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INVITED REVIEW

Clinical Utility of In Situ Hybridization Assays in Head and Neck Neoplasms

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Abstract
Head and neck pathology present a unique set of challenges including the morphological diversity of the neoplasms and presentation of metastases of unknown primary origin. The detection of human papillomavirus and Epstein–Barr virus associated with squamous cell carcinoma and newer entities like HPV-related carcinoma with adenoid cystic like features have critical prognostic and management implications. In salivary gland neoplasms, differential diagnoses can be broad and include non-neoplastic conditions as well as benign and malignant neoplasms. The detection of specific gene rearrangements can be immensely helpful in reaching the diagnosis in pleomorphic adenoma, mucoepidermoid carcinoma, secretory carcinoma, hyalinizing clear cell carcinoma and adenoid cystic carcinoma. Furthermore, molecular techniques are essential in diagnosis of small round blue cell neoplasms and spindle cell neoplasms including Ewing sarcoma, rhabdomyosarcoma, synovial sarcoma, biphenotypic sinonasal sarcoma, dermatofibrosarcoma protuberans, nodular fasciitis and inflammatory myofibroblastic tumor. The detection of genetic rearrangements is also important in lymphomas particularly in identifying 'double-hit' and 'triple-hit' lymphomas in diffuse large B cell lymphoma. This article reviews the use of in situ hybridization in the diagnosis of these neoplasms.

Keywords In situ hybridization · Head and neck neoplasms · Squamous cell carcinoma · Salivary gland neoplasms · Soft tissue and bone neoplasms · Lymphoma

Introduction

The surgical pathologist is an integral part of the multidisciplinary team in the management of head and neck tumors [1]. Their role is not only to make the diagnosis, but also to provide critical prognostic and predictive information that will guide management decisions. Head and neck pathology presents several challenges, particularly due to the anatomic complexity of this region and close association of numerous critical structures. Most of the structures also have diverse histologic components that can give rise to a large number of primary epithelial, mesenchymal and haematolymphoid neoplasms. Many of these lesions can show diagnostically challenging morphologic variants and some can initially present as metastases of unknown primary origin. Thus a wide range of differential diagnoses with overlapping morphologic and immunophenotypic characteristics need to be considered. The difficulty is further compounded by the availability of limited diagnostic material from fine needle aspiration or small biopsy specimens. The role of accurate diagnosis in preventing under or over treatment in the head and neck region cannot be underestimated. Thus clinical, radiologic correlation, histopathology and immunohistochemistry (IHC) often need to be complemented by diagnostic techniques such as in situ hybridization (ISH) to assess for specific genetic alterations. Recent advances

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in molecular techniques have identified several recurrent genetic changes in a variety of neoplasms and ISH techniques are increasingly playing a larger role in diagnostic pathology. This manuscript reviews the role of ISH in head and neck neoplasms. A summary of the entities discussed are listed in Table 1.

General Principles of ISH

ISH involves the use of single-stranded DNA probes that bind to complementary target sequences in the DNA or mRNA of the cells. In fluorescence in-situ hybridization (FISH), the DNA probes are labelled with a fluorochrome which allows visualization using a dark field fluorescence microscope. The probes are designed to work with routine formalin fixed paraffin-embedded tissue thus allowing them to be easily integrated into the routine workflow of a diagnostic laboratory.

Three types of FISH probes are commercially available (Fig. 1) and several manufacturers support the design and development of novel probes for diagnostic purposes. Break-apart probes and dual-fusion probes are used to detect chromosomal translocations that result in gene rearrangements. Break-apart probes include two different colored probes (usually one red and one green) that bind separately to sequences flanking the known breakpoint region in the gene of interest [2]. Both the signals appear fused in normal cells, whereas a translocation will result in separation of the signals. Separation of the signals indicates presence of gene rearrangement but does not provide information regarding the position of the translocated gene or its fusion partner [2]. On the other hand, dual-fusion probes consist of two different colored probes that bind to different genes on different chromosomes. Therefore these signals appear separate under normal conditions and a translocation results in fusion of the signals. Thus fusion probes can aid in the identification of specific fusion partners [2]. The third probe type is enumeration probe used to detect gene amplification or deletion. This type consists of two different colored probes, one binding to the gene of interest and another binding to the centromere of a chromosome as a control. A normal cell shows two copies of each signal while gene amplification results in an increased number of signals or signal clusters of the gene of interest [2]. A gene deletion usually shows loss of at least one signal corresponding to the gene region deleted. All three types of FISH probes are used in diagnostic work.

Chromogenic and Silver In Situ Hybridisation

Alternatively, these probes can be labelled with a chromogen (CISH) or with reagents that generate a silver precipitate (SISH) to allow visualization with bright field microscopy. The CISH and SISH probes are primarily used for enumeration as fusion signals and isolated signals are often difficult to distinguish under standard bright field microscopes [3], making them less sensitive in detecting gene rearrangements.

Virus Associated Head and Neck Squamous Cell Carcinoma

Human Papilloma Virus Associated Oropharyngeal Carcinoma

Human papillomavirus (HPV) is a cause of a molecularly distinct subgroup of oropharyngeal squamous cell carcinoma (SCC), particularly in the 5th and 6th decade [4]. Typically, oropharyngeal SCC are associated with high risk HPV (HR-HPV) subtypes 16, 31 and 33. HPV-associated oropharyngeal SCCs have better prognosis and better response rates to both surgery and chemoradiotherapy as compared with smoking or alcohol associated oral cavity SCC [5].

HPV-associated oropharyngeal SCCs are histologically distinct from HPV-negative tumors. The majority of HPV-associated SCCs are non-keratinizing with minimal cytoplasm and spindle to ovoid hyperchromatic nuclei and demonstrate nuclear and cytoplasmic staining with p16 in $\geq 75\%$ of tumor cells [6]. However, many morphologic variants such as papillary, adenosquamous, lymphoepithelial-like, spindle cell and ciliated cell exist that can cause diagnostic difficulties in lymph node metastases [4]. Indeed, when faced with these unusual variants in lymph node metastases of unknown primary, molecular tests to identify the presence of HPV in addition to IHC for p16 is immensely helpful in predicting an oropharyngeal primary (Fig. 2). This is important as p16 is a tumor suppressor gene and immunohistochemical p16 expression is a highly sensitive but not specific surrogate marker for HPV. Several other malignancies such as cutaneous SCC and adenoid cystic carcinoma can show nuclear and cytoplasmic p16 staining in $>75\%$ of the neoplastic cells [7].

Two methods are available for directly testing for HPV using formalin fixed paraffin embedded (FFPE) material in clinical laboratories: ISH for HR-HPV DNA, and messenger RNA (mRNA) based RNAScope [8]. DNA ISH detects HPV DNA integrated in the tumor cells while RNA ISH detects transcriptionally active HR-HPV E6 and E7 mRNA. HPV ISH probes are labelled with a non-fluorescent molecule (usually biotin or digoxigenin) that are then detected using antibodies and visualized using a chromogenic reaction and bright field microscopy. This has the added benefits of ensuring that HPV is detected in neoplastic cells and the slides can be stored in the conventional archives [8]. While both DNA and RNA ISH are independently prognostic, DNA ISH has lower sensitivity

Table 1 Currently commercially available ISH probes and their diagnostic utility in head and neck neoplasms

ISH probe	Diagnosis	Differential diagnoses	Caveats
Oropharyngeal and sinonasal neoplasms			
HPV DNA or mRNA	HPV associated oropharyngeal carcinoma	Metastatic deposits of non-keratinising squamous cell carcinoma from non-oropharyngeal sites such as oral cavity, larynx, skin or basaloid squamous cell carcinoma that may show cytoplasmic and nuclear p16 expression in more than 75% of the tumor cells	HPV ISH is sometimes useful on cell block material with limited number of viable neoplastic cells
HPV DNA	HPV associated carcinoma with adenoid cystic like features	Adenoid cystic carcinoma, sinonasal non-keratinising squamous cell carcinoma	Critical to ensure that the ISH probe cocktail includes HPV 33
Epstein Barr virus encoding region (EBER)	Nasopharyngeal carcinoma (NPC)	Nonkeratinising squamous cell carcinoma from the oropharynx or other sites	Hodgkin and several non-Hodgkin lymphomas can also show EBER expression. These can be easily distinguished from NPC by using appropriate epithelial and hematolymphoid IHC markers
NUT break-apart	NUT midline carcinoma	Poorly differentiated squamous cell carcinoma, sinonasal undifferentiated carcinoma, small round blue cell sarcomas such as Ewing or rhabdomyosarcoma, high grade non-Hodgkin lymphomas	IHC for nuclear protein in testis (NUT) is also available
EWSR1 break-apart *EWSR1-FLI1* fusion	Adamantimoma-like EFT	Sinonasal undifferentiated carcinoma, small round blue cell sarcomas such as Ewing or rhabdomyosarcoma, high grade olfactory neuroblastoma	Currently there is limited experience about whether these cases should be treated as carcinomas or with Ewing sarcoma chemotherapy protocols
Salivary gland neoplasms			
PLAG1 and *HMGA2* break-apart	Pleomorphic adenoma	Epithelial myoepithelial carcinoma, adenoid cystic carcinoma	Rearrangement is seen in up to 70% cases only
MYB break-apart	Adenoid cystic carcinoma	Epithelial myoepithelial carcinoma, pleomorphic adenoma, polymorphous adenocarcinoma	*MYB* rearrangement is seen in approximately 50% of morphologically unequivocal adenoid cystic carcinoma, thus only useful when positive. *MYB* rearrangement may not be seen in adenoid cystic carcinoma with high grade transformation
MAML2 break-apart	Mucoepidermoid carcinoma	Warthins tumor with metaplasia, hyalinizing clear cell carcinoma, squamous cell carcinoma, salivary duct carcinoma	
ETV6 break-apart	Secretory carcinoma	Acinic cell carcinoma, mucoepidermoid carcinoma	
EWSR1 break-apart	Hyalinizing clear cell carcinoma	Mucoepidermoid carcinoma, squamous cell carcinoma	Interpretation in the context of morphologic and immunohistochemical features is very important as *EWSR1* rearrangement is seen in a large number of morphologically unrelated lesions in the head and neck (angiomatoid fibrous histiocytoma, clear cell sarcoma and admantinoma like EFT for example)

Table 1 (continued)

ISH probe	Diagnosis	Differential diagnoses	Caveats
Soft tissue neoplasms			
EWSR1 break-apart *EWSR1-FLI1* fusion	Ewing sarcoma	Other round blue cell sarcomas including rhabdomyosarcoma, CIC-DUX4 sarcoma, BCOR-CCNB3 sarcoma, desmoplastic small round cell tumor	
CIC break-apart *CIC-DUX4* fusion	CIC-DUX sarcoma	Other round blue cell sarcomas	
BCOR break-apart *BCOR-CCNB3* fusion	BCOR-CCNB3 sarcoma	Other round blue cell sarcomas	
FOXO1 break-apart *PAX3-FOXO1* fusion *PAX7-FOXO1* fusion	Alveolar rhabdomyosarcoma	Other round blue cell sarcomas	
SS18 break-apart	Synovial sarcoma	Spindle cell sarcomas including malignant peripheral nerve sheath tumor, dermatofibrosarcoma protuberans, leiomyosarcoma	
PAX3 and *MAML3* break-apart	Biphenotypic sinonasal sarcoma	Spindle cell rhabdomyosarcoma	
PDGFB breakapart *COL1A1-PDGFB* fusion	Dermatofibrosarcoma protuberans	Dermatofibroma, fibromatosis, synovial sarcoma	Presence of *PDGFB* rearrangement can also be used for targeted therapy
MDM2 amplification	Well differentiated liposarcoma	Lipoma with reactive changes	
	Low grade osteosarcoma	Fibrous dysplasia	Care should be taken to use chelating agents for decalcification to allow ISH studies
DDIT3 break-apart	Myxoid liposarcoma	Round cell and myxoid sarcomas	
RB1 deletion	Spindle cell lipoma	Well differentiated liposarcoma	
USP6 break-apart	Nodular fasciitis	Dermatofibroma, fibromatosis, spindle cell sarcomas	Care should be taken to assess FISH signals in lesional spindle shaped cells and not in the associated inflammatory component and endothelial cells which can be prominent enough to mask lesional cells
ALK break-apart	Inflammatory myofibroblastic tumor	Nodular fasciitis, fibromatosis, spindle cell sarcomas	
Haematolymphoid neoplasms			
MYC, BCL2, BCL6 break-apart *MYC-IGH* fusion	Double hit/triple hit lymphomas	Diffuse large B-cell lymphoma, Burkitt lymphoma, high grade B-cell lymphoma, NOS	
MYC break-apart *MYC-IGH, MYC-IGK, MYC-IGL* fusion	Burkitt lymphoma	High grade B-cell lymphoma, NOS, diffuse large B-cell lymphoma	
BCL2-IGH fusion	Follicular lymphoma	Other low grade NHL, reactive lymphoid hyperplasia	
CCND1-IGH fusion	Mantle cell lymphoma	Other low grade NHL, reactive lymphoid hyperplasia	
MALT1 break-apart	Extranodal marginal zone lymphoma	Other low grade NHL, reactive lymphoid hyperplasia	

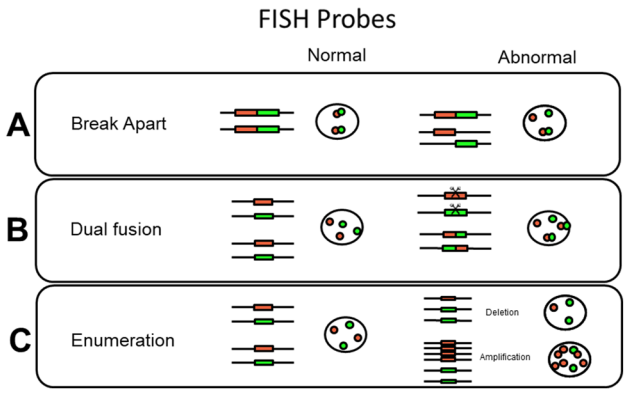

Fig. 1 Three different types of probes commonly used in clinical practice. **a** Break-apart probes use probes of different colors to flank the 3′ and 5′ regions of the breakpoint of interest. Thus these signals appear separated in cases with rearrangement. The signals should be separated by a distance equal to the diameter of one signal. **b** Fusion probes use probes of two different colors to cover the break points of interest. In normal cells these breakpoints are located on different chromosomes and thus the signals appear to be separated. In cases of rearrangement, the genes at the breakpoint translocate towards each other and appear to be fused. **c** Enumeration probes use a probe to label the centromeric region of the chromosome of interest and another colored probe to label the gene of interest. Thus normally, there are two copies of each of the probe. Increase in the copy number of the gene of interest with two copies of the centromeric signal indicates amplification. While decrease in the copy number of the gene of interest with two copies of the centromere indicates deletion. Both of these numerical changes use ratios of the copy numbers of both the genes of interest and the centromeric region and various thresholds are specified in the literature for different malignancies. On the other hand increase in the copy number of both the centromeric region as well as the gene of interest indicates aneuploidy

and the majority of p16 positive oropharyngeal tumors that are DNA ISH negative tend to be positive with RNA ISH [9]. Evidence of transcriptionally active virus rather than simply the presence of the virus is also biologically more relevant. Thus RNA ISH has been considered as the gold standard by some [10].

Metastatic SCCs with marked cystic change in levels II and III lymph nodes are most likely to be of oropharyngeal origin. The cystic nature can make obtaining a diagnostic yield of viable cells particularly challenging during the diagnostic fine needle aspiration procedure. While p16 IHC on a cell block is useful, its sensitivity can be affected by degenerate squamous cells which show reduced p16 staining [4]. Furthermore, unlike histology the threshold for interpreting p16 on cytology is not well established [4]. HPV ISH has been found to be a highly specific marker for oropharyngeal origin and it is very useful in limited material obtained from fine needle aspiration biopsies thus improving the diagnostic sensitivity and specificity [11].

The current College of American Pathologists guidelines recommend routinely performing p16 IHC on all primary oropharyngeal SCCs while HPV ISH is recommended in cases where IHC is equivocal (50–75% staining) [12]. Furthermore, p16 IHC should be performed on all metastatic SCC of unknown primary in level II and III lymph nodes and positive cases with keratinizing morphology should be additionally tested with HPV ISH to exclude the possibility of metastatic SCC unrelated to HPV overexpressing p16 [12].

Epstein Barr Virus Associated Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) most commonly occurs in patients from Southeast Asia. Histologically, World Health Organisation (WHO) classifies it into keratinizing, non-keratinizing and basaloid SCC [13]. The vast majority are of the non-keratinizing subtype. Approximately 90% of non-keratinizing NPCs are associated with Epstein–Barr virus (EBV). Cells with latent EBV infection express a limited number of viral proteins as well as two small noncoding

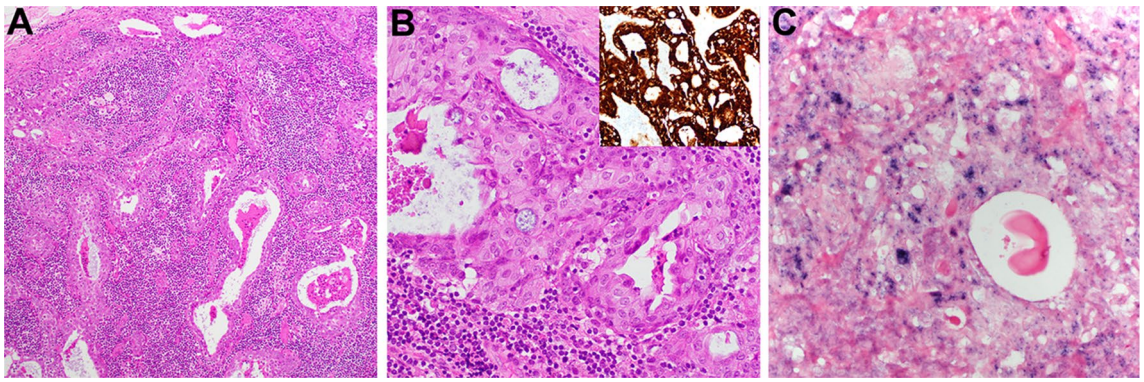

Fig. 2 A 42 year old male presented with left level II cervical lymph node metastasis of unknown primary. **a, b** Histology showed an adenosquamous carcinoma with intermixed squamoid and glandular areas containing mucin. The tumor was (**b** inset) p16 positive and also **c** HPV ISH positive, suggesting oropharyngeal origin

RNAs Epstein–Barr encoding region (EBER)-1 and EBER-2. EBER is amplified at greater than 10^6 copies per infected cell making it an ideal molecular target for detection. EBER in situ hybridization (EBER ISH) is therefore the gold standard for detecting cells with latent EBV infection in tissue sections [14]. Similar to the HPV ISH, EBER ISH probes are labelled with a molecule that can be visualized using a chromogenic reaction therefore assessment is performed by bright field microscopy [14].

NPC has a high metastatic rate with lymph node metastasis often being the initial presentation and more than 70% of patients present at advanced stages [15]. In lymph node metastases of unknown primary, EBER ISH is useful in identifying nasopharynx as the primary site particularly when endoscopic examination and biopsy of the nasopharynx fails to detect the primary tumor as can happen in approximately 15% of cases [16]. Studies have reported up to 100% sensitivity and specificity in surgical as well as fine needle aspiration specimens [17]. EBER ISH positivity is specific for nasopharyngeal primary in the context of carcinoma of unknown primary as SCCs from other sites such as oropharynx and oral cavity have been shown to be negative [16]. Although lymphoepithelial carcinomas arising from non-nasopharyngeal sites tend to be negative for EBER ISH [18], some primary sites such as lung are positive especially in Asian populations [19]. Therefore clinical and radiological correlation is essential in excluding a non-nasopharygeal primary.

A pitfall in the diagnosis of metastatic NPC is the rare lymphoepithelial-like variant of HPV-associated SCC, accounting for approximately 3% of HPV-associated SCCs [20]. This creates a particularly difficult diagnostic dilemma due their overlapping morphologies. These tumors originating from the oropharynx are not related to EBV [21]. Furthermore, it is now recognised that a minority of carcinomas arising in the nasopharynx and the sinonasal tree in the non-EBV endemic Caucasian populations are EBV negative but p16 and HPV ISH positive (Fig. 3) [22]. Thus a combination of HPV ISH (positive) and EBER ISH (negative) is helpful in both these instances in addition to the appropriate clinical and radiologic correlation. Accurate detection of HPV or EBV in metastatic SCC is particularly important in patients that initially present with cervical lymph node metastases as presence of HPV or EBV directs clinical and radiologic examination of the oropharynx or the nasopharynx and is also of therapeutic and prognostic significance [23, 24].

Salivary Gland Neoplasms

Salivary gland neoplasms are rare, accounting for 3–6% of head and neck cancers and 0.3% of all cancers [25]. In 2017, the WHO published the most recent classification of head and neck tumors which includes 11 types of benign and 20 types of malignant salivary gland tumors. These tumors often pose a diagnostic challenge for the pathologists, primarily due to their low incidence, variant morphologies and morphologic mimics. This makes the commercially available FISH probes an important adjunct in challenging cases.

Pleomorphic Adenoma

Pleomorphic adenoma (PA) is the most common neoplasm seen in salivary glands. It is a benign biphasic tumor composed of epithelial and myoepithelial elements typically with chondromyxoid stroma [26]. The tumors are called 'pleomorphic' because they show a wide spectrum of cell

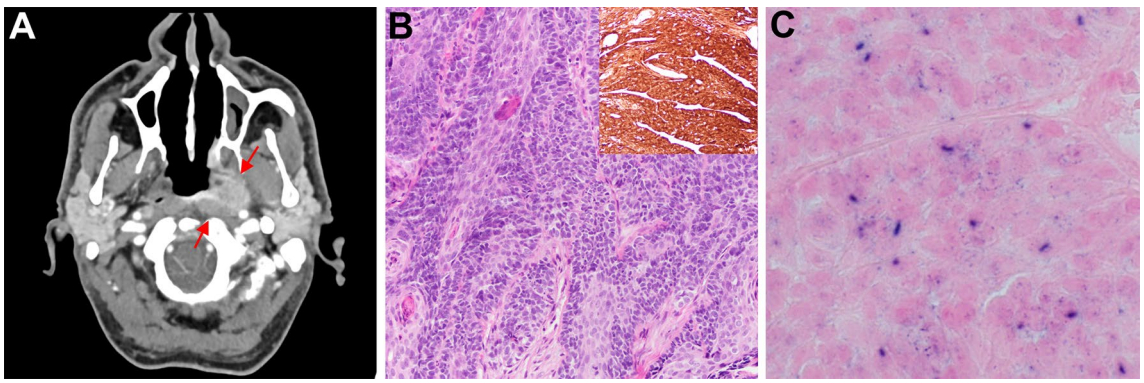

Fig. 3 A 53 year old Caucasian male presented with a nasopharyngeal mass. **a** CT showed an enhancing soft tissue lesion in the left fossa of Rosenmuller with associated bone destruction of left medial pterygoid plate and base of left sphenoid sinus. **b** Biopsy showed a squamous cell carcinoma. The tumour was composed of cells with scanty cytoplasm and spindle to ovoid hyperchromatic nuclei. The cells were arranged in lobules and focal keratinisation was seen at the periphery of the lobules. The tumour histologically resembled a squamous cell carcinoma of oropharyngeal origin and the tumour cells were (inset) p16 positive and negative for EBER ISH. **c** HPV ISH confirmed HPV integration in the tumour nuclei

morphology including plasmacytoid, spindle and clear cells, as well as squamous, oncocytic and mucinous appearance. Whilst the majority are stroma-rich, cellular PAs also occur. Such a wide morphologic spectrum can make it difficult to distinguish PA from tumors such as adenoid cystic carcinoma, epithelial myoepithelial carcinoma, polymorphous adenocarcinoma and basal cell neoplasms particularly on preoperative fine needle aspiration material and occasionally on histology. Distinguishing PAs from malignant mimics, particularly adenoid cystic carcinoma, can be difficult on limited material such as fine needle aspiration as both can show cribriform structures and stroma. This distinction however carries important clinical implication since PAs can be treated with simple excision while adenoid cystic carcinoma requires radical surgery with adjuvant radiotherapy [25].

Cytogenetic studies have identified karyotypic abnormalities in up to 70% of PAs, the most common being rearrangements in the developmentally regulated zinc finger gene *PLAG1* in 8q12 accounting for about 39% of cases [27]. Rearrangements in 12q13–15 involving the *HMGA2* gene or other clonal aberrations such as trisomy 8 are seen in 23% and the remainder have normal karyotype [27]. *PLAG1* translocations include t(3;8)(p21;q12) and t(5;8)(p13;q12) which causes *PLAG1* to be fused with the *CTNNB1* and the *LIFR* gene respectively [27]. *PLAG1* fusion with *FGFR1* has also been described. In addition, some cases with normal karyotype have been shown to harbour *PLAG1* fusion with *TFIIS* gene as a result of cryptic rearrangements [28].

Break-apart FISH probes for both *PLAG1* and *HMGA2* are commercially available. The genetic alteration is seen in both epithelial and myoepithelial cells of the tumor [29]. *PLAG1* or *HMGA2* FISH is highly specific for PA as histologic mimics such as adenoid cystic carcinoma, polymorphous adenocarcinoma, epithelial myoepithelial carcinoma and basal cell neoplasms lack these rearrangements [30], making FISH diagnostically useful in exclusion of these entities. Interestingly, rearrangement of *PLAG1*/*HMGA2* is more common in PAs with abundant stroma than cellular PAs and thus is of limited utility in distinguishing a cellular PA from its mimics [30].

A further utility of *PLAG1* FISH is the diagnosis of carcinoma ex-pleomorphic adenoma (CA ex-PA). The histological diagnosis of CA ex-PA requires the presence of a co-existing PA component or a history of a prior PA excised from the same site. However, the benign component can be overgrown by the malignant component making it difficult to identify the remaining PA. Studies report 63–100% of CA ex-PA show *PLAG1* rearrangement regardless of the histologic subtype, which includes salivary duct carcinoma and myoepithelial carcinoma [30–32]. In addition, 5–18% of CA ex-PA harbor HMGA2 rearrangement in the absence of *PLAG1* rearrangement [30–32]. In contrast, de novo carcinomas lack abnormalities in *PLAG1* or *HMGA2*. Although salivary duct carcinoma have rarely been reported to harbour *PLAG1*/*HMGA2* rearrangement, these are likely to represent CA ex-PA with PA component that was completely obscured by the malignant component or not sampled [30].

Adenoid Cystic Carcinoma

Adenoid cystic carcinoma (ACC) is a relatively common primary salivary gland malignancy accounting for approximately 10% of salivary gland cancers [33]. Histologically, ACC is biphasic and consists of epithelial and myoepithelial cells with hyperchromatic angulated nuclei showing tubular, cribriform and/or solid growth. Grading systems have been proposed for ACC with the presence of a significant component of solid growth designated as high grade and worse prognosis [34]. ACC is typically locally aggressive and recurs locally followed by hematogenous metastases. Treatment options are limited to radical surgery and adjuvant radiotherapy while chemotherapy is of limited value [25].

A well-known cytogenetic abnormality in ACC is the t(6;9)(q22–23;p23–24) translocation resulting in fusion between the *MYB* and *NFIB* genes [35]. *MYB* rearrangement can be detected using break-apart FISH in 28–50% of ACC [36–38]. This translocation causes *MYB* overexpression, resulting in transcriptional activation of target genes associated with cell cycle progression, cell growth and angiogenesis [39]. *MYB* rearrangement has low sensitivity for ACC as nearly 50% of morphologically unequivocal ACC may be negative for *MYB* rearrangement. However, it is highly specific as it has not been found in the most common differential diagnoses such as myoepithelial carcinoma, polymorphous adenocarcinoma and epithelial myoepithelial carcinoma. Thus the presence of *MYB* rearrangement can help exclude these mimics or confirm the diagnosis of ACC at a metastatic site in scanty diagnostic material.

Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm of the salivary gland, consisting of varying proportions of mucous, intermediate and squamoid cells. In addition, clear cell and oncocytic cell variants have been described [40]. A specific cytogenetic abnormality (t(11;19)(q21;p13)) is observed in MEC resulting in fusion between exon 1 of *MECT1* on chromosome 19 and exons 2–5 of *MAML2* on chromosome 11 [27]. Fusion of *MAML2* with *CRTC3* has also been described in a small proportion of MECs [41]. *MAML2* gene encodes a transcriptional coactivator for Notch receptors which plays a key role in cell proliferation and tumorigenesis [27].

Break-apart FISH probes for *MAML2* are commercially available. The diagnostic utility of *MAML2* FISH stems from its high specificity for MEC. Studies have shown

MAML2 rearrangement detectable by FISH in 55–82% of MECs [40–43]. In particular, clear cell and oncocytic variants of MEC have also been demonstrated to be positive on FISH [40]. On the other hand, histological mimics of MECs including sialadenitis with squamous metaplasia, Warthins tumor, oncocytoma, squamous cell carcinoma and salivary duct carcinoma do not show *MAML2* rearrangement [40, 43]. Thus *MAML2* FISH is a useful confirmatory tool in tumors with variant morphologic features (Fig. 4), low grade tumors with predominant mucin and tumors obscured by inflammation or procedural and processing artefacts. It is also a useful diagnostic tool in cases with limited material such as fine needle aspiration cell block preparations.

Secretory carcinoma

Secretory carcinoma (formerly known as mammary analogue secretory carcinoma) was described by Skalova et al. in 2010 [44]. It typically occurs in younger patients [45]. Histologically, secretory carcinomas typically demonstrate variably sized cysts containing colloid-like eosinophilic secretions with peripheral scalloping lined by cells with finely vacuolated eosinophilic cytoplasm and uniform central round nuclei [46]. It shares immunohistochemical and genetic features with secretory carcinoma of the breast. Both secretory carcinoma of the breast and salivary glands harbour a balanced translocation t(12;15)(p13;q25), leading to *ETV6-NTRK3* fusion gene [47]. This translocation encodes a chimeric oncoprotein in which the *ETV6* transcription factor is fused with the tyrosine kinase domain of neurotrophin-3 receptor *NTRK3* activating the oncogenic signaling pathways including Ras and PI3K [48]. A small proportion of secretory carcinoma (2–5%) harbour *ETV6* fusion with an unknown partner and these may be associated more invasive histology [49]. *ETV6* break-apart FISH probes are commercially available and studies have found that all secretory carcinomas have this translocation [44–46]. Furthermore, it is highly specific as morphologic mimics including acinic cell carcinoma are negative for the translocation [45, 46]. Therefore it is a useful adjunct to IHC in difficult cases. Testing for *NTRK3* rearrangement may also be of potential therapeutic value, particularly in cases of high grade transformation, unresectable recurrence or metastases as therapy targeting NTRK3 is currently available [50].

Hyalinizing Clear Cell Carcinoma

Hyalinizing clear cell carcinoma (HCCC) is a low grade carcinoma that mostly arises from minor salivary glands, accounting for less than 1% of all salivary gland neoplasms. The tumors are composed of a mixture of cells with clear cytoplasm and cells with pale eosinophilic cytoplasm, forming cords and nests within a hyalinized basement membrane-like stroma. The main differential diagnoses include other tumors that can exhibit clear cell change, including clear cell variants of MEC, epithelial myoepithelial carcinoma, myoepithelial carcinoma and SCC. The hallmark genetic change of HCCC is t(12;22)(q13;q12) resulting in *EWSR1-ATF1* fusion [51]. However, this genetic aberration is not specific for HCCC and it is shared with soft tissue tumors including angiomatoid fibrous histiocytoma and clear cell sarcoma [52]. Nevertheless, *EWSR1* rearrangement can be detected on FISH in 82–87% of HCCC and not found in other clear cell salivary gland tumors including MEC, epithelial myoepithelial carcinomas, myoepitheliomas or in clear cell SCC or metastatic clear cell renal cell carcinoma [51, 53]. Therefore *EWSR1* break-apart FISH is a useful adjunct in confirming the diagnosis of HCCC in the appropriate morphological context (Fig. 5).

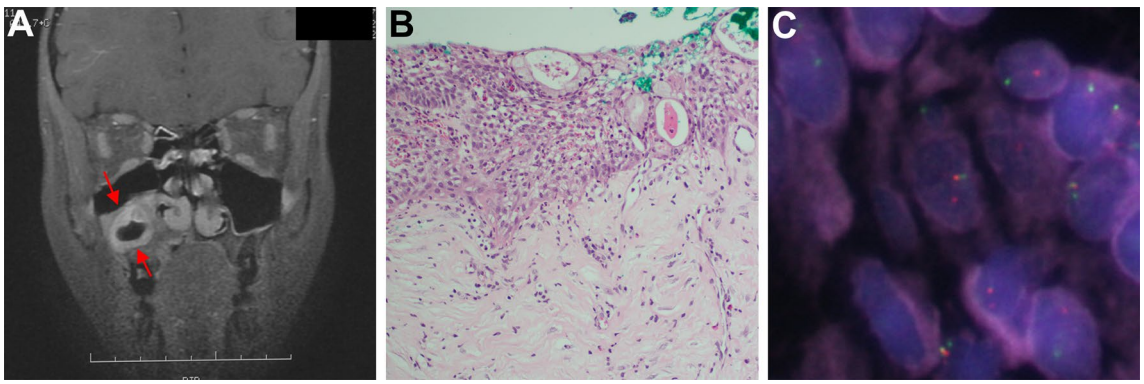

Fig. 4 An 11 year old female presented with an expansile lesion in the right maxillary sinus with mucous discharge in the palate. **a** CT showed a cystic lesion with opacification of the sinus. **b** Biopsy of the lesion showed the cyst wall to be lined by squamoid epithelium with interspersed goblet cells with intracytoplasmic mucin as well as small cysts lined by squamoid cells and goblet cells. The differential diagnosis included mucoepidermoid carcinoma and glandular odontogenic cyst. **c** FISH for *MAML2* rearrangement with a break-apart probe was positive confirming mucoepidermoid carcinoma

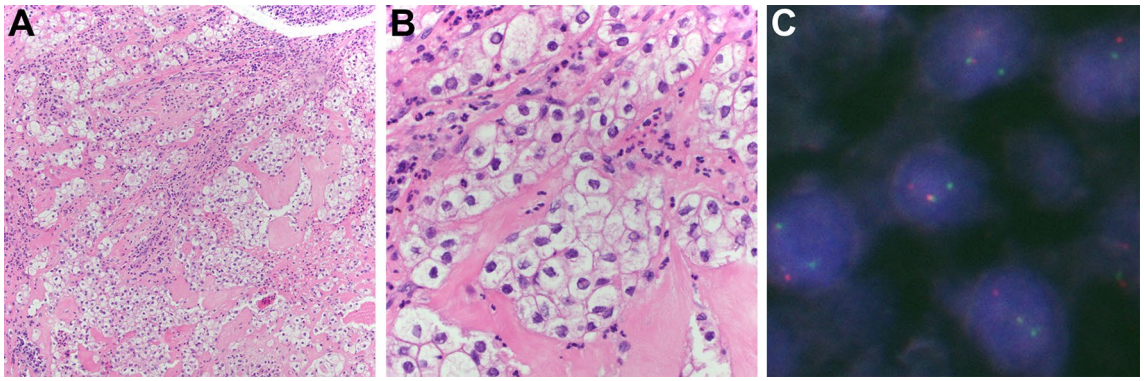

Fig. 5 A 30 year old male presented with left neck lymph node metastasis. **a, b** The tumour was composed of nests of clear cells with uniform nuclei showing occasional nucleoli. The nests were separated by hyalinised to sclerotic stroma. The features were of a metastatic clear cell carcinoma. Immunohistochemistry was used to exclude kidney, adrenocortical and lung origin however differential diagnosis included various salivary gland tumours with clear cell morphology. **c** FISH for *EWSR1* rearrangement using a break-apart probe was positive confirming hyalinizing clear cell carcinoma. Subsequent radiologic investigations revealed a minor salivary gland lesion in the soft palate

Interestingly, clear cell odontogenic carcinoma (CCOC), a rare odontogenic tumor most commonly arising from the posterior mandible, also shows *EWSR1* rearrangement in approximately 80% of cases [54]. In addition to the genetic link, CCOC and HCCC have considerable morphologic and immunophenotypic overlap and may therefore be related tumors arising in different locations.

Salivary Duct Carcinoma

Salivary duct carcinoma (SDCa) is a high grade malignant salivary gland neoplasm which was initially under-recognised due to its wide morphologic spectrum. Typically, the tumors resemble high grade breast ductal carcinoma in situ and have a prominent apocrine morphology. Less commonly, solid, papillary and micropapillary growth patterns are also seen. With increasing awareness and availability of immunohistochemical markers such as GATA3 and androgen receptor, the incidence of SDCa has been reported to be between 1.8–12% of all salivary gland cancers [55, 56]. SDCa have extremely poor prognosis due to the high incidence of locoregional and distant metastases at presentation. Systemic treatment is essential to address the distant metastases [25].

Novel treatment options being investigated include androgen deprivation therapy and trastuzumab as nearly 90% of SDCa express androgen receptor and 30% demonstrate HER2 amplification. Assessment of *HER2* amplification by FISH may play an important therapeutic role, particularly in the setting of metastatic disease as several studies have demonstrated response to trastuzumab in combination with chemotherapy [57, 58].

Soft Tissue and Bone Neoplasms

Benign and malignant primary soft tissue and bone neoplasms of the head and neck are rare. Sarcomas of head and neck account for approximate 1% of all head and neck malignancies Histologically, the differential diagnoses of malignant spindle cell tumors in the head and neck include spindle cell carcinoma, melanoma, leiomyosarcoma, synovial sarcoma, spindle cell rhabdomyosarcoma, biphenotypic sinonasal sarcoma, malignant peripheral nerve sheath tumor and pseudosarcomas such as nodular fasciitis. Similarly, small round blue cell tumors in the head and neck raise a broad list of differential diagnoses including poorly differentiated carcinomas, melanoma, lymphomas and sarcomas such as Ewings sarcoma and rhabdomyoscarcoma. The most common sarcomas in adults are angiosarcoma, pleomorphic sarcoma, osteosarcoma, Ewings sarcoma and dermatofibrosarcoma protuberans whereas rhabdomyosarcoma is most common in children [59].

The use of IHC is usually sufficient to identify carcinoma, melanoma and lymphomas, however FISH is often necessary to confirm the diagnosis of sarcomas, in particular Ewing sarcoma family of tumors and alveolar rhabdomyosarcoma. Making the correct diagnosis is important because the primary treatment for sarcomas is often chemotherapy with the most common agents being doxorubicin and ifosfamide [60]. The primary treatment for carcinomas and melanomas is surgical and metastatic melanomas are increasingly treated with targeted therapy and immunotherapy [61].

Ewing Sarcoma

Ewing sarcoma (EWS) of head and neck account for 4–9% of all EWS and most commonly found in skull, maxilla and mandible [62]. The clinical characteristics of EWS in the head and neck are similar to EWS in other anatomical sites. However, EWS in the head and neck has a slightly better survival, likely due to earlier detection and small tumor volume at presentation [62]. EWS harbour characteristic chromosomal translocations resulting in fusion of the *EWSR1* gene with an ETS family of transcription factors. This causes the production of chimeric nuclear proteins that deregulate gene expression and induce oncogenesis [63]. The chromosomal translocation in approximately 85% of the cases is t(11;22)(q24;q12) resulting in *EWSR1-FLI1* fusion while 10% show t(21;22)(q22;q12) resulting in *EWSR1-ERG* fusion. Many less common translocations have now been described, including t(7;22)(p22;q12) resulting in *EWSR1-ETV1*, t(17;22)(q12;q12) resulting in *EWSR1-ETV4* and t(2;22)(q33;q12) resulting in *EWSR1-FEV* fusion.

Whilst commercially available *EWSR1* break-apart probes are a good adjunct to confirm the diagnosis of EWS (Fig. 6), this must be interpreted in the appropriate clinical, radiological, histologic and immunohistochemical context given the large number of soft tissue and epithelial neoplasms that can show *EWSR1* rearrangement. *EWSR1* is a 'promiscuous' gene and known to have many different fusion partners in other soft tissue tumors including extraskeletal myxoid chondrosarcoma, desmoplastic small round cell tumor, angiomatoid fibrous histiocytoma, myxoid liposarcoma and clear cell sarcoma of soft tissue [64].

There are also rare subsets of undifferentiated round cell sarcomas that share histologic similarities with EWS but lack *EWSR1* rearrangement. These occur exclusively in the soft tissue, commonly in the neck but also in the scalp, submandibular region and tonsil [65]. The histology of these sarcomas differs slightly from EWS in that they often show geographic necrosis, mild nuclear pleomorphism with prominent nucleoli, clear cell areas, and focal myxoid matrix. Unlike EWS, these tumors typically show only focal staining for CD99. These tumors may show t(4;19)(q35;q13.1) or t(10;19)(q26;q13) resulting in fusion of *CIC* on chromosome 19, with either *DUX4* on chromosome 4 or its paralog *DUX4L* on chromosome 10 [66]. Other translocations include t(X;19)(q13;q13.3) resulting in *CIC-FOXO4* fusion and X-chromosomal paracentric inversion resulting in *BCOR-CCNB3* fusion. *CIC* and *DUX4* break-apart FISH probes as well as *CIC-DUX4* and *BCOR-CCNB3* fusion probes are commercially available for confirmation of diagnosis. *CIC-DUX* sarcomas tend to show highly aggressive clinical course and optimum management protocols are under investigation.

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Approximately 40% of cases are primary in the head and neck region. The majority of the head and neck RMS are embryonal RMS and alveolar RMS is rare in the head and neck [67]. Embryonal RMS are characterised by loss of heterozygosity on the short arm of chromosome 11 (11p15.5) and inactivation of a tumor-suppressor genes in that region [68]. A FISH test is not available for embryonal RMS, on the other hand approximately 80% of alveolar RMS harbour one of two characteristic chromosomal translocations: t(2;13)(q35;q14) and t(1;13)(p36;q14) resulting in fusion of *FOXO1* with *PAX3* and *PAX7* respectively [68]. *PAX3-FOXO1* fusion is more common, accounting for approximately 55% of alveolar RMS while *PAX7-FOXO1* accounts for approximately 20%.

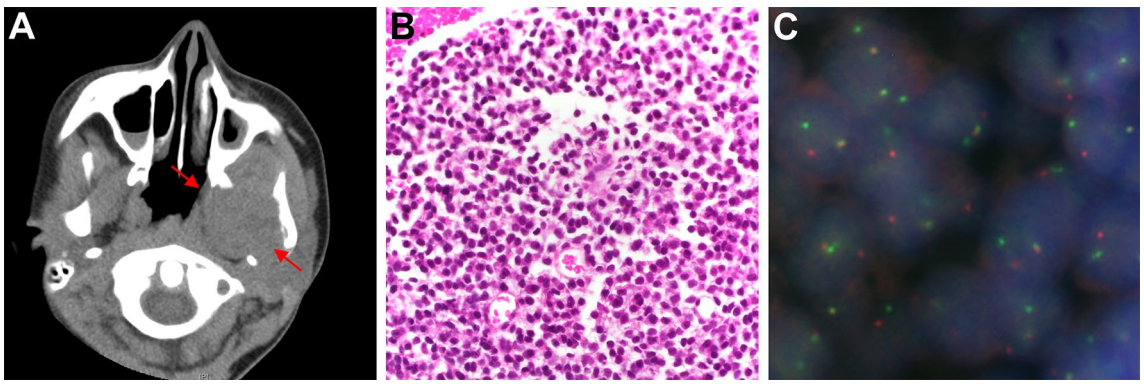

Fig. 6 A 20 year female presented with 12 months of intermittent progressive numbness over left chin and tongue as well as 2 months of trismus and jaw pain. **a** CT showed a parapharyngeal mass with destruction of mandibular ramus. **b** Biopsy of the mass showed a small round blue cell tumor composed of small to intermediate sized cells showing round monotonous nuclei with fine chromatin. Immunohistochemistry was positive for FLI1 and also showed membranous CD99 staining. Myoid, lymphoid, epithelial and melanoma markers were negative. **c** FISH for *EWSR1* rearrangement with a break-apart probe was positive confirming Ewing sarcoma

Much less common translocations have also been described including t(2;2)(p23;q35) and t(2;8)(q35;q13) resulting in *PAX3-NCOA1* and *PAX3-NCOA2* fusions respectively [69]. Thus the use of break-apart FISH probe for the detection of *FOXO1* rearrangement has a sensitivity of up to 88% and 100% specificity for alveolar RMS [70, 71]. In particular, this rearrangement is not seen in morphologic mimics including embryonal RMS, EWS and desmoplastic small round cell tumors.

Synovial Sarcoma

Approximately 3–10% of synovial sarcomas originate in the head and neck, particularly from the parapharyngeal area [72]. Prognosis is generally poor with local recurrence and late metastases. It has similar morphological, immunohistochemical and molecular characteristics as synovial sarcoma in other parts of the body (Fig. 7). Biphasic tumors that show epithelial and spindle cell components can be easily recognised however, the majority of the tumors are monophasic with spindle cells only, raising a broad list of differential diagnoses. Synovial sarcomas harbour a balanced translocation t(X;18)(p11.2;q11.2) in over 90% of cases. This results in *SS18* on 18q11.2 to be fused with one of three related genes, *SSX, SSX2*, or infrequently *SSX4* on Xp11.2 [73]. FISH probes to detect *SS18* rearrangement are commercially available with high sensitivity and specificity and are negative in other spindle cell tumors including solitary fibrous tumor, malignant peripheral nerve sheath tumor, leiomyosarcoma and low-grade fibromyxoid sarcoma.

Biphenotypic Sinonasal Sarcoma

Biphenotypic sinonasal sarcoma (BSNS) is a newly described rare sarcoma with both myogenic and neural differentiation exclusive to the sinonasal region. It is frequently misdiagnosed as solitary fibrous tumor or haemangiopericytoma. BSNS occurs in females in their 4th decade and is restricted to nasal cavity and paranasal sinuses. Histologically, BSNS is infiltrative and composed of hypercellular fascicles of monotonous spindle cells with focal herringbone pattern. Tumor cells show indistinct cell borders with moderate amount of eosinophilic cytoplasm and bland overlapping hypochromatic nuclei. Focal rhabdomyoblastic differentiation may be present. BSNS shows immunoreactivity for smooth muscle actin, S-100 and nuclear β-catenin while SOX10 is negative. Focal desmin and myogenin staining may be present. *PAX3–MAML3* fusion has identified in approximate half of the cases while one-third show *PAX3* rearrangement without *MAML3* involvement or vice versa [74]. *PAX3* and *MAML3* break-apart FISH probes are commercially available and may be a useful diagnostic adjunct but their role has yet to be established since the diagnosis of BSNS can be established based on morphology and IHC.

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is a rare locally aggressive dermal tumor that commonly occurs on the trunk but 10–15% of DFSP occur in the head and neck. It typically has a high rate of recurrence after surgery but metastasizes only in 0.5–5% of the cases that undergo fibrosarcomatous transformation [75]. Histologically the tumor is composed of monomorphic spindle cells often arranged in a storiform pattern and the subcutaneous fat resulting in a honeycomb appearance. It presents significant diagnostic challenges in the head and neck as the excision requires wide margins that may not be achievable given the complex anatomy of the head and neck. Also, generally the initial biopsies on which treatment decisions are based sample small amounts

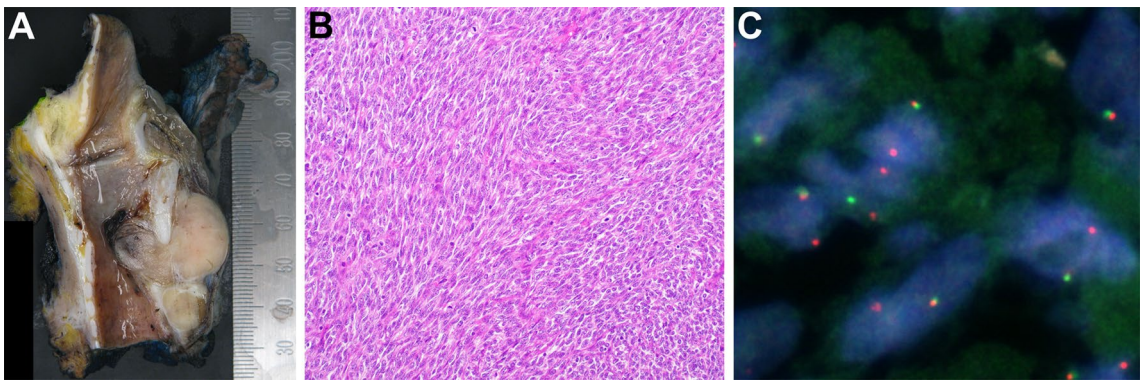

Fig. 7 A 22 year old female presented with a mass in the larynx. **a** A pharyngolaryngectomy was performed. Macroscopically a 22 mm submucosal nodule was seen involving the anterior wall of the cervical oesophagus extending into the soft tissue of the posterior subglottis. **b** Histology showed a tumor composed of fascicles of relatively monotonous spindle cells with scanty cytoplasm and ovoid to spindle shaped nuclei. Immunohistochemistry showed focal patchy EMA staining. S100, cytokeratin, CD34 and desmin were negative. **c** FISH for *SS18* rearrangement with a break-apart probe was positive confirming synovial sarcoma

and superficial aspects of the lesion where the classic storiform patterns of involvement of the subcutaneous tissues may not be obvious. The majority of DFSP harbour t(17;22)(q22;q13) translocation which results in fusion of the *COL1A1* gene from chromosome 17 with the *PDGFB* gene on chromosome 22. This leads to constitutive upregulation of PDGFβ production and activation of signaling pathways promoting cell growth and proliferation [76]. Both *PDGFB* break-apart and *COL1A1-PDGFB* fusion FISH probes are available commercially. Gene rearrangement can be detected on FISH in 86–96% of DFSP [77, 78]. Furthermore, this gene rearrangement is not detected in the close differential diagnosis of dermatofibroma, nodular fasciitis or scar tissue [79] making it highly specific (Fig. 8).

Although surgery is the mainstay of treatment, inoperable or metastatic tumors can now be treated with targeted therapy with tyrosine kinase inhibitor imatinib which blocks PDGFβ receptor signaling [76]. However, this therapy is only effective if *COL1A1-PDGFB* fusion is present therefore FISH carries important therapeutic implications as well in this context.

Osteosarcoma

Osteosarcoma of maxilla and mandible is rare and account for approximately 6% of all osteosarcomas. Compared to osteosarcomas of long bones, patients typically present at later age (around fourth decade) and have longer median survival and rare metastases [80]. The majority are chondroblastic and osteoblastic osteosarcomas that do not pose diagnostic challenges as high grade atypical cells producing osteoid matrix are relatively easy to recognise. However low grade central osteosarcoma are often misdiagnosed as a benign lesion such as fibrous dysplasia as both entities can show spindle cell fibroblastic proliferation without significant atypia [81]. Low-grade osteosarcomas have supernumerary ring chromosomes with amplification of chromosome 12q13–15 which includes the *CDK4* and *MDM2* genes. FISH studies have found that all low grade osteosarcomas have amplified *MDM2* while benign fibro-osseous lesions do not show this amplification [82]. Therefore, commercially available *MDM2* enumeration FISH probe is valuable adjunct in separating low grade osteosarcoma from benign fibro-osseous lesions. However, care must be taken to ensure appropriate decalcification during specimen processing so that molecular tests can be performed. Routinely used decalcification using strong acid solutions damages DNA and renders the specimen unsuitable for molecular tests such as FISH. On the other hand, EDTA-based decalcification solutions or formic acid help preserve the DNA integrity and are more suitable for both primary and metastatic bone lesions that potentially require molecular tests [82].

Lipomatous Neoplasms

Lipomas generally do not pose a challenge however some variants of lipomas can cause diagnostic difficulties including spindle cell and pleomorphic lipomas. Although liposarcoma is one of the most common soft tissue sarcomas in adults accounting for 17–23% of all soft tissue sarcomas, it rarely occurs in the head and neck and accounts for only 1% of head and neck sarcomas [83].

Spindle cell lipomas and pleomorphic lipomas share the same genetic abnormality of 13q deletion with or without 16q deletion. 13q deletion involves the 13q14 locus which contains the *RB1* gene encoding the Rb protein [84]. All spindle cell lipomas and pleomorphic lipomas show loss of Rb expression on IHC while histologic mimics including well differentiated liposarcoma, solitary fibrous tumors,

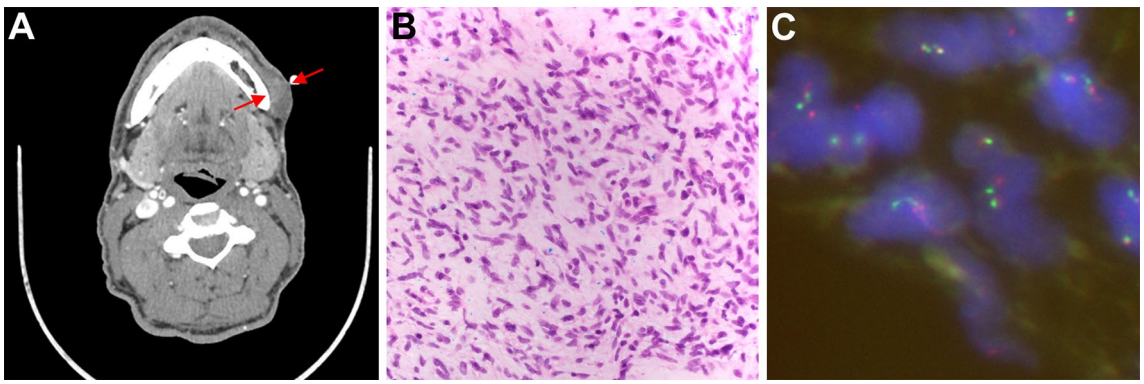

Fig. 8 A 42 year old male who was involved in a motor vehicle accident 20 years ago had facial reconstruction at the time and scar formation. He presented with a newly palpable nodule in the scar. **a** CT showed a 31 mm non-enhancing soft tissue mass overlying the left mandibular body with ill-defined margins. **b** Biopsy of the mass showed a tumor composed of uniform spindle cells with a vague storiform growth pattern. Immunohistochemistry was positive for CD34, and negative for S100, cytokeratin and desmin. **c** FISH for *PDGFB* rearrangement with a break-apart probe was positive confirming DFSP

and myxoid liposarcomas retain Rb expression [85]. Similar, all spindle cell lipomas show either monoallelic or biallelic deletion of *RB1* on FISH [85].

On the other hand, well differentiated liposarcoma are characterised by amplified chromosomal region 12q14–15 contain *CDK4* and *MDM2* genes [86]. FISH for *MDM2* amplification has almost 100% sensitivity and specificity for well differentiated liposarcoma [87]. Benign lipomatous tumors, in particular spindle cell lipoma and pleomorphic lipoma, are negative for *MDM2* amplification on FISH.

Myxoid liposarcomas generally involve the lower extremity and are known for their propensity to metastasize to unusual sites. Metastatic myxoid liposarcomas are rarely seen in the head and neck and present a significant diagnostic challenge, primarily as these tumors are not expected in this region. Myxoid liposarcoma are characterized by a specific translocation, t(12;16)(q13;p11) resulting in *DDIT3-FUS* fusion while a minority show t(12;22)(q13;q12) resulting in *DDIT3-EWSR1* fusion instead [88]. FISH for *DDIT3* rearrangement has been shown to have 100% sensitivity and specificity for myxoid liposarcoma [88]. DDIT3 FISH is useful for excluding other myxoid neoplasms including intramuscular myxoma, low-grade fibromyxoid sarcoma, extraskeletal myxoid chondrosarcoma, and myxofibrosarcoma.

Myofibroblastic Neoplasms

Nodular fasciitis (NF) and inflammatory myofibroblastic tumor (IMT) are myofibroblastic neoplasms that may be confused with high grade neoplasms such as sarcomas and spindle cell carcinomas. Accurate identification of the myofibroblastic proliferations is important to avoid radical surgeries. NF is a benign self-limited neoplasm that typically presents as rapidly growing mass in the upper extremities, trunk and head and neck. Histologically they typically show uniform plump spindled cells in irregular short fascicles and stellate cells in myxoid stroma with tissue culture-like appearance (Fig. 9) [89]. More than 90% of NF harbor *USP6* rearrangement with 65% being t(17;22)(p13;q13) resulting in *USP6-MYH9* fusion [90]. The remaining fusion partners have yet to be elucidated. Break-apart FISH for *USP6* has 86–92% sensitivity for NF and 100% specificity in excluding other spindle cell neoplasms [89, 90].

IMT is classified by the WHO as a neoplasm of intermediate malignant potential with a metastatic rate of less than 2% [91]. It typically has a polypoid appearance clinically and is composed of myofibroblastic-type spindle cells associated with lymphoplasmacytic inflammatory infiltrate and can occur at any anatomic site including the oral cavity, larynx and trachea. Approximately 50% of IMT harbor a translocation at 2p23 locus that involves the *ALK* gene [92]. Multiple fusion partners with *ALK* have been identified in IMT, including *TPM3, TPM4, RANBP2* and *EML4*. Immunohistochemical expression of ALK can be seen in approximately 50% of the cases and *ALK* rearrangement in IMT is detectable by FISH in up to 56% of cases [93]. Therefore ALK protein expression and FISH are useful in differentiating IMTs from other spindle cell neoplasms, particularly the high grade malignant mimics such as carcinomas and sarcomas.

Hematolymphoid Neoplasms

Lymphomas account for approximately 5% of head and neck malignancies and both Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) are seen. NHL is much more common and account for approximately 75% of head and neck lymphomas. Cervical lymphadenopathy is the most common presentation for both HL and NHL, frequently involving lower cervical or supraclavicular lymph nodes

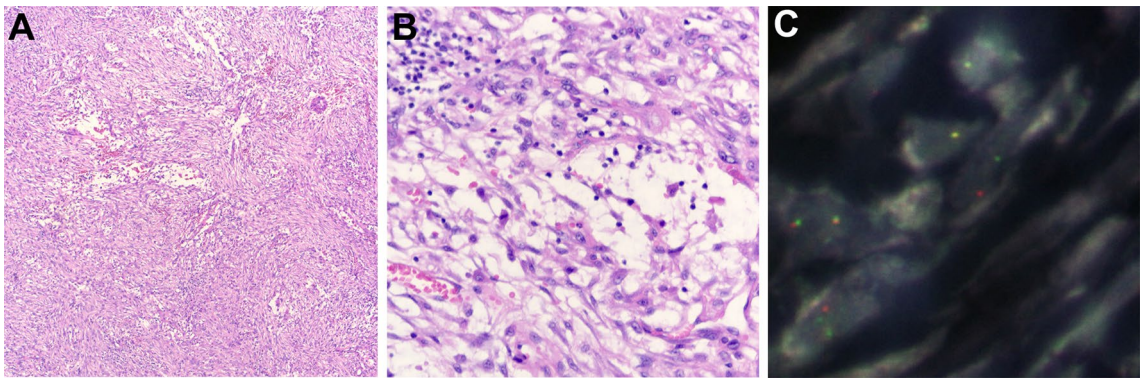

Fig. 9 A 39 year old female presented with a tumor in the right cheek. **a, b** It was excised showing fascicles of spindle cells with indistinct cell membrane and oval nuclei. Areas of tissue culture appearance were seen. Lymphocytes and extravasated red blood cells were present in the background. **c** FISH for *USP6* rearrangement with a break-apart probe was positive confirming nodular fasciitis

[94]. Extranodal lymphomas most commonly involve Waldeyer's ring while lymphoma accounts for 2–5% of salivary gland neoplasms with the parotid being most common. The most common NHL are B-cell lymphomas including diffuse large B-cell lymphoma (DLBCL). While not common, Burkitt lymphoma has a propensity to occur in the jaw, particularly in endemic regions [94]. Burkitt lymphoma is defined by a chromosomal breakpoint affecting the *MYC*/8q24 locus with 70–80% being t(8;14)(q24;q32) while 10–15% harbour t(2;8)(p12;q24) or t(8;22)(q24;q11). These translocations result in fusion of *MYC* with the enhancer elements of an immunoglobulin gene and thereby deregulate the expression of MYC which promotes cell cycle progression and cellular transformation [95]. Approximately 5–15% of DLBCL have been found to harbour *MYC* rearrangement in combination with *BCL2* rearrangement, usually at (14;18)(q32;q21), and/or BCL6/3q27 locus chromosomal translocation. These lymphomas were historically called 'double hit' or 'triple hit' lymphomas. Under the 2016 WHO classification, these are now classified as 'high-grade B-cell lymphomas, with *MYC* and *BCL2* or *BCL6* translocations' [96]. Making a correct and early diagnosis of these lymphomas has important prognostic and therapeutic implications because they behave more aggressively than other DLBCL and do not respond to standard chemotherapy used to treat DLBCL [97]. In this regard, the use of FISH is essential. Both break-apart and *IgH* dual-fusion probes are available for *MYC, BCL2* and *BCL6*. Break-apart probes will detect the rearrangement regardless of the fusion partner therefore has higher sensitivity and is useful for the less common translocations, however, commercially available break-apart probes for *MYC* do not cover all known breakpoints [97]. In addition, false-positive results can rarely occur as normal signals can appear to be slightly separated due to the localization and secondary structure of the DNA. On the other hand, the advantage of dual fusion probes is that they produce virtually no false-positive results but they will not detect variant fusion partners [98]. Approximately one-third of DLBCL show protein overexpression of MYC without gene rearrangement, which also predicts poorer prognosis [99]. 40% tumor cell staining has been used as a threshold and MYC IHC can be used as a screening tool for selecting cases for FISH [99]. Other NHL that can affect the head and neck region also may also harbor genetic alterations that can be detected by FISH including follicular lymphoma (*BCL2* gene rearrangements), mantle cell lymphoma (*cyclin D1* gene rearrangements) and extranodal marginal zone lymphoma (*MALT1* rearrangements) [100]. While FISH may be a useful adjunct for diagnosis of these NHLs in some settings, diagnosis can usually be achieved based on morphology, IHC and clonality studies alone.

Recently Described Entities

HPV-Related Carcinoma with Adenoid Cystic Like Features

It is now recognised that there is a subset of HPV positive sinonasal carcinomas with histological similarities to ACC but are molecularly unrelated, known as HPV-related carcinoma with adenoid cystic like features. They are characterized by nested growth of basaloid cells showing myoepithelial differentiation and forming cribriform or microcystic spaces (Fig. 10). A minor epithelial component with ductal structures is present and may be accompanied by squamous differentiation restricted to the surface epithelium [101]. They are almost exclusively related to HPV type 33 and do not show *MYB* rearrangement. Therefore they appear to

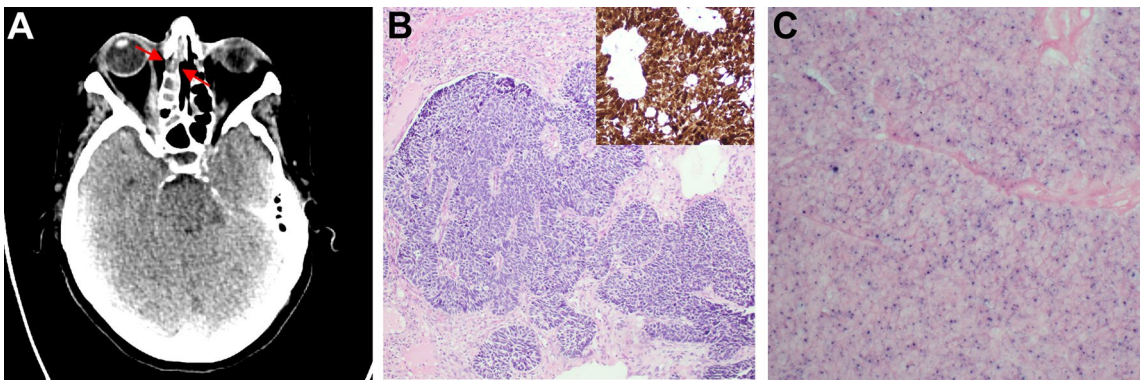

Fig. 10 A 67 year old female presented with epistaxis and nasal blockage. **a** CT showed a mass in the right superior nasal cavity. Functional endoscopic sinus surgery was performed. **b** Histology showed a malignant epithelial neoplasm consisting of infiltrative basaloid cells arranged in cords, trabeculae and cribriform nests. Differential diagnoses included HPV-related carcinoma with adenoid cystic like features, solid variant of ACC, non-keratinizing SCC and NUT midline carcinoma. The tumor was (**b** inset) p16 positive and **c** HPV 33 ISH positive, confirming the diagnosis of HPV-related carcinoma with adenoid cystic like features

be distinct from salivary gland ACC but due to their rarity, clinical significance remains to be determined.

NUT Midline Carcinoma

Another rare poorly differentiated malignancy in the head and neck is NUT midline carcinoma (NMC). Histologically they grow as nests and sheets of monotonous basaloid cells often with abrupt keratinization. This entity is defined by translocations involving *NUT* gene on chromosome 15q14 with the most frequent fusion partner being *BRD4* on chromosome 19 [102]. *NUT* translocation can be detected using *NUT* break-apart FISH probe. Accurate diagnosis of NMC is important as it is associated with poor prognosis thus requires aggressive surgical resection and chemoradiotherapy [103].

Adamantimoma-Like Ewings Family Tumor

Another newly described entity important in the differential diagnosis for undifferentiated malignancies is a rare type of tumor in the EWS family of tumors (EFT) known as adamantimoma-like EFT [104]. These tumors have been reported to arise from the sinonasal tract, parotid and thyroid glands. Similar to typical EWS, adamantimoma-like EFT show a small round blue cell appearance but have more basaloid appearance with peripheral nuclear palisading and interlobular fibrosis as well as histologic and immunophenotypic evidence of squamous differentiation, including squamous pearls, intracellular bridges, diffuse p40 and high molecular weight cytokeratin expression. Similar to EWS, they show diffuse CD99 staining and all of them harbor *EWSR1-FLI1* rearrangement. Due to its rarity, it is uncertain whether it is epithelial or mesenchymal in origin and further data is needed to determine if it will respond to the chemotherapy regimen used for EWS.

Conclusion

With advances in molecular biology, viral driven malignancies have gained recognition and tumors have become more molecularly defined. Accordingly, diagnostic requirements in head and neck pathology have become more refined. Molecular diagnostic modalities, in particular ISH, have become the cornerstone of diagnosis in tumors with challenging morphology. ISH is an important adjunct to the diagnosis of HPV and EBV driven malignancies. Furthermore, FISH is helpful in establishing diagnosis in a variety of salivary gland neoplasms including pleomorphic adenoma, mucoepidermoid carcinoma, secretory carcinoma, hyalinizing clear cell carcinoma and adenoid cystic carcinoma. Soft tissue tumors including small round blue cell neoplasms and spindle cell neoplasms are difficult due to morphological overlap between different entities and FISH is often essential in the diagnosis of Ewing sarcoma, rhabdomyosarcoma, synovial sarcoma, biphenotypic sinonasal sarcoma, dermatofibrosarcoma protuberans, nodular fasciitis and inflammatory myofibroblastic tumor. The use of FISH is also important in separating high-grade B-cell lymphomas with *MYC* and *BCL2* or *BCL6* translocations from DLBCL. FISH plays a critical role in the diagnosis of newer entities such as HPV-related carcinoma with adenoid cystic like features and adamantimoma-like EFT while we still gain experience and familiarity with these evolving concepts.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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