCC)

AdCC is the second most common salivary gland malignant neoplasm after MEC. It is composed of epithelial and myoepithelial cells arranged in varying architectural patterns including cribriform, tubular and solid patterns. They are slow growing tumors with a higher propensity for local recurrence, perineural invasion and distant metastasis [1].

Key mutation is t(6;9) (q22-23;p23-24) resulting in fusion of v-myb avian myeloblastosis viral oncogene homolog (*MYB*) and nuclear factor 1B-type (*NFIB*). It has been reported in 29–84% cases. Another similar but mutually exclusive t (8;9) involving *MYBL1-NFIB* fusion has been reported in 9–14% cases [1, 3, 15]. Ho et al. found *NOTCH1*, *KDM6A*, *MLL3*, *ARID1A*, *ARID1B*, *BCOR*, *MLL2*, *CREBBP*, *NOTCH 2*, *NOTCH3*, *NOTCH4* mutations to be increased in recurrent and metastatic AdCC compared to primary tumour. They also found *TERT* promoter mutations in *MYB/MYBL1-NFIB* translocation negative AdCC, indicating *TERT* mutations are an alternate mechanism of AdCC pathogenesis. *NOTCH1* and *TERT* mutations were mutually exclusive. Based on *MYB*, *NOTCH* and *TERT* mutations four molecular subgroups have been proposed: $MYB^{+}NOTCH1^{+}$, $MYB^{+}/other$, $MYB^{WT}TERT^{+}$ and $MYB^{WT}NOTCH1^{+}$ [16].

Diagnostic role

Smaller biopsies often cause a diagnostic dilemma when they show of architectural pattern such as cribriform which overlaps with other basaloid salivary gland tumors eg: pleomorphic adenoma (PA), basal cell adenoma (BCA), polymorphous adenocarcinoma (PAC) (Fig. 3d, e). Certain high grade tumors with a predominant solid component also create a challenge on biopsies (Table 2; Fig. 3a,b,c). Since *MYB/MYB1* rearrangements are unique to AdCC so its expression helps to distinguish AdCC from other salivary gland tumours. It can also help to diagnose AdCC at uncommon anatomic locations like lung, breast, trachea, sinonasal tract etc. [15–17]. It can be detected by MYB IHC, *MYB* Rearrangements (by FISH), *MYB-NFIB* Fusion (by FISH), *MYB-NFIB* Fusion (by RT-PCR).

Conventionally, IHC using epithelial and myoepithelial markers can be helpful in demonstrating the dual cell population in AdCC. AdCC can show luminal ckit positivity,

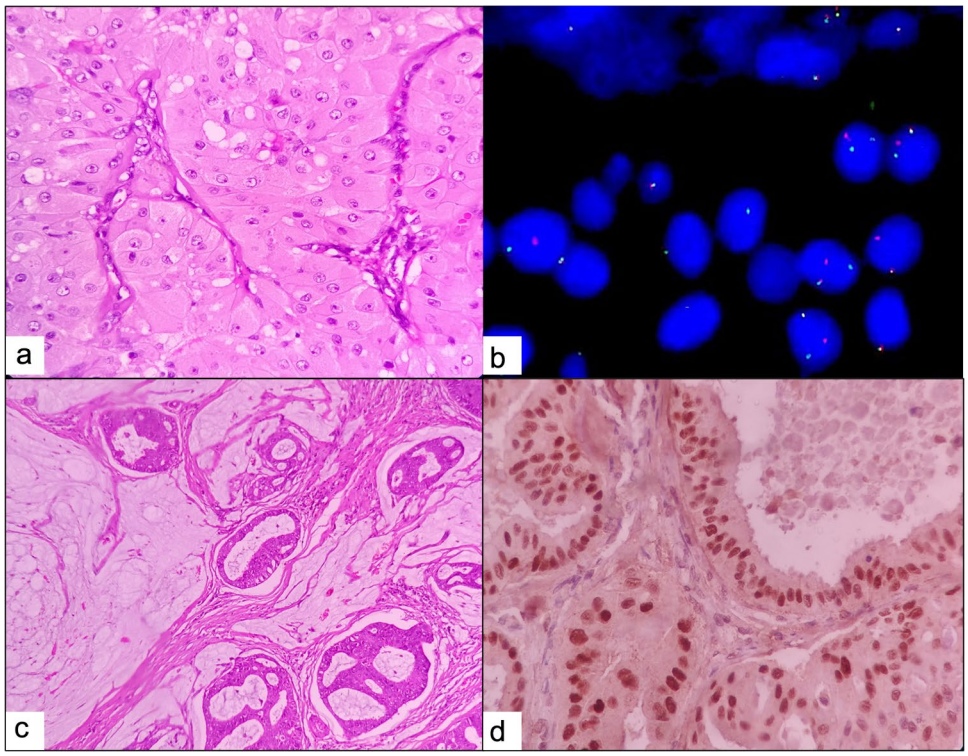

Fig. 2 Tumours with unusual morphology where molecular testing has a key diagnostic role: **a** Oncocytic variant of mucoepidermoid carcinoma. **b** FISH MAML2 breakapart probe shows split signals indicating MAML2 gene rearrangement in oncocytic MEC. **c** Salivary duct carcinoma mucinous variant. **d** Androgen receptor positivity in mucinous SDC

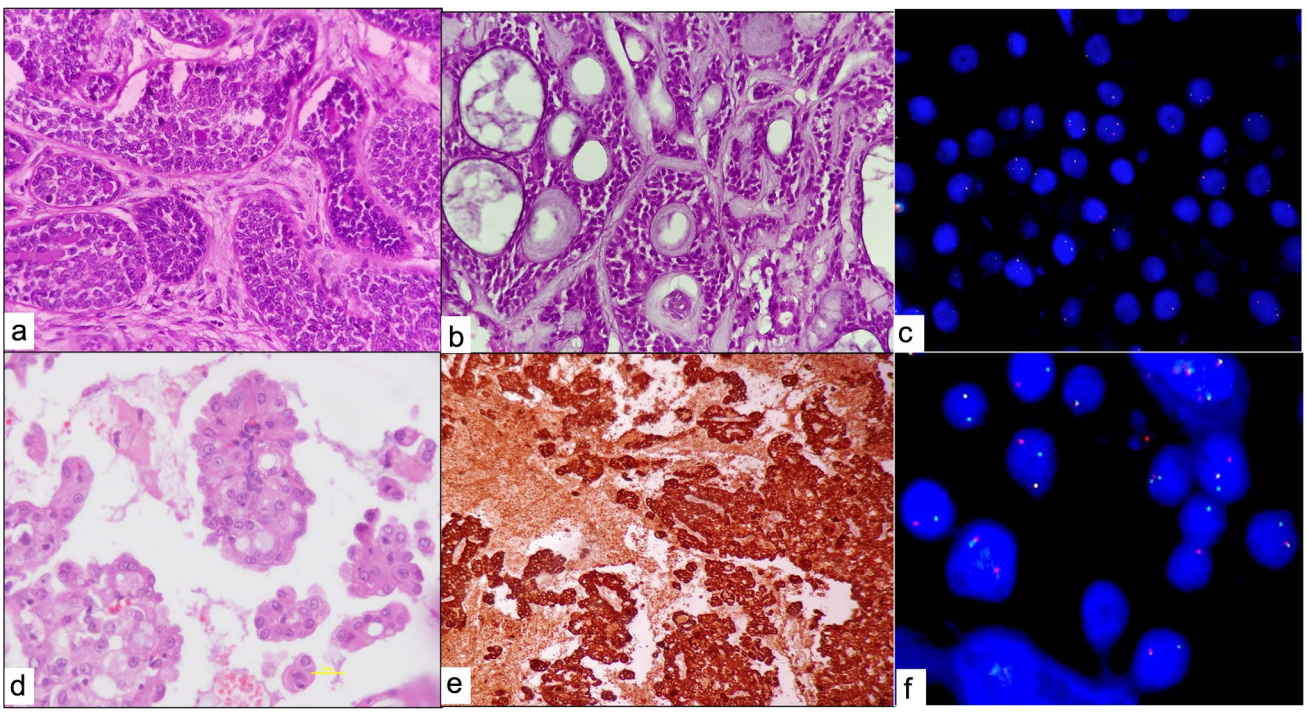

Fig. 3 **a** Basal cell adenocarcinoma. **b** Adenoid cystic carcinoma. **c** FISH MYB breakapart probe shows split signals indicating MYB gene rearrangement favoring adenoid cystic carcinoma over basal cell adenocarcinoma. **d** Secretory carcinoma (SC). **e** Positive mammoglobin and **f** FISH ETV6 breakapart probe shows split signals indicating ETV6 gene rearrangement favouring secretory carcinoma and rules out acinic cell carcinoma

Table 2 Differential diagnosis of basaloid neoplasms in salivary glands AdCC: Adenoid cystic carcinoma, PA: pleomorphic adenoma, PAC: polymorphous adenocarcinoma, BCA: basal cell adenoma, BCAC: basal cell adenocarcinoma,

Tumour	IHC	IHC as molecular surrogate	Remarks
AdCC	p63+/p40+ abluminal cells S100± SOX10+	cKIT in luminal cells MYB	MYB IHC has Sensitivity: 65–82% and Specificity: 86% In equivocal cases, perform MYB, MYBL1 and NFIB FISH
PA	p63+/p40+ myoepithelial S100±	PLAG1 HMAG2	Combination of PLAG1 and HMGA2 IHC can detect 85% of PA
PAC	p63+/p40− S100+	cKIT±	–
BCA	p63+/p40+ abluminal cells	Nuclear β-catenin	IHC for β-catenin sensitivity:82% Specificity: 96% Not seen in membranous BCA or BCAC
BCAC	p63+/p40+ abluminal cells	cKIT± Nuclear b-catenin±	
Metastatic basaloid squamous cell carcinoma	CK5/6, p40,p63 in all tumour cells, SOX10± SMA, Calponin, S100 negative	cKIT± p16±	MYB, MYBL1 and NFIB FISH negative
HPV related multiphenotypic sinonasal carcinoma	P40/p63+ SMA, calponin, S100+	P16+ MYB IHC can be positive	Positive for HPV 33 Negative for MYB / MYBL1 / NFIB translocation by FISH

but is neither sensitive nor specific. Nearly 90% of AdCC show luminal cKIT expression on IHC but no mutations were detected by PCR, except for an occasional point mutation, which too were found to be functionally inactive in the cultured cells [18].

AdCC exhibits a strong diffuse nuclear expression of MYB by IHC in both myoepithelial and ductal cells while some authors reported expression only in myopeithelial cells [19]. MYB expression can be useful in distinguishing AdCC from other rare basaloid salivary gland tumours i.e. cribriform type basal cell adenoma, basal cell adenoma with incomplete capsule, sialoblastoma and intercalated duct hyperplasia. This has major treatment implications since AdCC is treated aggressively with a radical surgical excision with or without postoperative radiation [2]. MYB IHC is not specific and expression has been reported in sailoblastoma, PA, BCA, myoepithelioma, PAC, myoepithelial carcinoma, MEC, SDC and other non-salivary gland tumors such as lymphoma, melanoma, head and neck squamous cell carcinoma. FISH translocation studies are more definitive. FISH using a break-apart probe for the *MYB* gene is one of the commonest methods used (Fig. 3a,b,c). However, this may be of limited use in cases with atypical patterns of *MYB/MYBL1* alterations. In such cases, fusion variants can be detected by using Reverse Transcriptase PCR [19, 20].

Prognostic value

MYB expression showed no correlation with TNM stage or tumor grade. Ho et al. found $MYB^{WT}NOTCH1^{+}$ and $MYB^{+}NOTCH1^{+}$ to be associated with worst prognosis [16].

Predictive value

MYB activation in AdCC represent a potential therapeutic target for AdCC. *MYB* targeted anti-neoplastic therapy using Dovitinib (an FGFR inhibitor, FGF2 ligand is a downstream target of *MYB*), Vorinostat, and Chidamide (a HDAC inhibitor that drives the down-regulation of *MYB*) are under development. Multiple kinase inhibitors such as Sorafenib, Lenvatinib, and Axitinib have also showed promising response in advanced AdCC. In a subset of AdCC, Notch signaling pathway inhibitors and PI3K/IGF/FGFR1 pathway inhibitors are also potential therapeutic targets. Brontictuzumab a humanized monoclonal antibody against the Notch1 protein is under trial [2, 21].

Acinic cell carcinoma (AciCC)

It is a salivary gland malignancy with an acinar cell differentiation. Histologically composed of cells containing zymogen granules admixed with intercalated duct-type cells having variably clear, vacuolated, or oncocytic cytoplasm arranged in a solid, papillary, follicular or microcystic pattern [1].

In 2019, a recurrent translocation t(4; 9) (q13; q31) resulting in fusion of Secretory Ca-binding phosphoprotein (*SCPP*) gene cluster on 4q13 and *NR4A3* on 9q31 was reported in 84% of AciCCs. *NR4A3* gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily: Nuclear Receptor Subfamily 4 Group A Member 3 (*NR4A3*) protein. This protein acts as transcriptional activator and binds the NGFI-B response element. t(4;9) brings highly active enhancer chromatin regions from *SCPP* in close proximity to upstream regions of the *NR4A3* gene locus. This doesn't result in new fusion protein but leads to the upregulation of the *NR4A3* transcription factor [3, 22]. In 4–5% cases, *MSANTD3 (Myb/SANT-like DNA-binding domain containing 3)—HTN3* (*Histatin 3*) translocation has been described as strongly associated with solid pattern and absence of lymphoid stroma in AciCC [3, 23].

Diagnostic role

Although, AciCC has a characteristic histomorphological appearance but cases with predominance of zymogen-poor cells or high-grade transformation, additional testing for *NR4A3* will guide the diagnosis. *NR4A3* rearrangement can be targeted by IHC, FISH, PCR and NGS and can help to differentiate AciCC from mimickers like secretory carcinoma (SC) and MEC [1, 3, 22].

NR4A3 moderate to strong nuclear staining by IHC had reported 98% sensitivity and 100% specificity for primary, metastasis, recurrent and with high grade transformation. *MSANTD3-HTN3* translocation AciCC also showed positive nuclear NR4A3. Huller et al. reported weak to moderate cytoplasmic positivity in the striated duct cells in normal parotid parenchyma but not in the serous acinar cells. In multiple studies, SC was consistently negative for NR4A3 as were other salivary gland tumors like MEC. However, they may show nonspecific weak focal cytoplasmic staining. NR4A3 IHC has shown better sensitivity than DOG1 in differentiating AciCC from SC [22, 24].

FISH uses dual-color break-apart probe for *NR4A3* and Huller et al. reported 84% sensitivity for FISH compared to IHC for the presence of genomic rearrangements of the *NR4A3*. This lower sensitivity compared to IHC is because unlike other translocation associated salivary gland tumors, t(4;9) doesn't produce an oncogenic fusion gene but chromatin enhancer regions of *SCPP* can increase activation of *NR4A3* gene locus even from a relatively long distance of up to several hundred kbps. Hence, the 9q31 genomic breakpoint can be located far upstream of the *NR4A3* gene locus which may be beyond the range of fluorescent probe while NR4A3 upregulation and nuclear accumulation can still be detected by IHC [22, 24].

Secretory carcinoma (SC)

The entity of mammary analogue secretory carcinoma was first described in 2010 and was renamed as SC by WHO in 2017. Histologically, SC shares nearly identical growth patterns to AciCC but cells with true zymogen granules are absent (Fig. 3d) [1].

Recurrent balanced translocation t(12;15)(p13;q25) leads to fusion of *ETV6-NTRK3* in >95% of SC. Neurotropic receptor tyrosine kinase 3 (*NTRK3*) gene is located on chromosome 15 and encodes Trk membrane receptor kinase. The chimeric *ETV6-NTRK3* gene fuses exon 4, 5 or 6 of *ETV6* with the kinase domain of *NTRK3*, thus, encoding a constitutively activated membrane receptor TRKC kinase protein which promotes cell proliferation through activation of the Ras-MAP kinase and PI3K-Akt pathways. An NGS-based study has identified *ETV6-RET, ETV6-MAML3* and *ETV6-MET* translocations fusion in a subset of SC. The *ETV6-NTRK3* fusion gene has not been demonstrated in any other salivary gland tumor [3, 4, 25].

Diagnostic role

ETV6 gene rearrangements are found exclusively in SC, amongst the salivary gland tumors. Hence, identifying *ETV6* fusion is a useful marker to differentiate SC from its histologic mimickers, such as AciCC (most important), adenocarcinoma not otherwise specified, intraductal carcinoma (IC) (Fig. 3d,e,f). Though both SC and AciCC are indolent but differentiation is important as SC has higher lymph node metastasis and availability of targeted therapy for NTRK3 mutations [1, 3, 4]. Hence, *NTRK* fusion gene is targeted for detection by FISH, RT-PCR, and both RNA-based and DNA-based next generation sequencing (NGS). Nuclear pan-TRK IHC is considered a surrogate of *NTRK3* fusions [26]. Pan-Trk IHC is used target an amino acid sequence common to TRKA, TRKB and TRKC. Some authors consider nuclear staining even in ≥1% of tumor cells as positive. It is 64% sensitive and reported specificities range from 92 to 100% [27].

Weak cytoplasmic expression of pan-TRK in tumors need further evaluation by other molecular/cytogenetic methods to ensure that fusion is present, especially if the patient is being considered for targeted therapy. FISH uses dual color break apart probes for either *NTRK3* or *ETV6* [2, 26].

Prognostic role

ETV6-RET, *ETV6-MAML3*, and *ETV6-MET* and atypical non exon5-15 *ETV6-NTRK3* translocations have been found to be associated with aggressive biological features [25].

Predictive role

The *NTRK* fusion can be targeted by Trk inhibitors such as larotrectinib and entrectinib, both approved by FDA. Mechanisms of acquired resistance to larotrectinib have been described with an on-target mutation in the drug-binding site. Selitrectinib, a second-generation Trk inhibitor, have been designed to overcome the acquired resistance to the first-line treatment. Cases with *ETV6-RET* or *MET* translocation are eligible for *RET*-targeted or *MET*-targeted therapies. So, it is important to identify specific partners of *ETV6* [25, 26].

Clear cell carcinoma (CCC)

Clear cell carcinoma (CCC) is a rare malignancy arising mainly in intraoral minor salivary glands and less commonly in the base of tongue, tonsil, nasopharynx, and lung. Histomorphologically, it is characterized by nests of clear cells embedded in hyalinizing stroma. A broad list of differentials include other clear cell tumours i.e. clear cell variant of MEC, clear cell squamous cell carcinoma, epithelial-myopeithelial carcinoma (EMC), clear cell myopeithelial carcinoma (CCMC), clear cell oncocytoma, clear cell variant of AciCC, clear cell odontogenic carcinoma, clear cell variant of calcifying epithelial odontogenic tumour, sinonasal renal cell-like adenocarcinoma and metastatic renal cell carcinoma.

In 2011, t(12;22) (q13;q12) translocation was discovered in CCC and is now reported in nearly 85–90% of the tumors. This results in fusion of *EWSR1* exon 11 on 22q12 with *ATF1* exon 3 on chromosome 12 [28, 29]. In 2018, *EWSR1-CREM* fusions were identified in a subset of CCC. *CREM* and *ATF1* both belong to the CREB family of transcription factors [3, 30].

Diagnostic role

EWSR1-ATF1 fusion is specific for CCC. Its detection is important diagnostically in cases with limited biopsy material and minimal clear cell changes. Mutations can be detected using a dual color break apart *EWSR1* probes by FISH. List of *EWSR1* translocated tumours is increasing with each passing day and include clinically diverse tumours. Apart from CCC, amongst salivary gland tumors, EWSR1 rearrangements have been reported in subset of clear cell myoepithelial carcinoma (CCMC), MEC, and epithelial myoepithelial carcinoma EMC, but with fusion partners other than ATF1 (Table 3) [31, 32].

EWSR1-FISH positive CCMC failed show rearrangements of *EWSR1* on NGS, rather fusions involving *PLAG1* were identified including *LIFR-PLAG1*, *CTNNB1-PLAG1* and *CHCHD7-PLAG1*. Hence, *EWSR1* abnormality in CCMC may represent a passenger mutation [32]. *EWSR1-POU5F1* which is detected in a subset of MEC is also found in soft tissue myoepithelial carcinoma but not in salivary gland myoepithelial carcinoma [6, 31]. Skálová A found one *EWSR1* rearrangement on FISH out of 11 EMC studied but partner gene was not evaluated [31]. Urano et al. evaluated 87 EMC and found *HRAS, PIK3CA* and *AKT1* to be chief mutations but *EWSR1* was not evaluated [33]. More studies are needed for exact incidence of *EWSR1* status in EMC. Another major differential is clear cell odontogenic carcinoma which arises from gingiva but shares same histomorphology, IHC and molecular genetics of *EWSR1-ATF1* with CCC. Therefore, currently clear cell odontogenic carcinomas are considered analogue of CCC [29].

Due to overlapping mutations in differentials of CCC, molecular diagnosis needs careful selection with the type of technique used, interpretation and the sensitivity of the technique used. Evaluation of partner fusion genes of *EWSR1* using *ATF1* breakapart probes by FISH or *EWSR1/ATF* fusion transcript by qRT-PCR or NGS becomes mandatory.

Detection of *EWSR1* rearrangements in salivary gland tumors currently has no prognostic or predictive value [4].

Pleomorphic adenoma (PA) and carcinoma ex pleomorphic (CA ex-PA)

Pleomorphic adenoma (PA) is the most common neoplasm of the salivary gland. PA can show variable morphology which sometimes makes it difficult to distinguish it from its malignant mimickers, particularly AdCC and PAC in cases with limited biopsy material and overlapping morphology (cribriform architecture and hyaline globules). This distinction however carries major clinical implications [1].

The pleomorphic adenoma gene 1 (*PLAG1)* gene rearrangements are detected in >50% of PA. It is located on chromosome 8q12 and encodes a transcription factor with DNA binding zinc-finger protein domains. PA also show fusion of *PLAG1* with various partners, most common being t(3;8) resulting in promoter swapping between the *PLAG1* gene and the constitutively expressed gene for beta-catenin (*CTNNB1*) which have a role in cell–cell adhesion and the Wnt signaling pathway. Another is t(5;8)(p13;q12) involving ubiquitously expressed gene for the leukemia inhibitory factor receptor (*LIFR*) and *PLAG1*. Other reported fusion partners are *FGFR1*, *TCEA1*, *CHCHD7*, *TGFBR3*, *GEM*, *ACTA2*, *ND4*. These translocations result in *PLAG1* overexpression which ultimately activates *HRAS* and Wnt pathways [3, 34, 35].

High-mobility group AT-hook 2 (*HMAG2*) located on chromosome 12q14 and encodes non-histone protein components of chromatin. *HMGA2* fusion with partners like *NFIB,*

Table 3 Differential diagnosis of clear cell lesions in salivary gland MEC: mucoepidermoid carcinoma, EMC: epithelial myoepithelial carcinoma, CCC: clear cell carcinoma, AciCC: acinic cell carcinoma

Diagnosis	IHC	IHC surrogate for molecular alteration	EWS1 breakapart fish	Remarks
Clear Cell Variant Of MEC	p63/p40 strong positive, SOX10 negative S100negative	–	POSITIVE IN A SUBSET	*MAML2* FISH is useful for clear cell and oncocytic variants of MEC but few have *EWSR1-POU5F1*
Clear Cell Squamous Cell Carcinoma	P63, p40 positive	–	NEGATIVE	–
EMC	Myoepithelial component: p63/p40 and S100 Epithelial component: EMA	RAS Q61R	NEGATIVE	One case reported in literature with EWSR1 positivity
Clear Cell Myopeithelial Carcinoma	p63, p40+ S100, actin and calponin,	–	POSITIVE IN 27% CASES	NO EWSR1 rearrangements on NGS. LIFR-PLAG1, CTNNB1-PLAG1 and CHCHD7-PLAG1
CCC	CK7+, P63+, S100, SMA, Calponin -	–	POSITIVE IN > 80%	EWSR1-ATF1
Clear Cell Variant of AciCC	DOG1	NR4A3	NEGATIVE	–
Clear Cell Odontogenic Carcinoma	CK7,S100, SOX10, SMA -CK19+/p63+	–	POSITIVE	EWSR1-ATF1; considered as analogue of CCC
Clear Cell Variant of Calcifying Epithelial Odontogenic Tumour (Pindborg tumour)	CK8+ Amyloid material	–	NEGATIVE	–
Sinonasal Renal Cell-Like Adenocarcinoma	CK7+, CD10, PAX8 SMA, calponin neg	–	NEGATIVE	–
Metastatic Renal Cell Carcinoma	CD10+, PAX8 positive, p63, S100−	–	NEGATIVE	–

WIF1, FHIT has been identified in 10–20% of PA. *PLAG1* and *HMGA2* aberrations are most likely mutually exclusive [36].

Carcinoma ex-pleomorphic adenoma (CA ex-PA) is a malignancy arising in association with PA. Any type of salivary gland and non salivary malignancy can occur in CA ex-PA. But distinction from de novo counterparts is important since CA exPA is more aggressive and carries worse prognosis. *PLAG1* and/or *HMGA2* rearrangements have been identified in 63–88% of CA ex PA. Additionally, amplification of *MDM2* and *HMGA2, TP53* mutations, *MYC* amplification can be detected in CA ex PA compared to PA [3, 37].

Diagnostic role

Occasionally CA ex PA completely overrides PA component or only a hyalinized nodule without epithelial components is noted, making the diagnosis nearly impossible. In such cases, molecular markers are useful. Also, *PLAG1* or *HMGA2* fusions are useful biomarkers to distinguish PA from its morphological mimickers like AdCC, myoepithelioma, BCA, myoepithelial carcinoma, PAC and MEC. Due to *PLAG1* or *HMGA2* rearrangements involving multiple partners, FISH using *HMGA2* and *PLAGA2* break apart probes is amongst the most viable clinical method for detecting mutations but RT-PCR and NGS can also be used.

PLAG1 and *HMGA2* rearrangements cause an overexpression of Plag1 and Hmga2 proteins, which can be used as surrogate IHC markers for underlying *PLAG1* or *HMGA2* fusion [3, 38, 39].

PLAG1 IHC has sensitivity of 96 and 60% respectively for diagnosing PA and CA ex-PA [38].

PLAG1 IHC is more sensitive than FISH since it can detect PLAG1 in fusions formed by intrachromosomal rearrangements. FISH fails to identify *TCEA1-PLAG1* or *CHCHD7-PLAG1* fusions formed by intrachromosomal rearrangement. Specificity of PLAG1 IHC is much less than FISH since it also has been reported in MEC, basal cell adenocarcinoma, EMC, myoepithelioma. But it is usually negative in AdCC, AciCC, PAC and SC. Thus, negative PLAG1 IHC is useful in excluding PLAG1 fusion but a

positive PLAG1 IHC may not always predict the existence of PLAG1 fusion [3, 38].

HMGA2 IHC have been reported in 33.9% of PAs and 24.3% of CA ex-PA. HMGA2 expression has low-sensitivity of 29.8% and specificity of 96.2%. Mito et al. considered SDC with HMGA2 as unrecognized cases of CA ex PA. HMGA2 positivity have been reported occasionally in BCA, myoepithelioma and EMC [39].

Prognostic and predictive role

SDC ex PA is associated with Her2 neu amplification which is a potential therapeutic target [2–4].

Polymorphous adenocarcinoma (PAC) and cribriform adenocarcinoma of minor salivary glands (CAMSG)

Polymorphous adenocarcinoma was earlier called as polymorphous low-grade adenocarcinoma but WHO 2017 dropped the "low grade" terminology. This tumour is characterized by architectural diversity but cytological uniformity with a mixture of tubular, cribriform, papillary, fascicular and solid growth patterns. Targetoid neurotropism is characteristic feature which along with cribriform pattern makes its differentiation from AdCC difficult and other major differential is pleomorphic adenoma [1].

In the current WHO classification, CAMSG is classified as a subtype of PAC. Controversy exists as whether these tumors represent separate entities or variants of one tumour. CAMSG is characterized by cribriform, papillary and glomeruloid patterns. Tumour cells show nuclear clearing similar to papillary thyroid carcinoma [1, 3, 4, 40].

Both PAC and CAMSG have molecular alterations in the activating protein kinase D (PRKD) gene family. Nearly 75% of PAC shows missense point mutations in exon 15 at nucleotide 2130 (c.2130A>T and c.2130A>C mutations) of *PRKD1* gene. Both mutations result in amino acid change from glutamic acid to aspartic acid in codon 710 (E710D). This mutated PRKD1 protein has increased catalytic activity of the kinase domain. CAMSG shows translocations involving the *PRKD1-3* gene including t(1;14) *ARID1APRKD1* and *DDX3X-PRKD1* gene fusions. However, such molecular events were not exclusive as 7% of PAC had *PRKD1* fusion but *PRKD2-3* genes alterations are never found in PAC [3, 4, 40, 41].

Diagnostic role

PRKD1 E710D hotspot mutation and *PRKD1/2/3* gene rearrangements can be identified by FISH, PCR and sequencing and used to differentiate PACs from other salivary gland tumors [3, 4, 40, 41].

Salivary duct carcinoma (SDC)

SDC is an aggressive salivary gland tumor with histomorphological features similar to high-grade ductal carcinoma of the breast. Nearly 40–50% of SDC arise as Ca ex PA. The genetic profile of SDC varies with the absence or presence of pre-existing PA. SDC ex PA show rearrangements in *PLAG1* or *HMGA2* along with higher rate of *TP53* and *Her2neu* mutations while de novo SDC show mutations of *HRAS* and *PIK3CA*. Chiosea et al. found that Her2neu amplification was not seen in de novo salivary duct carcinoma [42]. Few case reports have identified rare translocations in SDC like *NCOA4-RET*, *ETV6-NTRK3*, *BCL6-TRADD* and *ABL1-PPP2R2C* [3].

Diagnostic role

AR positivity by IHC is noted in about 83% of SDC. Non-SDC carcinoma can show AR immunoexpression but it is infrequent and the staining is focal. It is very difficult to differentiate metastatic invasive ductal carcinoma of breast from SDC, which can be done only on clinical grounds. Tumor with strong ER, PR positivity in absence of sialodochodysplasia favors a metastatic tumor [43].

Prognostic role

TP53 and *Her2 neu* are associated with bad prognosis [7].

Predictive role

Androgen deprivation therapy (leuprolide and bicalutamide) is considered in AR positive tumours with metastasis. Her2 targeted therapy involving traztuzumab, emtansine, pertuzumab is considered in recurrent/unresectable or metastatic tumours [2]. Complete response to anti-HER2 therapy is rare in SDC and there is evidence that additional mutations involving *TP53*, *HRAS*, or loss of *PTEN* may decrease the efficacy of anti- HER2 therapy [4].

Epithelial-myoepithelial carcinoma (EMC)

It is rare low grade salivary gland malignancy characterized by biphasic tubular structures composed of inner ductal and outer myoepithelial cells. Majority of EMC show *HRAS* mutations primarily in codon 61 followed by *PIK3CA* and/

or *AKT1* mutations. None of EMC ex pleomorphic adenoma cases showed *HRAS* mutations [33].

Diagnostic role

HRAS mutations were not identified in any salivary gland tumors manifesting EMC-like features, including AdCC, PA, BCA and myoepithelial carcinoma [33]. RAS Q61R mutant-specific IHC show diffuse cytoplasmic/membranous staining in myoepithelial cells in *HRAS Q61R* mutation positive EMC. IHC has reported high sensitivity and specificity [44].

Intradutcal carcinoma (IC)

Previously called as "low-grade cribriform cystadenocarcinoma", but recent WHO classify it as "intraductal carcinoma". Histologically, it is similar to ductal carcinoma in-situ, low-grade [1]. Intercalated duct type IC show *NCOA4-RET* fusion, hybrid type IC show *TRIM27-RET* fusion while Apocrine IC show mutations in *PIK3CA* and *HRAS* similar to SDC [3, 45].

Basal cell adenoma (BCA) and basal cell adenocarcinoma (BCAC)

BCA and BCAC show similar cyto-morphologic features being composed of uniform basal cells forming nests and glands with or without dense basement membrane material. They are classified into adenoma and adenocarcinoma based on the presence or absence of tumor invasion [1].

Tubular or tubulotrabecular patterns of BCA are associated with *CTNNB1* mutations in nearly 85% of tumours. *CTNNB1* is present on chromosome 3p21 and encodes β-catenin. Gain-of-function mutations in the *CTNNB1* gene inhibits the degradation of β-catenin and results in activation of the Wnt pathway. Membranous-type BCA is associated with alterations in the *CYLD* mutations in both sporadic tumors and in the setting of Brooke-Spiegler syndrome. *CYLD* is a tumor suppressor gene at 16q12-13 and encodes a deubiquitinating enzyme, which removes ubiquitin from various signaling proteins, and regulates the activities of many cellular processes, including the nuclear factor-κB and Wnt/β-catenin pathways [3, 46]. BCAC appears to have more complex genetic profile with activating mutations in *PIK3CA* and absent *CTNNB1* mutations [3].

Diagnostic role

BCA is extremely difficult to differentiate on core biopsies from AdCC and PA. *CTNNB1* mutations can be detected by using nuclear β-catenin IHC as surrogate marker. Nuclear β-catenin is used as a specific marker for BCA and helps to distinguish it from AdCC. Expression is prominently seen in the basal component and in stroma. BCAC show variable expression of β-catenin. Normal salivary epithelium and most salivary gland tumours including AdCC and PA show diffuse membranous β-catenin. Despite *CTNNB1* mutation nuclear translocation of β-Catenin is not observed in PA [47]. EMC can show focal nuclear β-catenin. LEF-1 is a nuclear transcription factor of the Wnt/β-catenin signal pathway, and its expression can be detected by IHC [3, 46, 47].

CYLD mutations can be detected using direct sequencing, or probe-based FISH or linkage analysis. CYLD protein can also be detected by cytoplasmic CYLD IHC [48].

Microsecretory adenocarcinoma

Microsecretory adenocarcinoma is novel entity described in 2019, previously grouped under adenocarcinoma NOS. Histologically, tumour is composed of anastomosing microcysts and tubules lined by eosinophilic cells with prominent basophilic secretions and myxohyaline stroma. Recurrent *MEF2C-SS18* fusion that is highly specific for this tumour which can be detected by FISH for *SS18* rearrangement or RNAseq. Molecular analysis is useful in distinguishing it from PAC since both occur in oral cavity and show similar immunophenotype of diffuse S100+/p63+/p40−ve [49].

Intraductal papillary mucinous neoplasm (IPMN)

IPMN was described in 2018 and characterized by prominent papillary–cystic proliferations. They were found to harbor *AKT1 E17K* mutations [49].

Sialadenoma papilliferum

Sialoadenoma papilleferum is a benign salivary gland tumor resembles to syringocystadenoma papilliferum of the skin. Histologically consist of exophytic squamous epithelium and endophytic intraductal papillary growth. They are characterized by *BRAF V600E* [49].

Salvglandx panel

Recently a comprehensive 27 gene NGS- RNA sequencing panel has been proposed, for the detection of mutations, fusions and gene expression levels on RNA level. It includes most of the recurrent gene aberrations known to date and assists in better diagnosis and identification therapeutic

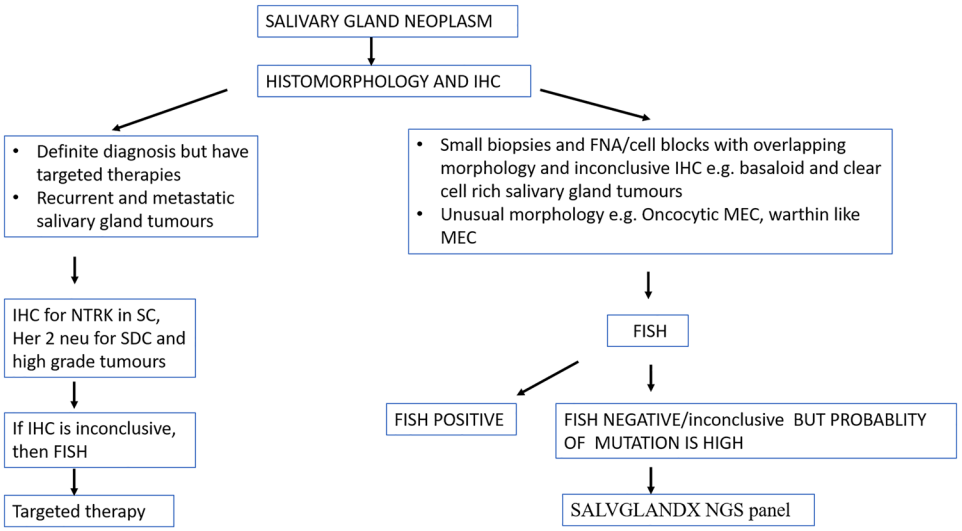

Fig. 4 General workflow for the pathological diagnosis of salivary gland neoplasms

targets. This panel is a useful all-in-one tool to diagnose salivary gland malignancies especially on fine needle aspiration (FNA) or small biopsies where the differentiation between benign and low-grade malignant tumors can sometimes be impossible based on morphology alone. NGS has led to the identification of many novel morphological variants of commonly known tumours such as spindle cell and pseudoangiomatoid adenoid cystic carcinoma, purely oncocytic mucoepidermoid carcinoma [12]. More studies are needed for its validation of SALIVGLANDX panel for widespread use.

The widespread clinical implementation of NGS based techniques has increased in recent times but NGS is still an expensive and time-consuming tool, thereby rendering it a challenge to implement in most laboratories. There is also need for proper interpretation and verification using a gold standard sequencing techniques such as Sanger sequencing when encountering a novel fusion. Hence, we recommend a cost-effective approach for pathological diagnosis of salivary gland tumours where the use of molecular techniques is decided by pathologist on the case-to-case basis (Fig. 4).

Conclusion

Constantly evolving knowledge of biology and pathogenesis of salivary gland tumours resulted in gaining insight on mechanisms of initiation and progression of these cancers. The ability to detect these characteristic chromosomal rearrangements with FISH and even with IHC makes their incorporation into routine histopathology feasible. More sophisticated diagnostic techniques such as NGS should be applied on case-by-case basis by the pathologist. It must be stressed, that, despite the outlined benefits of molecular diagnostics, their results should not be interpreted in a vacuum and the combination of histology, ancillary IHC and molecular results renders the final diagnosis.

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Declarations

Conflict of interest There is no conflict of interest to disclose.

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