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Contemporary Assessment and Management of Head and Neck Cancer Surgical Margins

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1. Introduction

Head and neck cancer (HNC) is the sixth most common cancer worldwide,[1] which includes cancers of the aerodigestive tract, including lip, oral cavity, nasal cavity, paranasal sinuses, pharynx, larynx, oropharynx, hypopharynx, salivary glands, and local lymph nodes.[2] More than 90% of these are head and neck squamous cell carcinomas (HNSCC), arising from the mucosal lining in these regions.[3, 4]

Although oral squamous cell carcinomas (OSCC) can arise de novo from clinically normal appearing mucosa,[5, 6] they are typically preceded by clinically apparent changes in the tissue, termed oral potentially malignant lesions (OPML) and include leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen planus and actinic keratosis.[7-9] Oral epithelial dysplasia (OED) is a histopathologic diagnosis that describes this tissue transformation and is characterised by cellular and morphological changes similar to those in OSCC but are limited to epithelial cells and remain non-invasive, hence termed premalignant or potentially malignant.[10] The histological grading system developed by the World Health Organisation is used widely to describe the degree of OED in oral mucosa – mild, moderate and severe dysplasia, and carcinoma in situ.[10] The histopathological diagnosis of OED, and its severity as interpreted by pathologists, is used as a predictor of a lesion's risk of malignant transformation, and also the type of intervention required – surgical treatment or watchful waiting.[11] However, a recent study found that this system was not useful for predicting patient outcomes or determining management strategies and recommended definitive treatment of all OED until a more reliable progression/transformation system is developed.[10] In addition, the presence of a non-homogeneous mucosal lesion has been shown to be a significant independent clinical indicator of underlying OED.[8]

Despite the reported transformation rate of 31.4% of OPMLs to OSCC,[12] clinical and histological characteristics have limited potential as predictors of transformation and do not aid in early diagnosis of HNSCC.[5, 13] It has been shown that as many as 50% of HNSCCs may arise from apparently clinically normal mucosa, thus posing an inherent diagnostic challenge.[5, 6] Although it is established that OPML and OED are statistically more likely to progress to cancer, the actual underlying mechanisms are poorly understood, and it is not inevitable that a dysplastic lesion will progress to cancer.[5, 6] Thus upon clinical diagnosis of HNSCC, the disease staging is often advanced with worsened prognosis.[5, 11]

The diagnostic process for OPML, OED or suspected HNSCC involves visual and tactile inspection using white light and other adjunctive visual aids, histopathological assessment of a biopsy sample, and one or more diagnostic imaging methods by radiography or molecular methods (positron emission tomography (PET), computerised tomography (CT), magnetic resonance imaging (MRI)).[14] All these approaches are necessary to aid in accurate tumour staging which directs therapeutic planning, and have to overcome significant challenges including delineation of tumour volume and accurate location, cervical lymph node involvement, distant metastasis, and presence of second primary tumours.[15] The main treatment modality for HNSCC, determined at the stage of diagnosis, continues to be surgical resection in combination with chemoradiotherapy depending on anatomical location.[16] Stratified treatment approaches exist based on HPV status. Newer treatment modalities involve drug/molecular targets used in conjunction with radionuclide tracers leading to personalised medicine.[14, 17] However, despite advanced techniques for early detection and management of HNSCC, the 5-year survival rate of smoking associated HNSCC is still 30-50%, with survivors experiencing poor quality of life.[4, 18] Overall, patients with advanced disease continue to have a poor prognosis and high locoregional and distant recurrences,[19] supporting the need for hybrid technologies both pre-, post-, and during surgery to attain maximum information in minimum time.

2. Surgical margin assessment

The aim of cancer surgery is to remove as much diseased tissue and retain as much healthy tissue as possible.[20] The key issue with surgical management of OSCC is predicting the risk of locoregional relapse, reported to occur in up to 20% of cases, accounting for ongoing modest survival rates.[21-24] One key predictor of locoregional relapse is the presence of carcinoma in or close to the surgical margins of the primary tumour, which is currently not reliably possible despite surgeon's conventional gross assessment (limited to white light tissue reflectance assessing colour and texture) and thorough histopathological examination, as relapse can occur in cases with clear margins.[19, 20, 24] Histopathological examination reports margins as clean/clear (>5 mm between carcinoma and margin), involved (carcinoma exists within 1 mm of the margin), or close (carcinoma exists between 1 to 5 mm from the margin). [24-26] Even though this method has a reported accuracy ratio of >95%, around 30% of patients with histologically negative margins undergo treatment failure raising concerns about its sensitivity.[19, 27-30] In addition, intraoperative histopathological assessment relies on the

quality of samples and degree of sampling, extends the time of operation and yields incomplete results.[20]

While in some cases histopathological examination shows tumour cells in the surgical margins thus implying that residual tumour cells could still exist in patients, most patients with local relapse have histologically clear surgical margins.[24] In these cases, relapse may be due to minimal residual disease (MRD) or field cancerisation.[24] In the case of MRD, small clusters of histopathologically undetectable tumour cells proliferate leading to local recurrence.[24] The field cancerisation model describes a field of premalignant epithelium, which may be rather expansive due to the process of lateral cancerisation, containing preneoplastic cells from which the primary carcinoma may have developed, and second primary tumours (SPT) can develop, following additional genetic hits.[24, 31] Differentiation between SPT and local recurrence due to MRD must be made where possible in order to determine appropriate therapeutic measures – while the latter may be treated with post-surgical radiotherapy or resection, treatment of the former is more complicated.[24] Surgery is not feasible due to the large extent of disease and radiotherapy may even be contraindicated as it could aid in the progression of preneoplastic cells into neoplasia, and thus more intensive surveillance during follow-up may be the best option.[24]

Most studies demonstrate an association between involved or close margins and a worse prognosis,[21, 32] with involved margins resulting in shorter disease-free survival,[21, 23, 32] and shorter overall survival.[33] The presence of close margins has prognostic significance, with a recent study finding margins at a cut-off of ≤ 1.6 mm from the tumour to be prognostic of shorter disease-free survival and shorter overall survival.[32] However, molecular changes indicating early tumour development have been demonstrated in surgical margins of tumours from the larynx, pharynx, and oral cavity considered histologically 'normal'. [23, 34, 35] The rate of local recurrence (and thus failure of treatment) even in margins diagnosed as tumour-free is quoted in studies to be anywhere from 6.9-22%.[36]

It has been hypothesized that the majority of genetic alterations may occur during the early cancer progression process and can precede the observation of certain cytological changes.[37] It is thus believed that if given a reliable set of molecular or genetic biomarkers of epithelial transition/progression to malignancy, the subsequent removal of the altered tissue may prevent the future development of malignancy at that site.[38, 39] Such molecular biomarkers may also be used to assess the margins of tumours subsequent to surgical resection,[24, 40] allowing a means of objective assessment which may detect MRD, and predict potential for local recurrence in the surrounding tissues. Furthermore, molecular studies on genetic markers have shown there is a clonal relationship between the primary tumour and premalignant epithelium adjacent to the tumour,[24] suggesting that molecular analysis of histologically negative surgical margins may be a more sensitive method for detecting malignant transformed cells.[19] Ultimately we would like to propose a shift from conventional histopathological assessment of surgical margins to molecular analysis, either through laboratory testing or imaging techniques.

3. Biomarkers in HNSCC

Since there is a need to reconsider methods of surgical margin assessment during SCC resection, margins should not only be examined macroscopically and microscopically, but also at a molecular level for dysregulated gene expression, which is also applicable in the diagnosis of OED and OPML. It is currently accepted that genetic and epigenetic changes within a clonal population of cells drives carcinogenesis by influencing oncogenes and tumour suppressor genes (TSG).[41-43]

Current modelling postulates that the development of cancer is driven by the accumulation of genetic and epigenetic changes within a clonal population of cells.[44] These genotypic alterations can affect hundreds of genes, leading to phenotypic changes in critical cellular functions such as resistance to cell death, increased proliferation, induction of angiogenesis, and the ability to invade and metastasize.[45] The mechanisms underlying these genetic and epigenetic aberrations can include genomic instability through chromosomal rearrangement, amplification, deletion, methylation, or mutation.[45]

These genetic alterations have been shown to contribute directly to cancer development and progression, and have a direct effect upon oncogenes and TSGs as well as the phenotypes they regulate.[41-43] There has been considerable investigation into the genotypic and phenotypic alterations observed in HNC,[42] and many studies have attempted to identify the genetic and molecular aberrations occurring in HNC surgical margins as a means of predicting local recurrence and relapse.

De Carvalho *et al.* examined 55 HNSCC patients undergoing operative therapy with histologically negative surgical margins and found 36.4% of these patients (20/55) showed overexpression of one of three genes they reported to be overexpressed in tumour samples – *MMP9*, *EPCAM* and *PTHLH*, with *MMP9* overexpression correlating with the risk of developing SPT.[19] Santhi *et al.* showed that both cytoplasmic and nuclear NF- κ B proteins had a significant negative correlation from tumour to surgical margin to extra margin (2 cm away from the actual surgical margin), with COX-2 paralleling its expression, suggesting that these molecules are involved in tumour progression and may be used as markers in assessing MRD.[46] In a later study, they compared the expression microRNAs (miRs) in oral tumour progression and oral surgical margins.[47] They reported a decreased expression of I-miR-125a, I-miR-184, and I-miR-16 and an increased expression of I-miR-96 in the progression from normal mucosa to OED to OSCC, supported by the same pattern of expression retrogressing from extra margin samples to margin samples to tumour samples.[47]

Potential molecular markers for OSCC or OED include: protein markers (e.g. *TP53*,[35, 48-53] *MMP9*,[54] *CDKN2A* (p16),[49, 55-57] *EIF4E*[58]), epigenetic markers (promoter hypermethylation), [Sinha, 2009 #1258, 59-61] microRNA expression (e.g. miR-16, miR-125a, miR-184),[47] DNA copy number changes, [50, 62-65] and loss of heterozygosity (e.g. 3p, 9p, 13q, 11q, 17p).[63, 66-68] Table 1 summarises studies that have investigated molecular markers with the potential to predict local relapse. Table 2 details the list of studies investigating the performance of molecular markers in HNSCC surgical margin analysis using a case (with relapse) and control (without local relapse) approach, with the development of local relapse being the end-point.

Study	Marker (analysis method)	Patients and Tumours	Samples studied	Results (number of positive/total)	Notes
Jin et al.[292]	TP53 mutation (PCR-SSCP)	Laryngeal carcinoma (n = 20)	Tumour-adjacent tissue histologically normal	5/20	
Cruz et al.[48]	TP53 mutation (IHC)	OSCC (n = 42)	Tumour-adjacent tissue non-malignant mucosa	7/42	
Tunca et al.[51]	TP53 mutation (PCR-SSCP and sequencing)	HNSCC (n = 15)	Surgical margin tissue	5/15	
Van der Toorn et al.[293]	TP53 mutation (IHC)	OSCC (n = 20)	Tumour-free surgical margin	11/20	
Blide et al.[49]	TP53 mutation, CDKN2A (p16), CHEK2, LAMA5 (via IHC)	OSCC (n = 16)	Tumour-free surgical margin	12/16 (TP53) 11/16 (p16) 1/16 (CHEK2) 0/16 (LAMA5)	
Shin et al.[294]	TP53 mutation (IHC)	HNSCC (n = 31)	Tumour-adjacent normal epithelium	6/31	
Van Houten et al. [94]	TP53 mutation (IHC)	HNSCC (n = 30)	Tumour-free surgical margin	19/30	Only margin samples with TP53 mutation in tumour were investigated
Nathan et al.[54]	TP53 mutation, 4E, MMP-9 (via IHC)	HNSCC (n = 52)	Tumour-free surgical margin	- 23/52 (TP53) - 27/52 (4E) - 28/52 (MMP-9)	
Tabor et al.[82]	Microsatellite (LOH)	HNSCC (n = 28)	- Tumour-adjacent non-malignant mucosa samples (n = 140) - Tumour-free surgical margins (n = 42)	- 10/28 - 7/28	
Szukala et al.[295]	Microsatellite (LOH at 13q)	Laryngeal carcinoma (n = 65)	Cancer-free surgical margin	8-20/65	
Poh et al.[68]	Microsatellite (LOH at 3p and 9p)	OSCC (n = 20)	Tumour-adjacent tissue, cancer free (n = 32)	15/32	
Breiger et al.[67]	Microsatellite at 3q26	HNSCC (n = 20)	- Biopsy 1cm from tumour (n = 20)	- 4/20 - 3/20	

Study	Marker (analysis method)	Patients and Tumours	Samples studied	Results (number of positive/total)	Notes
			- Biopsy 2cm from tumour (n = 20)		
Bremmer et al.[63]	Microsatellite (LOH at 3p, 9p, 11q and 17p), DNA ploidy, MLPA	HNSCC (n = 10)	Cancer-free surgical margin	- 10/10 - 4/10 - 10/10	Only margins with TP53 mutations were analysed
Martone et al.[55]	Promoter hypermethylation of MGMT, CDKN2A (p16), DAPK1	HNSCC (n = 11)	Cancer-free surgical margin	5/11 (MGMT) 3/11 (CDKN2A (p16)) 8/11 (DAPK1)	
Goldenberg et al. [34]	Promoter hypermethylation MGMT and CDKN2A (p16)	HNSCC (n = 6)	Surgical margins	3/6	Intraoperative margin analysis
Wong et al.[57]	Promoter-methylation CDKN2A (p16) and p15	HNSCC (n = 73)	Tumour-adjacent mucosa histologically normal (n = 29)	5/29 (CDKN2A (p16)) 18/29 (p15)	
Supic et al.[61]	Promoter hypermethylation of p16, DAPK, RASSF1A, APC, WIF1, RUNX3, E-cad, MGMT, hMLH1	OSCC (n = 47)	Tumour-adjacent mucosa histologically normal	44/47 (any marker) 27/47 (p16) 14/47 (DAPK) 17/47 (RASSF1A) 6/47 (APC) 19/47 (WIF1) 11/47 (RUNX3) 6/47 (E-cad) 7/47 (MGMT) 6/47 (hMLH1)	
Shaw et al.[296]	Promoter hypermethylation of p16, CYGB (via PMA)	OSCC (n = 20)	Deep margins histologically tumour-free	11/20 (p16) 17/20 (CYGB)	Possible contamination from adjacent tumour

Study	Marker (analysis method)	Patients and Tumours	Samples studied	Results (number of positive/total)	Notes
Roh et al.[60]	Promoter hypermethylation of p16, DCC, KIF1A, EDNRB (via qMSP)	HNSCC (n = 12)	Deep margins grossly tumour-free	8/12 (any marker)	
Kato et al.[72]	Promoter hypermethylation of p16, MGMT (via MSP)	OSCC (n = 51)	Tumour-adjacent mucosa histologically normal (n = 22)	6/22 (p16) 9/22 (MGMT)	
Barrera et al.[297]	Chromosome imbalance (Interphase-FISH)	HNSCC (n = 10)	Cell brushings of clinically normal tumour-adjacent margins	10/10	Possible contamination from adjacent tumour
Voravud et al. [298]	Chromosome imbalance (Interphase-FISH)	HNSCC (n = 20)	Epithelium adjacent to tumour, histologically normal	8/20	
Ott et al.[62]	Chromosome imbalance (Interphase-FISH)	HNSCC (n = 20)	Tumour-adjacent margins	Most cases/20 (any genomic change)	Various chromosomes targeted
Stafford et al.[64]	Chromosome imbalance (CGH)	HNSCC (n = 19)	Clinically normal tumour-adjacent mucosa	0/19	
Fabricius et al. [299]	Telomerase (DNA-PCR)	HNSCC (n = 40)	Tumour margin biopsy	13/40	
Preuss et al.[65]	DNA Ploidy	HNSCC (n = 20)	- Biopsy 1cm from tumour (n = 20) - Biopsy 2cm from tumour (n = 20)	Greater DNA irregularity at 1cm than 2cm	
De Carvalho et al. [19]	Expression of PTHLH, EPCAM, MMP-9,	HNSCC (n = 55)	Tumour-adjacent mucosa histologically normal	20/55 (any marker) 13/55 (MMP-9) 6/55 (EPCAM) 5/55 (PTHLH)	

Study	Marker (analysis method)	Patients and Tumours	Samples studied	Results (number of positive/total)	Notes
Graveland et al. [300]	LY6D (qRT-PCR)	HNSCC (n=55)	'Clean' or 'close' deep margins histologically tumour-free	12/55	
Dasgupta et al. [301]	Mitochondrial DNA (mtDNA) mutation	HNSCC (n = 50)	Histologically normal margins (n = 24)	17/24	Only margins with mtDNA mutation in tumour were assessed
Santhi et al.[47]	microRNA expression	OSCC (n = 84)	- Surgical margin tissues (histologically mild to moderate dysplastic) - Extra margin tissue (histologically normal) (n = 56)	- Increased expression (miR-125a, miR-184, miR-16) in margin vs. tumour - Decreased expression (miR-96) in margin vs. tumour	

Table 1. Molecular markers at HNSCC surgical margins with potential to predict local relapse

Study	Marker (analysis method)	Patients and margins	(Number of patients with positive molecular margins/total patients)	Sensitivity (positive margins/ number of cases)	Specificity (negative margins/ number of controls)	Significance	Notes
Brennan et al. [93]	TP53 mutation (Sanger sequencing)	HNSCC (n = 30) Evaluated (n = 25) Margins (n = 72)	13/25 (48%)	5/5 (100%)	12/20 (60%)	Yes (KM-logrank)	
Van Houten et al.[97]	TP53 mutation (Sanger sequencing)	HNSCC (n = 179) Evaluated (n = 76) Margins (4 to 5 per tumour, 3-4 superficial)	50/76 (66%)	9/9 (100%)	25/62 (40%)	Yes (KM-logrank)	

Study	Marker (analysis method)	Patients and margins	(Number of patients with positive molecular margins/total patients)	Sensitivity (positive margins/ number of cases)	Specificity (negative margins/ number of controls)	Significance	Notes
		and 1 deep mucosal margin)					
Bergshoeff et al.[50]	TP53 mutation (IHC), Chromosome instability (CIN) (via Interphase-FISH)	OSCC (n = 20) Evaluated (n = 19)	8/19 (42%)	3/4 (75%) – TP53 4/4 (100%) - CIN	10/15 (67%) - TP53 11/15 (73%) - CIN	No – TP53 (Fisher-exact test) Yes - CIN (Fisher-exact test)	
Huang et al.[302]	TP53 mutation (Sanger sequencing)	OSCC (n = 58) Evaluated (n = 25)	16/25 (64%)	11/13 (85%)	7/12 (58%)	Yes (KM-logrank)	
Partridge et al.[35]	TP53 mutation (p53 phage plaque assay, immunocytochemistry, FASAY)	OSCC (n = 18) Evaluated (n = 11)	6/11 (55%)	4/5 (80%)	4/6 (67%)	Not performed	
Nathan et al.[110]	TP53 mutation (IHC), eIF4E (IHC)	Laryngeal carcinomas (n = 54)	6/54 (11%) - TP53 32/54 (59%) – EIF4	6/23 (26%) – TP53 21/25 (84%) – EIF4	31/31 (100%) – TP53 18/29 (82%) – EIF4	Yes (KM-logrank)	
Graveland et al.[52]	TP53 mutation (IHC), LOH (PCR), Ki-67 (IHC)	HNSCC (n = 35)	17/35 (49%) - LOH	11/16 (69%) – LOH (75%) – positive TP53 staining (62%) – positive TP53 with >5% total epithelium positive (62%) - LOH 9p present (88%) – LOH 9p and/or >5% TP53 staining positive	6/19 (31%) – LOH (47%) – positive TP53 staining (84%) – positive TP53 with >5% total epithelium positive (74%) - LOH 9p present (63%) – LOH 9p and/or >5% TP53 staining positive	Yes - LOH 9p and/or >5% TP53 staining positive No – Ki-67 (KM-logrank)	

Study	Marker (analysis method)	Patients and margins	(Number of patients with positive molecular margins/total patients)	Sensitivity (positive margins/ number of cases)	Specificity (negative margins/ number of controls)	Significance	Notes
Pena Murillo et al.[95]	TP53 mutation, Ly-6D (qRT-PCR)	OSCC (n = 142) Evaluable (n = 102) Carcinoma-free resection margins	51/102 (50%) – TP53 14/51 (27%) – Ly-6D	(92%) – TP53 (42%) – Ly-6D (70%) – Combined TP53 and Ly-6D	(56%) – TP53 (81%) – Ly-6D (70%) – Combined TP53 and Ly-6D	Yes – TP53 (KM-logrank) No – Ly-6D (KM-logrank)	46 cases received post-op radiotherapy Only wild-type TP53 positive margins were analysed for Ly-6D
Yi et al.[96]	TP53 mutation, Cyclin D1, eIF4E (ISH)	Laryngeal carcinoma (n = 115)	47/115 (41%) – TP53 34/115 (30%) – D1 35/115 (30%) – eIF4E	21/33 (64%) – TP53 17/33 (51%) – D1 28/33 (85%) – eIF4E	56/82 (68%) – TP53 65/82 (79%) – D1 75/82 (91%) – eIF4E	Yes (Chi-square)	
Nathan et al. [58]	eIF4E (IHC)	HNSCC (n = 65)	36/65 (55%)	20/22 (91%)	27/43 (63%)	Yes (KM-logrank)	
Ogbureke et al. [303]	Bone sialoprotein (BSP), Dentin sialophosphoprotein (DSSP), Osteopontin (OPN), MMP-9 (via IHC)	OSCC (n = 20) Surgical margins (histologically negative) (n = 200)	10/20 (50%) – BSP 14/20 (70%) – DSSP 14/20 (70%) – OPN 16/20 (80%) – MMP-9	6/9 (67%) – BSP 8/9 (89%) – DSPP 7/9 (78%) – OPN 6/9 (67%) – MMP-9	4/11 (36%) – BSP 6/11 (55%) – DSPP 7/11 (64%) – OPN 10/10 (100%) – MMP-9	Yes (KM-logrank)	
Reis et al. [304]	4-gene signature of MMP-1, COL4A1, P4HA2, THBS2 (via qRT-PCR)	OSCC (n = 30) Margins (n = 136)	Not reported	Not reported	Not reported	Yes (KM-logrank)	All four genes were up-regulated in margins of patients with disease recurrence compared to those without recurrence.
Montebugnoli et al. [305]	Ki-67 expression	OSCC (n = 42)	13/42 (30%) – High Ki-67 values	2/4 (50%) – High Ki-67 values	25/38 (65%) – High Ki-67 values	Yes (KM-logrank)	

Study	Marker (analysis method)	Patients and margins (n =)	(Number of patients with positive molecular margins/total patients)	Sensitivity (positive margins/ number of cases)	Specificity (negative margins/ number of controls)	Significance	Notes
			29/42 (69%) – Low Ki-67 values	1/4 (25%) - Low Ki-67 values	9/38 (24%) - Low Ki-67 values		
Sardi et al.[86]	Microsatellite analysis (MSI and LOH)	HNSCC (n = 41)	11/25 (44%)	7/8 (88%)	13/17 (76%)	Yes (KM- logrank)	
Temam et al. [306]	Microsatellite analysis (MSI)	HNSCC (n = 76) Evaluated (n = 26) Margins (n = 113)	7/26 (27%)	5/5 (100%)	19/21 (90%)	Yes (KM- logrank)	
Handschel et al.[307]	DNA ploidy	HNSCC (n = 40)	16/40 (40%)	14/20 (70%)	18/20 (90%)	Not reported	
Zhao et al. [308]	CD44v6, BIRC5 (survivin) (via IHC)	Laryngeal carcinoma (n = 146) Evaluated (n = 112)	35/112 (31%) 44/112 (39%)	20/41 (49%) 26/41 (63%)	56/71 (79%) 53/71 (75%)	Yes (univariate cox- proportional hazard)	
Schaaij-Visser et al. [309]	KRT4 (cytokeratin 4), CRNN (cornulin) (via IHC)	HNSCC (n = 46)	23/46 (50%) 23/46 (50%)	17/23 (74%) 16/23 (70%)	17/23 (74%) 16/23 (70%)	Yes (KM- logrank)	
Sinha et al.[56]	Methylation of CDKN2A (p16)	OSCC (n = 30)	13/30 (43%)	5/6 (67%)	16/24 (67%)	Yes (KM- logrank)	
Tan et al.[59]	Methylation of CDKN2A (p16, CCNA1, DCC)	HNSCC (n = 42) Evaluated (n = 27)	11/27 (41%)	5/5 (100%)	16/22 (73%)	Yes (KM- logrank)	

Table 2. Performance of molecular markers in HNSCC surgical margin analysis of patients with (cases) and without (controls) local relapse [adapted and modified from Braakhuis *et al.*[24]

3.1. Epigenetic events

Unlike genetic alterations, epigenetic changes are heritable and potentially reversible.[69] Epigenetic changes refer to any heritable modifications in gene expression without alterations of the DNA sequence; they occur more frequently than gene mutations and may persist for the entire cell life and even for multiple generations.[43] The transcription of each gene may change from high-level expression to complete silencing, depending on the influence of the “epimutations” which interfere with the action of activators and suppressors on specific promoters in the chromatin context.[41] Epigenetic inheritance includes DNA methylation, histone modifications and RNA-mediated silencing.

Promoter hypermethylation is a well-documented mechanism for tumour-specific alteration of suppressor gene activity in human malignancy, including head and neck cancer.[70] In normal tissues, unmethylated cytosine is found in high densities in CpG islands; areas with high concentration of cytosine and guanine that map close to a promoter region in 40% of mammalian genes.[41] This unmethylated state is associated with a high rate of transcriptional activity; vital for maintaining TSG levels. Where hypermethylation of TSG occurs (via the enzyme DNA methyltransferase), stable transcriptional silencing of tumour suppressor activity occurs.[42, 69]

Studies have shown that methylation of the p16INK4a gene is a frequent event in primary HNC, with hypermethylation occurring in 50-73% of cases.[34, 71] In an analysis of 22 OSCC cases where paired cancerous tissues and the surrounding normal mucosa were simultaneously analysed, methylation of p16 and O6-methylguanine-DNA-methyltransferase (MGMT; a gene which produces a DNA repair enzyme essential for removing adducts caused by alkylating agents) were shown in 27-40% of specimen margins considered ‘normal’.[72] In a recent study on the prognostic significance of tumour-related gene hypermethylation in cancer-free surgical margins of OSCC, Supic *et al.* selected a number of genes involved in a wide range of cellular processes for analysis.[61] These included cell cycle control (*p16*), apoptosis (*DAPK* and *RASSF1A*), *Wnt* signalling (*APC*, *WIF1* and *RUNX3*), cell-cell adhesion (*E-cad*), and DNA repair (*MGMT* and *hMLH1*).[61] Results showed that whilst DNA hypermethylation in histologically negative surgical margins is a frequent event, hypermethylation of p16 did not have prognostic significance.[61] Sinha *et al.* found that patients with positive molecular margins for p16 hypermethylation had a 6.3-fold increased risk of having local recurrence compared to patients with negative margins.[56] However, positivity of margins did not show any significant correlation with T classification, nodal status, histological presence or status of marginal dysplasia, or any other patient or tumour parameter.[56] However, in a recent systematic review and meta-analysis of literature, Rainsbury *et al.* concluded that based on 6 studies analysed, significantly better overall survival rates was seen in p16-positive oropharyngeal squamous cell carcinoma (OPSCC) tumours.[73] Differences in methodologies and cut-off points for analysis between the studies remain a limitation for analysis, and there remains inadequate evidence at this time to determine whether or not hypermethylation of p16 can be used as a biomarker for use in determination of clear surgical resection margins or to predict the risk of local relapse.

3.2. Loss of heterozygosity

Loss of heterozygosity (LOH) may occur when one copy of a polymorphic marker with two slightly different alleles is lost or amplified (allelic gain).[45] LOH in key chromosomal loci represents one of the more promising markers; consistently being identified as a potentially independent risk predictor, supported by data from several laboratories, including studies by Sidransky, Califano, Mao, Hong, Lippman, and Lee.[12, 74-78]

Califano and Sidransky developed genetic progression models based on their studies of gene alterations in squamous cell carcinoma of the head and neck.[79, 80] They reported LOH at 9p21, 3p and 17p13 in squamous hyperplasia, as well as LOH at 13q11, 13q21 and 14q31 in dysplasia, with loss of chromosomal region 9p21 being the most common genetic alteration in HNSCC (occurring in 70-80% of dysplastic lesions of the oral mucosa).[75, 79] Consensus has emerged that LOH at 3p and 9p provides evidence of the accumulation of genetic damage in potentially malignant lesions.[40, 81, 82] This has led to a number of investigations into the predictive value of LOH at these specific chromosomal loci in malignant risk of low-grade OED.[83-85] There is a general trend for lesions with greater disturbance in cellular architecture and organization to harbor more genetic alterations at 3p and 9p, however this is not noted in all studies. [40, 74, 76, 80]

Bremmer *et al.* implemented a range of genetic assays to screen for oral pre-malignant fields in histologically 'normal' mucosa, and concluded that LOH may be a valuable screening tool to detect oral pre-malignant fields in high risk patients.[63] The utility of LOH to evaluate risk of local recurrence in surgical margins was also noted by Sardi *et al.*[86] However, a study by Szukala *et al.* found no predictive value of LOH to determine risk of local relapse in laryngeal cancer patients, with a low frequency of LOH detected in collected 'clean' margin samples.[66]

The predictive and prognostic capacity of LOH at 3p and 9p to predict risk of transition from OED to malignancy has also been recently explored. A study by Zheng *et al.* in 2012 aimed to prospectively validate their retrospective "2000 LOH progression model" proposed by Rosin *et al.*[84, 87] Using a prospective cohort of 296 subjects with a histologic diagnosis of primary mild/moderate dysplasia, the authors first validated their original model for predicting progression to severe dysplasia, carcinoma in situ, or invasive cancer.[84, 88] High-risk (3p and/or 9p LOH) lesions were found to have a 22.6-fold increased risk of progression when compared with the low-risk (3p and 9p retention) lesions; findings consistent with the previous study.[84, 87] By further refining their model with the inclusion of two further markers (4q and 17p), prospective validation of the new model was performed. It demonstrated that low-grade lesions showing retention of 9p had approximately a 5% risk of progression over 5 years to severe dysplasia or more advanced disease.[87] This finding has important implications, as it could suggest that individuals falling into this category might not require aggressive treatment or monitoring despite having a histologic diagnosis of dysplasia. Conversely, a high-progression rate (approximately 65%) for high-risk lesions was found, and would suggest that this group should be aggressively monitored for clinical progression.[87]

From this study, a number of areas for future investigation arise. It is important to evaluate the capacity of LOH to predict risk of progression within the immediate surrounding field and

of secondary oral malignancy, given that a portion of HNSCC may not arise from the exact site of the visually distinguished pre-malignancy.[85, 88]

Ultimately, comparison amongst existing studies is hindered by methodological differences, adjustment for confounders, and controls. Whilst early evidence appears promising, the clinical utility of LOH in 3p and 9p as a predictive tool to screen for progression of OED at surgical resection margins still requires further long-term prospective validation and/or investigation.[45, 84]

3.3. p53 family

p53 is a TSG located on chromosome 17p13, and plays a major role in cell-cycle progression, cellular differentiation and DNA repair and apoptosis.[89, 90] Loss of p53 function impairs the regulation of cell cycle arrest and apoptosis, thus altering the ability of cells to respond to stress or damage (such as DNA damage, hypoxia, and oncogene activation).[89, 90] This can then lead to genomic instability, and the accumulation of additional genetic alterations.[91] Loss of p53 has long been implicated in early carcinogenesis, including HNSCC.[92]

Several studies have investigated the expression of p53 in HNSCC tumour resection margins.[24, 49] Three groups have used p53 mutation-specific probes to detect aberrant cells in the resection margins,[93, 94] with 100% sensitivity achieved in identifying the tumours that had a local relapse in two independent studies.[93, 94] However, the assays used had a relatively low specificity (40%),[94] and contamination of margin samples by mutated DNA derived from cells leaking from the tumour could not be excluded.[93, 94] In a recent study by Bilde *et al.*, immunohistochemistry was used to analyse surgical specimens from 16 consecutive OSCC patients with surgical margins deemed negative.[49] Histologically normal epithelium adjacent to oral carcinomas showed upregulation of both p53 and p16, but with very little overlap.[49] It was not possible to conclude whether the observed changes represented early malignant changes or simply a reaction to cellular stress.[49] In case-control comparisons using immunohistochemistry, p53 appears to have problems with respect to marker sensitivity, which may be due to the presence of mutations not resulting in protein overexpression.[83, 93, 95-97] Ultimately, there remains insufficient evidence to determine whether p53 alterations can be used as predictive markers to identify surgical margins at risk of local recurrence.

A number of studies have shown a correlation between p53 expression and early recurrence, risk for secondary recurrence, metastatic spread and more aggressive disease progression.[49, 98] Studies involving immunohistochemical staining for the p53 tumour suppressor protein, image cytometry of abnormal DNA content, and promoter methylation of the p16 tumour suppressor gene have all attempted to establish potential markers for malignant progression.[99-101] Inactivation of p53 has been associated with a reduction in post-surgical patient survival in OSCC.[102, 103] Suprabasal p53 staining was found to be correlated with increasing grades of dysplasia in a recent study by Vered *et al.*,[104] consistent with an earlier study by Bortoluzzi *et al.*[104, 105] However, Cruz *et al.*[99] did not find a correlation between grade of dysplasia and p53 expression, with Murti *et al.*[106] finding a similar level of p53 expression in biopsies of patients who did or did not progress to OSCC; thus concluding that p53 expression was not predictive of the risk of malignant transformation. p53, in combination

with p16INK4a and Ki-67 alteration, has been proposed as potential markers to define high risk leukoplakia, with further validation in larger sample sizes required.[107]

Due to the many differences in study design, methodology and laboratory techniques, there are currently conflicting reports regarding the value of p53 as a biomarker for the prediction of relapse in HNSCC surgical margins. There is yet to be sufficient validated evidence on its utility in adoption for predictive assessment of dysplastic progression.

The proto-oncogene eIF4E (eukaryotic initiation factor 4E) is a eukaryotic translation initiation factor.[108] eIF4E regulates the translation of cap-dependent mRNAs, and an aberrant increase in eIF4E shifts the balance in favour of translation of transcripts that promote cell proliferation and malignancy.[96, 108] eIF4E protein is commonly elevated in HNSCCs,[109] and its overexpression in surgical margins has been found in a number of studies associated with increased risk of local recurrence.[96] In an investigation into the prognostic value of p53 and eIF4E expression in laryngeal carcinoma surgical margins, Nathan *et al.* concluded that eIF4E overexpression appeared to be a more sensitive indicator of recurrence, and suggested that it may occur as an earlier event in the tumourigenesis process.[110] A recent study by Yi *et al.* investigating the prognostic value of p53, eIF4E and cyclin D1 in laryngeal carcinoma surgical margins found similar results, finding that eIF4E overexpression positivity of margins displayed a greater sensitivity than the other two studied factors.[96] Ultimately, further studies are still required to validate and assess the clinical utility of eIF4E in the surgical margin assessment of HNSCC.

Other genes in the p53 family have also been analysed, such as p63, p73 (both structurally and functionally related to p53) and CDK inhibitor (CDKI) p21. There is again insufficient data to determine the predictive value of p63 and p73 in the progression of dysplastic HNSCC lesions, and whilst general trends have been elucidated in the literature, there is no published data that correlates p63 or p73 expression with the prediction of progression to HNSCC.[104, 111, 112] There are conflicting reports on the expression of p21 in the progression of dysplasia in HNSCC, with Choi *et al.* concluding that whilst an increasing trend in p21 expression was detected in histological progression, there was no significant correlation or progression to OSCC.[113] Future studies aimed at assessing the p53 pathway as a whole may be beneficial to further explore the mechanisms of its deregulation in dysplastic progression to HNSCC.

3.4. microRNA

There has been increasing evidence of the role of non-coding microRNAs (miRs) in the regulation of fundamental processes such as cell cycle, differentiation and apoptosis; and by extension, the impact of their dysregulation on the process of carcinogenesis.[114-117] MiRs are single-stranded endogenous, non-coding RNA transcribed from DNA, ranging between 18 and 24 nucleotides in length. They have the ability to regulate expression of other genes on a post-transcriptional level through various processes by degradation or repression of target mRNA; influencing organ development, cell differentiation, proliferation, apoptosis and stress responses.[118, 119] Recent studies have suggested that miRs may also regulate mRNA targets through less stringent mechanisms, such as binding to non-complementary regions and binding to sites located within the coding regions of transcripts.[120] Given their pivotal

function as post-transcriptional regulators of gene expression, miRs affect almost every cellular process; and have been implicated in numerous disease types, including cancer.[116, 119, 121]

The role of miRs in cancer development was first established by Calin *et al.* in a study that reported a specific miR cluster (miR-15/16) was deleted and/or down-regulated in the majority of chronic lymphocytic leukemia (CLL) cases.[122] The link to cancer was further strengthened by the discovery that miR genomic positioning appeared to be non-random,[121, 122] and that a significant number of miR genes were located at fragile sites (unstable regions that have been shown to promote DNA instability in cancer cells) or genomic regions that have been linked to cancers.[123] *RAS*, *HMGA2*, and *MYC* oncogenes have been identified as let-7 targets, indicating significant tumour-suppressive importance for this family of miRs.[118, 123]

There have been many molecular studies investigating the expression and dysregulation of miRs in HNSCC.[13, 115, 116, 124-126] Using a candidate-gene approach, most have attempted to examine the role of expression and proposed targets of specific miRs in HNSCC cell lines compared to normal samples.[13, 125-128] The underlying process by which miR deregulation affects the process of transition from dysplasia to HNSCC has not yet been fully elucidated, with a main impediment being the multifactorial aetiology of HNSCC and wide heterogeneity of lesions. However, Zhang *et al.* reported that Dicer and Drosha, enzymes involved in processing miRNA, were upregulated in salivary gland pleomorphic adenomas.[129]

In HNSCC, Li *et al.* found an increased level of miR-21 expression that was negatively associated with low levels of tropomyosin 1 (TPM1) and phosphatase tensin homologue (PTEN); TSGs that mediate apoptotic and cell-cycle events.[126] In the same study, miR-21 was found to facilitate anchorage-independent growth of HNSCC cells, partly through the down-regulation of TPM1.[126] This finding has been validated in further studies observing elevated miR-21 expression contribution to neoplastic phenotypes.[130, 131] It is pertinent to note that most studies to date investigating miR expression profiles in HNSCC have used cancer cell lines, and only a few have been in solid tumour samples.[13, 132] Cell lines may not reflect the miR profiles of solid tumours, as particular culture conditions and clonal selection may radically change miR expression.[128] Of the few studies which have selected specific miRs for further investigation using patient samples, Childs *et al.* showed that miR-21 was also found in neoplastic head and neck cells, and may have potential as a prognostic marker in HNC.[124]

To date, there are limited studies investigating the role of miRs in surgical margins.[47] Santhi *et al.* analysed 72 miRs reported to be differentially expressed in OSCC and detected decreased expression of miR-125a, miR-184 and miR-16 and an increased expression of miR-96 in both progressive oral mucosal samples and dysplastic surgical margin samples.[47] Langevin *et al.* found miR-137 promoter hypermethylation to be associated with poor overall survival in patients with HNSCC, but found no significant associations with surgical resection margin positivity[133]. Further studies are required to define a broader set of miR profiles within a wider range of surgical samples, and to correlate results with patient outcomes. Ultimately, there remains insufficient evidence to determine whether these alterations could be used as predictive markers to identify dysplastic progression to HNSCC.

Despite the increasing number of studies into miR expression in HNSCC, there remain few publications that have investigated the deregulation of miRs in the transition process from dysplasia to malignancy. In an investigation of miR pre-cursors in oral leukoplakia (OL), Xiao *et al.* found up-regulation of both miR-31 and its passenger strand (termed miR-31*).[134] miR-31* was negatively associated with recurrent/newly formed OL, and they hypothesized that miR-31* may play an important role during OL progression via the regulation of fibroblast growth factor 3 (FGF3).[134] This was consistent with miR expression profile findings in a prospective translational study by Lajer *et al.*, who examined global miR expression in a series of consecutive tumours and biopsies obtained from patients with OSCC and OPSCC.[128] Of the one hundred and fourteen miRs differentially expressed between OSCC and normal epithelium, the upregulation of miR-31 and downregulation of miR-375 were found to be the most significant aberrations.[128] Thus there is evidence to suggest that the upregulation of miR-31 may be an early event in the transition process from dysplasia to OSCC; however, further elucidation of its role in the progression process and its predictive value still requires further investigation.

Ultimately, whilst early results of molecular prognostic indicators such as LOH and eIF4E appear promising, the routine use of these markers for HNSCC surgical resection margins assessment is yet to be validated. Further research is required which can ideally integrate the convenience of histopathology with the objectivity of molecular panel analysis, supported by a distinct outline of clinical parameters, baseline data, and sufficiently sizable homogeneous patient populations amenable to long-term follow-up.

4. Imaging techniques

It has now been established that molecular profiling of tissue changes enable clinicians to “visualise” more of the disease, indeed diagnose altered tissue early. While macroscopic changes may be detected under white light examination and tissue/cell level changes through histopathology, molecular dysregulation may be identified using special imaging techniques. While most current methods assess tissue in the plane parallel to the lesion, methods aiding assessment in the vertical cross-section (plane perpendicular to the mucosal surface) are required to detect lesions below the mucosal surface and evaluate submucosal tumour invasion.[135]

All optical imaging techniques detect and analyse backscattered photons from mucosa.[135] Visible light (400-700 nm) is used for conventional white light inspection, however shorter wavelengths in ultraviolet (UV) and longer wavelengths in the near-infrared (NIR) regions of the light spectrum can also be used for imaging. UV and blue light are absorbed by biomolecules to produce fluorescence.[135] In order to detect targeted tumour cells, the tumour-specific signal must be significantly discriminated from the non-specific background signals, thus optimising the signal-to-background ration (SBR).[136] The visible light spectrum has relatively short penetration depths useful for imaging (<100 µm) as it is mostly absorbed by haemoglobin, and is significantly associated with a high level of nonspecific surrounding

signals, resulting in a low SBR.[135, 136] NIR is less susceptible to tissue scattering and haemoglobin absorption, yielding penetration depths >1000 μm through the mucosa and a high SBR, with an optical imaging window of about 650-900 nm in which the absorption coefficient is at a minimum.[135, 136]

Optical imaging techniques using Optical Fluorescence Imaging (OFI) and Narrow Band Imaging (NBI) reflect tissue changes at the microscopic and molecular levels. Optical Coherence Tomography (OCT) and Angle-Resolved Low-Coherence Interferometry (a/LCI) non-invasively provide information in the vertical and axial planes. Raman spectroscopy is a point detection technique based on the inelastic scattering of light, also enabling molecular histopathological examination. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are methods traditionally used to detect carcinoma and metastasis (hence staging), and assess treatment response, providing anatomical and physiological information. Positron emission tomography (PET) is a true form of molecular imaging, opening the door for drug delivery and molecular surgical guidance. Hybrid imaging methods, PET/CT and PET/MRI, offer the best of both these imaging approaches. All these methods, collectively termed “optical biopsy”, are non-destructive *in situ* assays of mucosal histopathologic states using the spectral and spatial properties of scattered light to measure cellular and/or tissue morphology, providing an instantaneous diagnosis.[135, 137]

4.1. Optical imaging

4.1.1. Optical coherence tomography

Optical Coherence Tomography (OCT) is based on the principle of low-coherence interferometry.[135] It provides high resolution (~1-20 μm) cross-sectional images of tissue *in situ*, higher than conventional ultrasound, MRI, or CT, and comparable to conventional histology but being non-destructive, it aids real-time surgical diagnostics and an “optical biopsy” of the tissue.[138] Initial success with this modality was with retinal pathology[139] and bronchopulmonary diseases.[140] More recently, it has been deemed useful in diagnosing diseases of the oropharynx/larynx and other oral tissues.[138, 141, 142]

OCT is similar to ultrasound B-mode imaging except that OCT uses light instead of acoustic waves, measuring the echo time delay and intensity of backscattered light.[143] The system uses NIR light, split into reference and sample beams, and plots the back-reflected light from structures within the tissue against depth (up to 2-3 mm).[139, 143, 144] Since the velocity of light is extremely high, optical echoes cannot be measured directly by electronic detection, but instead uses low-coherence interferometry – the back scattered light waves interfere with the reference beam and this interference pattern is used to measure the light echoes versus the depth profile of the tissue *in vivo*. [144] OCT also uses fibre optic technology, allowing for low-profile imaging to be performed through small optical fibres attached directly to a scalpel, tissue probe, endoscope, or microscope.[144] The device is compact and portable.[144]

In healthy mucosa, the basement membrane can be easily identified at the junction of the bright lamina propria and the darker epithelium, which is lost in the presence of invasive cancer.[145] However, one study had inconsistent results, showing a deceptive change in the histological

layers when compared to conventional biopsy of oral lesions (various anatomical sites).[138] The authors also noted that OCT image analysis is unique, requiring special training, and associated with a wide range of variability when interpreting its parameters (mainly epithelium thickness and status of basement membrane).[138] The authors previously aimed to generate a bank of normative and pathological OCT data from oral tissues to identify cellular structures of normal and pathological processes, thus creating a diagnostic algorithm.[146]

While OCT is useful for clinical detection of OSCC and OPML,[147] it also has potential in evaluating surgical margins for MRD in HNSCC just as it has been proven useful in cancers of other tissues such as breast,[148, 149] skin,[150, 151] vulva,[152] and prostate.[153]

4.1.2. Angle-resolved low-coherence interferometry

Angle-resolved low-coherence interferometry (a/LCI) is a light scattering technique which isolates the angle scattering distribution from cellular nuclei at various tissue depths.[137] In doing so, it is able to provide biomarkers based on morphology that are highly correlated with the presence of dysplasia.[137] It measures the angular intensity distribution of light scattered by a tissue sample, quantifying subcellular morphology as a function of depth in the tissue.[137] For each depth layer, signatures from cell nuclei are extracted by collecting and processing the angular scattering signal using a Mie theory-based light-scattering model to produce measurements of average nuclear diameter with submicron-level accuracy.[137] Studies that have investigated the use of a/LCI have confirmed that neoplastic tissue transformation is accompanied by an increase in the average cell nuclei size,[137, 154-156] thus detecting potentially malignant lesions as well as malignant lesions. The diameter of a non-dysplastic epithelial cell nucleus is typically 5-10 μm , while dysplastic nuclei can be as large as 20 μm across.[157] When this is optimized to 11.84 μm for the classification of tissue health, a/LCI yields a sensitivity of 100%, specificity of 84%, overall accuracy of 86%, positive predictive value of 34% and negative predictive value of 100% in oesophageal epithelium *in vivo*. [137, 155] This technique has been studied in animal models, *ex vivo* human studies, and more recently in *in vivo* studies, predominantly associated with cases of Barrett's Oesophagus (which is associated with an increased risk of oesophageal adenocarcinoma) and oesophageal epithelium.[137] The system is portable and the probe can be used through the accessory channel of a standard endoscope, thus providing surgical guidance.[137]

a/LCI could have a role in assessing surgical margins in HNSCC by assessing size of nuclei in the margins although currently there are no studies that have investigated this.

4.1.3. Optical fluorescence imaging

The basis of optical imaging techniques is the ability of photons to travel through tissue and interact with tissue components.[158] Fluorescence is the property of certain molecules to absorb light at a particular wavelength and to emit light of a longer wavelength after a brief interval called fluorescence lifetime.[158] Fluorescence spectroscopy, a major form of optical imaging, is a non-invasive diagnostic tool that evaluates the biochemical composition and structure of tissue autofluorescence (AF).[159] It is relatively simple, fast, accurate, and can aid

in real-time cancer detection.[159] While microscopic imaging systems for intraoperative surgical margin assessment based on endogenous contrast or AF are useful, high resolution of the diseased tissue is limited to a small field of view, making it difficult to survey the entire surgical excision margin intraoperatively.[20] Extrinsic approaches are more effective, which use fluorescent dyes detected by probes.[20] The signals can also be integrated into the white light image, which enables real-time intraoperative visualisation.[20] OFI is advantageous and convenient as it can be used intraoperatively for surgical guidance in resecting malignant tissue and for pathological sampling.[160] Various devices implementing OFI, both commercially available as well as those developed by researchers, using visible light or NIR, with or without excitable dyes, have been investigated mostly in breast cancer,[160, 161] but is now being tested in HNSCC as well.[162-166]

Francisco *et al.*[159] recently showed that fluorescence spectroscopy could discriminate between oral mucosa, injury, margins, and areas of recurrence, using a homemade fluorescence spectroscopy system, at 406 nm wavelength [159] without using injectable dyes, and providing macroscopic visualisation of affected and unaffected tissue. The VELscope™ and Identafi™ are commercially available tools that use the principles of AF and tissue reflectance to discriminate between normal and abnormal tissue. These tools are described below in order to illustrate loss of AF (LAF) and diascopic fluorescence as indicators of tissue change, which provide the clinician with additional information aiding diagnosis. Miyamoto *et al.* investigated intraoperative molecular imaging (multispectral fluorescence images) to identify tumour extensions in a murine HNC model.[167] They reported 86% sensitivity and 100% specificity in the diagnostic accuracy analysis compared to histology, the gold standard. They also reported a 60-day improvement in survival rate when using molecular imaging during surgery, compared to standard surgery (37% versus 5% respectively). Thus fluorescence can be used both for diagnosis and for surgical guidance to improve patient outcomes.

The Visually Enhanced Lesion Scope (VELscope™; LED Medical Diagnostics Inc., Barnaby Canada) uses direct tissue AF to enhance oral mucosal abnormalities.[7] An external light source, in this case blue light excitation between 400-460 nm, is used to excite endogenous fluorophores (typically nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD)) in the oral epithelium and collagen cross-links in the underlying stroma, which absorb the extrinsic photons and emit lower energy photons which appear clinically as fluorescence.[168-173] Since each fluorophore is associated with specific excitation and emission wavelengths, changes in tissue architecture and concentrations of fluorophores (as in the case of mucosal abnormalities and neoplastic development) results in altered absorption and scattering properties of the tissue,[7] with decreased tissue AF being reported in OED and mucosal inflammation.[68, 169, 174] Under the VELscope™, normal oral mucosa appears pale green when viewed under a filter while abnormal tissue exhibits LAF and appears dark.

While the VELscope™ has assisted in the detection of OED and OSCC not visible by conventional oral examination (COE) warranting tissue biopsy and aiding in demarcating margins, [68, 175] clinicians have been advised to use the VELscope™ in conjunction with COE as LAF may also be displayed in tissues with mucosal inflammation.[176] Complete diascopic fluorescence, wherein tissues display normal fluorescence pattern with the application of

pressure, can differentiate inflammatory lesions from neoplastic lesions.[176] However, the challenge of completely blanching tissues and inter-operator variation in the interpretation of partial blanching (i.e. low specificity and variable sensitivity) grades the VELscope™ as a useful clinical tool for clinically visualising abnormalities but not an accurate discriminator of the condition of the mucosa under inspection.[176] Nevertheless, a recent clinical study suggested the use of a decision making protocol incorporating the VELscope™ in routine general dental practice allows for the detection of additional oral mucosal lesions requiring specialist referral.[177]

The Identafi™ (DentalEZ, PA, USA) is a multispectral screening device which uses direct fluorescence as well as tissue reflectance to visualise intraoral tissues by incorporating three different lights which are to be used sequentially.[1120;Bhatia, 2013 #729] The light emitting diode (LED) white light enables superior visualisation of oral tissues but cannot differentiate between OPML and other more benign abnormalities of the oral mucosa, in a manner similar to that displayed by Microlux/DL™.[7, 178] Visualisation of oral mucosa under violet light (405 nm wavelength) through the accompanying photosensitive filter glasses, assesses the AF properties of tissue, with normal mucosa exhibiting natural fluorescence and abnormal tissues displaying LAF in a similar fashion to VELscope™.[7] Despite the dubious sensitivity and specificity of this wavelength of light,[173] areas of LAF visualised were often larger than what was clinically visible which might be due to the visualisation of deeper neovascularisation and stromal changes that accompany lesion progression, thus having a potential application in the determination of surgical margins for lesion excision.[163, 164] The green-amber light (545 nm wavelength) uses the concept of reflectance spectroscopy to characterise the connective tissue vasculature.[7] The process of carcinogenesis involves angiogenesis resulting in altered vascular morphology and it has been suggested that these tissue changes can be used to determine the prognosis of oral lesions, enabling the differentiation between benign lesions and OPML.[7, 179-181] Reflectance spectroscopy uses light within the absorption spectrum of haemoglobin (400-600 nm) which would reflect the degree of angiogenesis in the tissue. A significantly reduced reflectance spectra is observed in OSCC and OPML due to greater light absorption from increased microvasculature density and oxygenated haemoglobin content in neoplastic tissue.[7]

The underlying principles have enormous potential for application. Ongoing clinical trials by our group have shown excellent lesion visibility compared to COE under incandescent light. Violet light examination provided improved lesion visibility compared to COE, and improved visualisation of lesion borders and slight increase in lesion size compared to incandescent and white light. It also has a high level of clinical utility for evaluating inflammatory pathology. However, a high level of clinical experience is required to interpret the results of AF examination as the violet light displays low sensitivity for detection of OED. The green light helps uncover subtle vascular and inflammatory patterns providing additional clinical information.

Both these technologies highlight the usefulness of detecting LAF and diascopic fluorescence, however, additional information is required to diagnose the tissue change. Molecular studies in this area aid in our understanding of the phenomena of AF, LAF and diascopic fluorescence in oral tissues, enabling more informed use of such devices and superior interpretation of

changes in AF patterns. One study in oral cancer patients showed that all their tumour samples (confirmed by histopathology) had displayed LOH intraorally when a simple hand-held device, similar to the VELscope™, was used.[68] Molecular analysis in this study on margins with low-grade or no dysplasia showed a significant association between LAF samples and LOH at 3p and/or 9p, which is strongly associated with tumour recurrence after tumour removal.[68] Furthermore, this study found that LAF extended beyond the clinical visible lesion, and these areas displayed dysplasia/cancer on histology and/or genetic alterations associated with molecular risk, thus showing that the VELscope™ can distinguish between dysplasia and normal oral mucosa.[68]

4.1.4. Narrow band imaging

Narrow Band Imaging (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) utilises the concept that the depth of light penetration is dependent on its wavelength to enhance mucosal surface texture and underlying vasculature.[182, 183] The spectral bandwidth of the filtered light is narrowed.[183] The system has two modes, white light and NBI.[7] In NBI mode, only blue light (400 – 430 nm) and green light (525 – 555 nm) are emitted in parallel which make blood vessels in the superficial mucosa appear brown, and the deeper larger vessels in the submucosa appear cyan.[183] Blue light (centred at 415 nm) penetrates shallowly and corresponds to the peak absorption spectrum of haemoglobin, while green light (centred at 540 nm) penetrates deeper.[183] In NBI mode, inflammatory lesions have an ill-demarcated border and can be differentiated from neoplastic lesions which appear as areas with scattered dark spots and a well-demarcated border.[184, 185] These scattered dark brown spots represent superficial blood vessels; interpapillary capillary loops (IPCL).[7] Visualisation of the vasculature, as well as the degree of dilation, meandering, tortuosity, and calibre of IPCLs all indicate the true extent of lesions and severity of pathology, thus guiding the position of biopsy and resection margins.[184, 186-188] Takano *et al.*'s[184] IPCL classification for oral mucosa is tabulated in Table 3. It has been recommended that lesions with Types III and IV IPCL patterns must always be biopsied.[189] The presence of keratinised tissue can pose a hindrance to optimal visualisation of the lesion itself.[7]

Gono *et al.*[183] used NBI in colonoscopy and upper gastrointestinal endoscopy and concluded that magnified NBI enhanced the capillary pattern and the crypt pattern on the mucosa, which are useful features for diagnosing early cancer.[190] Later, Muto *et al.* reported that carcinoma in situ at oropharyngeal and hypopharyngeal mucosal sites can be clinically recognised using magnified NBI endoscopy, confirming the usefulness of evaluating in situ angiogenesis in solid tumours in the head and neck region.[191, 192] Yoshida *et al.* validated the use of NBI with magnifying endoscopy in oesophageal lesions.[193] A case report by Katada *et al.* showed the usefulness of NBI combined with gastrointestinal endoscopy (GIE) in detecting OSCC in the floor of the mouth.[187] Further case reports and investigations also supported the use of NBI in the oropharynx,[194] nasopharynx,[195, 196] hypopharynx,[197] larynx,[198] and oesophagus.[199] Its use in determining tumour size and margins in gastrointestinal cancers of the bile duct,[200, 201] duodenal papilla,[202] stomach[203, 204] was also investigated, all with encouraging results boast-

ing higher sensitivities and specificities than the current detection method used at the time. All these studies hailed NBI as a method with improved detection and diagnostic accuracy of cancers in areas that are difficult to examine, and critically allowing for early diagnosis, having an impact on treatment options, quality of life and patient survival.[205] It was considered that NBI would develop into a useful tool in the future pre-, intra-, and post-operative endoscopic assessment of neoplastic lesions in the upper aerodigestive tract.[206]

Type	Description/Features
I	<ul style="list-style-type: none"> · Normal mucosa · Regular brown dots – when loops are perpendicular to the surface of the mucosa · Waved lines – loops are parallel · Study by Yang <i>et al.</i>[189] – 17% frequency of dysplasia in these lesions, hence remain cautious and utilise clinical judgement as well
II	<ul style="list-style-type: none"> · Non neoplastic and inflammatory lesions, but dysplasia present most of the time · Dilated and crossing IPCL pattern
III	<ul style="list-style-type: none"> · Non neoplastic lesions, but dysplasia is almost definitely present · Elongated and meandering IPCL pattern
IV	<ul style="list-style-type: none"> · Neoplastic lesions · Large vessels IPCL pattern destruction · Presence of angiogenesis

Table 3. Summary of IPCL classification for oral mucosa by Takano *et al.*[184]

In a multicentre, prospective, randomised controlled trial (n = 320), Muto *et al.*[207] found that NBI detected superficial cancer more frequently than white light imaging in both the head and neck region (100% vs 8%) and the oesophagus (97% vs 55%). They reported a sensitivity and accuracy of 100% and 86.7% in the detection of superficial cancer in the head and neck region using NBI, and 97.2% and 88.9% in the oesophagus, respectively.[207] Piazza *et al.*[208] showed that 27% (26 of 96) of patients with OSCC and OPSCC had a diagnostic advantage by applying NBI and high definition television (HDTV) compared to white light and HDTV. In a later study, [209] they concluded that NBI and HDTV were of value in defining superficial tumour extension, in the detection of synchronous lesions in the pre-/intra-operative settings, and in post-treatment surveillance for early detection of persistence, recurrence, and metachronous tumours.

Fielding *et al.* combined white light and AF to the bronchoscopic and laryngoscopic assessments of head and neck cancer patients, and reported improved sensitivity but low specificity, increasing the number of unnecessary biopsies.[210] A later study combined AF and NBI for the detection of mucosal lesions during panendoscopy in head and neck cancer patients, and reported higher specificity than when using AF or white light alone, thus directly impacting on patient management.[211]

A recently published systematic review by Vu & Farah[212] on the efficacy of NBI for detection and surveillance of OPML analysed data from a prospective cohort study by Piazza *et al.*,[209] and a retrospective cohort study by Yang *et al.*:[213] both of which aimed to evaluate the efficacy of NBI endoscopy compared to WL in oral mucosal examination. Vu & Farah concluded that based on available evidence, there is a demonstrable improvement in the ability of NBI visualization to stage tumours, assess margins and detect synchronous, metachronous and recurrent lesions compared to visualization using broadband WL.[212]

A prospective study by Nguyen *et al.* utilized white light (WL), NBI and AFAF to inspect the oral cavity, larynx/hypopharynx and bronchus of 73 patients with known HNSCC, patients with SCC of unknown primary origin, and surgically treated HNSCC patients requiring panendoscopy for suspected recurrent disease.[211] The authors found a significant improvement in the detection of moderate dysplasia or worse by NBI compared to WL, and that the combined use of AF and NBI had significant implications upon mapping and guiding the surgical resection borders of three assessed oral cases.[211] This study demonstrated increased specificity with NBI for the detection of mucosal lesions.[211]

While OFI and NBI can detect tissue and molecular changes in a localised region, imaging modalities such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) provide anatomical information, including nodal involvement and metastasis which influence staging and treatment protocol employed. Ultimately, multimodal imaging can provide additional diagnostic information than white light illumination or a single imaging modality alone.[214, 215]

Both CT and MRI involve 3D sectional imaging and have extremely high diagnostic value. [216] CT scans require ionising radiation (with shorter scan times) while MRI does not but has a longer scan time.[216] CT is currently the most commonly used modality for head and neck imaging, and can improve delineation of soft tissue pathologies with intravenously administered contrast media,[216] however MRI provides the most detailed view of soft tissues and is routinely used to visualise such tumours.[216]

4.2. Molecular imaging

Molecular imaging modalities have the potential to be indispensable in every aspect of cancer care, from early detection to staging, drug delivery, molecular surgical guidance and treatment response.[20, 217, 218] Oncological molecular imaging is defined as the non-invasive imaging of distinctive cellular and sub-cellular events in malignant cells.[20, 219] Molecular imaging probes target the production of genetically determined biomolecules by cancer cells by displaying these directly in or on individual malignant cells, in the extracellular matrix, or cells in the vicinity such as T cells, macrophages, dendritic cells, fibroblasts or endothelial cells.[158, 220, 221] For example, probes paired with positron emitters and novel target-specific anti-cancer drugs could be quantitatively imaged by PET, providing information on tumour biology, guiding drug development, and furthering personalized medicine.[14, 17] Diseased tissue may also be detected through this imaging modality based on hypoxia [222, 223] or pH changes.[17, 224] It is clearly useful to detect changes at the cellular and molecular level rather than rely on anatomical characteristics alone which is commonly the case at present.[20]

Tumours may be able to be characterised without biopsies or surgery, and allow for accurate staging, re-staging and drug response monitoring, paving the way towards true personalised medicine.[20] Molecular imaging modalities may also be used for intraoperative surgical guidance and evaluation of surgical margins, thus improving outcomes.[20]

4.2.1. Raman spectroscopy

Raman spectroscopy is a non-invasive technique that can analyse the molecular composition of a tissue, enabling surgeons to identify, examine and determine the quality of the tumour's molecular margins.[145] It is based on the phenomenon that intramolecular bonds cause light to scatter in a manner that is both measurable and predictable, albeit for a very short time constituting <1 part per million of the total reflected light.[145] Point detection techniques can be used to collect molecular information during endoscopy with optical fibre probes, and they have the potential to be extended to imaging.[135] Raman spectroscopy produces inelastic light scattering (returning photons have longer wavelength than the incident photons) and diffuse NIR photons (photons that return after several scattering events and are useful for measuring fine pathological structures) which aid molecular histopathologic examination.[135] It is performed by illuminating tissue with NIR photons that are absorbed by the vibrational/rotational nodes of molecular bonds associated with chemical functional groups specific to mucosal proteins, lipids, and nucleic acids. [135, 225, 226] Some of these photons are then inelastically scattered, forming detailed spectral patterns that can be reduced to the principal components using multivariate statistics. However, the Raman effect is much weaker than fluorescence and can be easily obscured by fluorescence from the tissue or optical fibre itself.[136]

Shim *et al.* demonstrated the use of CCD detector in collecting Raman spectra *in vivo* in the gastrointestinal tract.[227] Molckovsky and colleagues showed that Raman spectroscopy could be used to distinguish between adenomatous and hyperplastic polyps in the colon, with 100% sensitivity, 89% specificity, and 95% accuracy when used *in vivo*. [228] Haka *et al.* used Raman spectroscopy to examine breast tissue *in vivo*, and reported perfect sensitivity and specificity when using their diagnostic algorithm.[229] They highlighted the feasibility of using it for real-time intraoperative margin assessment during partial mastectomy surgery, which could be similarly used for intraoperative margin assessment in HNSCC cases. Stone *et al.* examined biopsy specimens of laryngeal mucosa using Raman spectroscopy and conventional histopathological analysis, and reported 92% sensitivity and 90% specificity for Raman spectra generated over 30 seconds in the diagnosis of invasive cancer (compared to reference spectra generated from histopathologically normal mucosa).[230] In membranous vocal cord specimens, Lau *et al.* reported 69% sensitivity and 94% specificity for invasive carcinoma using Raman spectra recorded over 5 seconds.[231]

Spatially offset Raman spectroscopy (SORS) has been shown to be an effective tool in recovering Raman spectra from up to several millimetres beneath the surface of turbid media.[232] Keller *et al.* found that, using source-detector separations of up to 3.75 mm, SORS can detect sub-millimetre-thick tumours under a 1mm normal layer, and tumours at least 1 mm thick can be detected under a 2 mm normal layer using the Monte Carlo simulation model of breast

tumour margin analysis.[232] Other recent developments within Raman spectroscopy include surface enhanced Raman spectroscopy (SERS), coherent anti-Stokes Raman spectroscopy (CARS), and stimulated Raman scatters (SRS),[165, 233] which could all have applications in HNSCC margin analysis. Visualising molecular information using Raman spectroscopy has also been shown to aid in identifying patients with prostate cancer who are at risk of cancer progression from those with no evidence of disease.[234]

Raman spectroscopy provides an objective analysis of the tissue's molecular structure compared to the *ex vivo* histopathological analysis and grading based on tissue morphology. It may provide a more clinically relevant measure of the tumour margin on which to guide surgical excision. It has been possible to stage and grade malignancies from a spectral measurement on the surface of bladder tissue using Raman spectroscopy.[235] Representative reference spectra need to be developed by analysing a large cohort of histologically diagnosed mucosal lesions, against which spectra captured *in vivo* can be compared and leading to algorithms that can quickly produce a diagnosis.[145]

4.2.2. Positron emission tomography and hybrid technologies

PET provides a 3-D image of the functional processes in the body, wherein (18F)-fluorodeoxyglucose (18F-FDG), a glucose analogue, is commonly used as the radiopharmaceutical delivering the positron-emitting radionuclide (tracer), thus reflecting tissue metabolic activity by regional glucose uptake, with cancer cells exhibiting increased use of glucose.[20, 236] 18F-FDG PET highlights metabolic differences between malignant and healthy cells and is the first true molecular imaging modality.[20] A hand-held PET probe to detect high-energy gamma rays during breast cancer surgery has been developed for intra-operative evaluation of tumour localisation and margin status.[162] PET is limited in its use due to high cost, use of ionising radiation, and relatively low spatial resolution (it is difficult to detect small tumours (<1 cm) using this hand-held probe).[20, 136] Since PET on its own provides low anatomical information, it is commonly used in conjunction with CT, and more recently, with MRI which has the advantage of greater soft tissue contrast and fewer artefacts.[14] The PET/MRI hybrid imaging technology combines the functional sequences of MR with the molecular information of PET to provide information about tumour biology and microenvironment [237] – hence the best of both worlds.

A number of studies have evaluated the effectiveness of PET/CT versus PET/MRI in HNSCC. Many studies have found no significant difference between diagnostic capability and anatomic localisation of lesions as detected by both modalities,[238-243] however, there is agreement for tailored use of PET/MRI in the head and neck region since higher soft tissue contrast would aid in diagnosis.[238, 242, 244, 245] Kanda *et al.* found that PET/MRI and PET/CT had equal sensitivities and specificities, and both these were more sensitive compared to MRI alone but less specific, when investigating these modalities in HNSCC.[244] Kubiessa *et al.*[239] showed that PET alone had the highest sensitivity while MRI alone showed best specificity when comparing PET/CT to PET/MRI in patients with HNSCC. Evangelista *et al.* evaluated contrast-enhanced CT (ceCT) with PET in HNSCC cases and found this to be particularly adept at diagnosing small lymph node metastases in positions usually difficult to interpret in the head

and neck region.[246] Lee *et al.* found PET/MRI to have acceptable accuracy in T staging compared to endoscopic ultrasound and even higher accuracy (although not statistically significant) than PET/CT in predicting N staging, demonstrating its value as preoperative diagnostic imaging tool in HNSCC.[245] PET/CT has been deemed useful in staging, identification of second primaries, and monitoring in the head and neck region aiding treatment planning.[216, 236, 244, 245, 247]

Hybrid technologies can be used to assess treatment response.[246, 248, 249] Adkins *et al.* found that PET/CT is better than CT in evaluating tumour response to cetuximab in patients with incurable HNSCC.[248] PET/CT has also exhibited high sensitivity to detecting distant metastases, assessing the response to chemotherapy or chemoradiation treatment and in predicting outcome.[246] PET/CT can be used post-therapeutically to assess treatment response, detect residual/recurrent tumours, and exclude distant metastases.[15] “Activatable molecular probes” or “smart” probes may be used to elicit a change in signal upon enzymatic activity or in response to specific biomolecular interactions.[20, 220] These allow very high signal-to-background ratios compared to conventional targeted contrast agents, and lead to the possibility of imaging intracellular targets.[220] Nguyen and Tsien summarise that the use of these fluorescent-labelled molecularly targeted probes would provide real-time, intraoperative distinction of the molecular edge between cancer and adjacent normal tissue, and could aid in discerning and preservation of vital structures such as nerves during surgery.[218] Bhatnagar *et al.* reviewed the use of different PET tracers in hybrid imaging modalities such as PET/CT and PET/MRI to detect and monitor biomarker status to assess effectiveness of therapy in patients with HNSCC.[250] They suggest that treatment strategies could be modulated and adapted by comparing an initial baseline measurement of biomarker expression before treatment to measurements taken during therapy, thus strategically addressing treatment response.[250] Some examples of biomarkers that may be used in this instance are tissue hypoxia, cell proliferation and apoptosis, and epidermal growth factor receptor (EGFR) status.[250] Examples of PET tracers that may be used in relation to biomarkers of response to therapy are described below.

4.2.3. Tumour hypoxia

Hypoxia has been established as an indicator of poor prognosis in HNSCC patients, causing radiation resistance in tumour cells by preventing irreversible damage to DNA by free radicals induced by ionising radiation (oxygen is required for the production of free radicals), thus allowing DNA repair and tumour cell survival.[251, 252] The critical partial pressure of oxygen below which solid tumours resist radiation therapy is about 10-15mm Hg.[252] In comparison, three times the amount of radiation needed to kill tumour cells in normoxic conditions is required to achieve the same in hypoxia. Hypoxia mapping can be performed with the use of molecular imaging to identify tumours that would benefit from hypoxia-reducing treatments.[250]

Fluoromisonidazole (FMISO) has been investigated widely as a PET imaging agent in HNSCC.[253-256] It has been shown that FMISO and FDG uptake do not necessarily correlate, thus representing different tumour properties, with high uptake of FMISO before

radiation therapy indicative of locoregional treatment failure and associated poor prognosis.[250, 255, 256] Nevertheless, FMISO may be used in HNSCCs to delineate hypoxic tumour volumes as an indicator to escalate radiation doses.[257-260] The key challenge though is that tumour hypoxia is a dynamic process with a constant change in relative contribution of acute and chronic hypoxia to the total hypoxic volume.[250] One study showed just 46% correlation between two sequential FMISO scans, just 3 days apart, in 20 HNSCC patients.[261] A smaller study with 7 HNSCC patients found correlation between hypoxic volumes on sequential scans in only three patients.[262] Further research is required to investigate the normal variation in FMISO uptake and changes in tumour oxygenation kinetics prior and during therapy, before FMISO imaging can clinically guide hypoxia-mediate intensity modulated radiation therapy (IMRT).[250, 259, 263]

Fluorine 18 fluoroazomycin arabinoside (FAZA) is also a hypoxia-specific PET agent that clears the blood more rapidly than FMISO, thus producing a higher target-to-background signal ratio.[264] Fluorine 18 fluoroerythronitroimidazole (FETNIM) is in theory a stronger indicator of hypoxia than FMISO due to its greater hydrophilia and better pharmacokinetics.[265] Both agents show promise as hypoxia radiotracers, but further research is needed, especially in comparison to FMISO.

Radioactive copper-labelled diacetyl-bis-(N4- methylthiosemicarbazone), or Cu-ATSM, is a neutral lipophilic compound that can permeate cell membranes.[250] In hypoxic conditions, Cu-ATSM molecules are reduced and negatively charged, while they wash out rapidly from normoxic cells, thus selectively accumulating in hypoxic cells resulting in a high SBR.[266] It has been shown that Cu-ATSM showed a significant difference in its uptake in HNSCC patients with residual or recurrent tumour compared to those without, which was not reflected in FDG uptake.[267] Others have shown that Cu-ATSM may be used to identify hypoxic subvolumes for IMRT.[268]

4.2.4. Tumour cell proliferation

While radiation therapy and chemotherapy can lead to a rapid decrease in the rate of cellular proliferation in responding tumours, which precedes a decrease in tumour size, accelerated tumour cell repopulation is an indicator of underlying radiation resistance and hence, treatment failure.[251, 269] Early identification of tumour cell repopulation as part of response assessment through imaging can identify target areas for dose escalation.[250] 3'-Fluoro-3' deoxythymidine (FLT)-PET is used widely to assess cellular proliferation, and unlike FDG, is only taken up by actively dividing cells and not surrounding inflammatory cells, allowing for specific detection of cellular division and subsequent dose escalation in these areas.[270, 271] Changes in the intensity of FLT uptake can be used to reflect cellular response to treatment, even prior to changes in tumour volume.[251, 271] However, FLT does not distinguish between benign and malignant abnormal cervical lymph nodes because its uptake by the germinal centres of reactive lymph nodes leads to a low positive predictive value.[272]

4.2.5. Apoptosis

Chemotherapy and radiation therapy rely on apoptosis to induce tumour cell death. Radiation resistance and treatment failure can result from mutations that lead to uncontrolled cellular

proliferation and dysregulation of apoptotic mechanisms.[273] Technetium 99m (^{99m}Tc)-labelled annexin V is a protein that binds to a major phospholipid constituent of cell membranes and has been investigated for imaging apoptosis in various malignancies including HNSCC.[274] The difficulty of radiolabeling annexin V with fluorine 18 has led to the development of other such tracers such as ^{18}F -ML-10 (2-[5-fluoro-pentyl]-2-methyl-malonic acid) (Aposense; Petach Tikva, Israel). This is a novel small-molecule probe designed to allow visualisation of apoptosis related cellular alterations, useful for differentiating between apoptotic and necrotic cells.[275]

4.2.6. Amino acid transport and protein synthesis

Carbon 11 (^{11}C) methionine is a PET tracer that has been investigated to assess amino acid transport and accelerated protein synthesis in malignant tissue.[276] ^{11}C -methionine allows for effective visualisation of HNSCC, demonstrating a good correlation with FDG demonstrating similar sensitivities and specificities for tumour detection, but does not distinguish between histological grade.[277, 278] It has been shown that there is a decline in ^{11}C -methionine uptake at tumour sites with histology-confirmed complete treatment response in HNSCC patients, in comparison with sites of residual tumour tissue after radiation therapy.[279] Early decrease of ^{11}C -methionine uptake correlates to final tumour volume reduction seen at MRI at the conclusion of treatment in HNSCC patients, suggesting that ^{11}C -methionine can be used for early treatment adaptation.[280] Conversely, Nuutinen *et al.* showed a substantial early decline in ^{11}C -methionine uptake in HNSCC patients after radiation therapy, but the rate of decrease in tracer uptake was comparable between patients with disease recurrence and those with preserved local control.[281] At present, there is no clear role for ^{11}C -methionine in the imaging of HNSCC.[250]

Fluorine 18 fluoroethyltyrosine (FET), an amino acid analogue that is taken up by tumour cells through amino acid transport systems, has shown high diagnostic accuracy in patients with brain tumours, but has lower sensitivity (64-75%) when compared to FDG (89-95%) in the evaluation of HNSCC, making it unsuitable to replace FDG in the initial assessment of HNSCC despite superior specificity (90-100%) than FDG (50-79%).[282-285] FET could still have a role in differentiating between residual tumour tissue and inflammatory tissue after therapy.[250]

4.2.7. Cell membrane synthesis

Choline is incorporated with phospholipids during cell membrane synthesis.[286] A preliminary study[287] using ^{11}C -choline in HNSCC patients found it to be just as effective as FDG for detecting malignant head and neck tumours with PET, however, another study[288] did not find ^{11}C -choline PET/CT to be superior to FDG PET/CT for the detection of recurrent HNSCC.

While this is useful, carcinogenesis and transformation of tissue in HNSCC involves elaborate modification of numerous biomarkers. It would be more useful to assess the overall imbalance in biomarker regulation rather than rely on one marker alone. This considerably complicates the process of molecular diagnosis and analysis through imaging and requires further research.

OFI techniques can be used intraoperatively, in conjunction with information from pre-operative MRI, CT or PET, providing a more holistic knowledge of macroscopic and molecular

level tissue alterations, enabling ideal surgical guidance.[289-291] This will ultimately improve patient outcome by decreasing MRD in surgical margins.

5. Conclusion

Methods for early detection, molecular assessment of margins and surgical guidance, and assessment of treatment response are instrumental to changing the rate of local recurrence and resultant reduced prognosis in patients with HNSCC by enabling personalised medicine. There are a number of biomarkers that alter expression as tissue transforms. These can be used to assess MRD in surgical margins. Furthermore, optical and molecular imaging techniques can be used to identify molecular changes in biomarker expression, enabling immediate intraoperative decisions on extent of lesion and margin status, reducing the need for repeat surgery and the risk of recurrence. Multimodal imaging will provide more information about diseased tissue, enabling the surgeon to visualise the tumour in terms of its molecular extent and not simply its visual extent (whether with white light or fluorescence). Further research into molecular biomarkers as potential targets for “smart” probes for assessment of MRD in surgical margins is required to enhance current molecular imaging modalities which have applications pre-operatively to delineate lesion location and volume, intra-operatively to assess surgical margins and for surgical guidance, and post-operatively to assess treatment response. Research is also required to assess projected improvements in overall recurrence rates following introduction of these technologies to reveal whether these technologies have improved outcomes in practice.

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