Emerging biomarkers in head and neck cancer in the era of genomics

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Abstract | Head and neck cancer (HNC) broadly includes carcinomas arising from the mucosal epithelia of the head and neck region as well as various cell types of salivary glands and the thyroid. As reflected by the multiple sites and histologies of HNC, the molecular characteristics and clinical outcomes of this disease vary widely. In this Review, we focus on established and emerging biomarkers that are most relevant to nasopharyngeal carcinoma and head and neck squamous-cell carcinoma (HNSCC), which includes primary sites in the oral cavity, oropharynx, hypopharynx and larynx. Applications and limitations of currently established biomarkers are discussed along with examples of successful biomarker development. For emerging biomarkers, preclinical or retrospective data are also described in the context of recently completed comprehensive molecular analyses of HNSCC, which provide a broad genetic landscape and molecular classification beyond histology and clinical characteristics. We will highlight the ongoing effort that will see a shift from prognostic to predictive biomarker development in HNC with the goal of delivering individualized cancer therapy.

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Introduction

Head and neck cancer (HNC) is a heterogeneous disease that can involve multiple sites and cellular origins within the head and neck region, such as the paranasal sinuses, nasal cavity, oral cavity, pharynx, larynx, salivary glands and the thyroid. The histology, molecular characteristics, treatment approaches and clinical outcomes vary widely across these cancers. For example, >90% of cancers arising from the oropharyngolaryngeal mucosa are squamous-cell carcinomas, whereas salivary gland cancers include a wide spectrum of histologies, such as adenoid cystic carcinoma, adenocarcinoma, mucoepidermoid carcinoma and salivary duct carcinoma.[1,2] Common thyroid cancer histologies are papillary, follicular, medullary and anaplastic carcinomas.[3] The squamous-cell carcinomas can be managed with primary surgical or nonsurgical approaches, but most salivary gland and thyroid cancers are treated with a surgical approach due to general resistance to chemotherapy and/or radiotherapy.[1–3] The scope of this Review is limited to discussion of the biomarkers pertinent to only carcinomas arising from the nasopharynx (nasopharyngeal carcinoma; NPC), oral cavity, oropharynx, hypopharynx and larynx (head and neck squamous-cell carcinoma; HNSCC).

Biomarkers are defined, according to the National Cancer Institute (NCI),[4] as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition." However, we have taken a broader definition and also included imaging features as biomarkers. Biomarkers are categorized as diagnostic, prognostic, or predictive, but we will focus on the last two categories. Prognostic biomarkers are associated with outcomes regardless of a given treatment, whereas predictive biomarkers are associated with outcomes to a specific treatment. These features are not mutually exclusive, meaning a biomarker can be both prognostic and predictive. Only a fraction of the numerous biomarkers in development will be translated into the clinic and applied to patient care as a routine test,[5,6] because a successful biomarker requires several components: robust clinical performance with high specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV), as well as a short turnaround time for results to be applicable in the clinical decision-making process. It is essential that biomarkers also provide useful information that is not routinely available in existing clinical practice, at a reasonable expense—considering the current state of health economics. We highlight these features in established biomarkers that are in clinical use as well as discussing emerging biomarkers that are in development.

Unmet clinical needs in HNC

Nasopharyngeal carcinoma

Although NPC is rare, a high incidence is observed among Asian individuals of southern Chinese descent (~25–30 cases per 100,000 individuals).[7] NPC pathogenesis is likely to be multifactorial involving viral, genetic and environmental factors. The association of Epstein–Barr virus (EBV) with nonkeratinizing NPC in

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The authors declare no competing interests.

Key points

- Epstein–Barr virus in nasopharyngeal carcinoma, human papillomavirus (HPV) in head and neck squamous-cell carcinoma (HNSCC), and PET-imaging features provide robust prognostic biomarkers that are actively being incorporated into clinical trials
- HPV-positive HNSCCs, most-commonly occurring in the oropharynx, have a better prognosis than HPV-negative tumours; HPV-positive status as a biomarker has facilitated efforts to de-intensify therapy in a subset of patients with a favourable prognosis
- Comprehensive genomic analyses of HNSCC show loss-of-function in tumour suppressor genes is more common compared with gain-of-function in oncogenes due to genetic aberrations or viral oncoproteins
- Although overall rates of DNA mutation and copy-number variation are low, HPV-positive tumours have a relatively higher rate of oncogene mutations than HPV-negative tumours
- For HNSCC, prognostic biomarkers are limited in their utility, and development of predictive biomarkers is desired
- Novel drug and biomarker developments have been focused on oncogenes, but more-common aberrations in tumour suppressors need to be further exploited

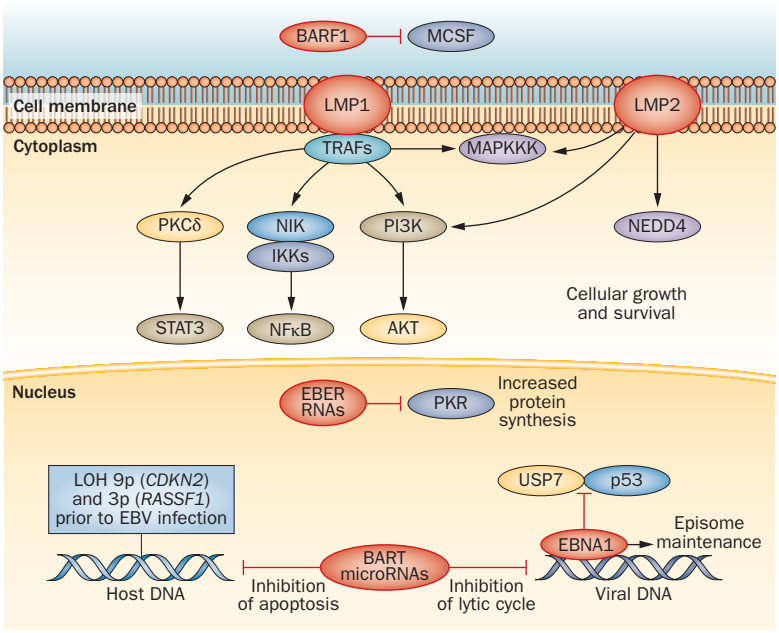

Figure 1 | Molecular pathogenesis of EBV-related NPC. EBV genome products are shown in red. In patients at high risk of NPC, LOH at 9p and 3p is found before EBV infection and might predispose to NPC.[7] After EBV infection, BART microRNAs inhibit transcription of genes related to apoptosis and viral lytic cycle, and untranslated EBERs block PKR, allowing for protein synthesis. EBNA1 binds to and maintains the viral episome and disrupts p53 stabilization caused by interaction of p53 with USP7. At the cell membrane, LMP1 and LMP2 activate many cellular growth and survival pathways including PI3K/AKT, MAPK, NFκB, STAT3 and the E3 ubiquitin-protein ligase NEDD4. LMP1 signalling is mediated primarily through TRAFs. An excreted EBV protein BARF1 might modulate the immune system by inhibiting MCSF.[10] Abbreviations: BARF1, BamH1 reading frame 1; BART, BamH1-A rightward transcript; EBERs, EBV-encoded RNAs; EBNA1, Epstein–Barr nuclear antigen 1; EBV, Epstein–Barr virus; IKKs, inhibitor of nuclear factor κB kinases; LMP1/2, latent membrane protein 1/2; LOH, loss of heterozygosity; MAPKKK, mitogen-activated protein kinase kinase kinase; MCSF, macrophage colony-stimulating factor 1; NFκB, nuclear factor κB; NIK, NFκB-inducing kinase; NPC, nasopharyngeal carcinoma; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; PKCδ, protein kinase Cδ; PKR, protein kinase RNA-dependent; STAT3, signal transducer and activator of transcription 3; TRAFs, tumour necrosis factor receptor-associated factors; USP7, ubiquitin-specific-processing protease 7.

Asian populations (former WHO types II–III) has been well established since the 1970s.[8] However, EBV is not clearly associated with keratinizing squamous-cell NPC observed in non-Asian patients (former WHO type I), and WHO type I is seen in <1% of Asian patients.[7,9] Latent EBV infection is found in NPC and high-grade dysplasia, but is not present in the normal nasopharyngeal epithelium.[9] The mechanisms of EBV entry, latency, and oncogenesis in epithelial cells remain uncertain; however, current data suggest that susceptible persons might harbour epithelial cells with genetic changes that expand and transform after EBV infection, and the carcinogenic role of viral proteins are becoming clearer (Figure 1).[10]

Most of patients with NPC present with locoregionally advanced disease, with more than 80% of patients harbouring cervical lymph-node metastases and <5% harbouring distant metastases. The definitive treatment for NPC is mostly nonsurgical owing to the anatomical location of the nasopharynx and frequent involvement of the base of skull and lateral retropharyngeal nodes. The standard of care is definitive radiotherapy for early stage disease and concurrent radiotherapy with cisplatin-based chemotherapy for locoregionally advanced disease. The development of biomarkers that would improve early detection of NPC in patients at high risk of the disease, prediction of NPC treatment response, and surveillance for recurrence in previously treated patients would be invaluable in the management of NPC.

HPV-related HNSCC

A subset of HNSCC is caused by human papillomavirus (HPV) and represents a biologically distinct entity.[11–13] Unlike cervical cancer, in which screening of abnormal cells by PAP smear for early detection is a routine clinical practice, screening is not possible in HNSCC because pre-malignant cells or lesions are not readily detectable and the presence of HPV DNA in the oral cavity or oropharynx is not an indication that patients will develop HNSCC.[14] The most-common sites of HPV-related HNSCC are the tonsils and base of tongue within the oropharynx, with a prevalence rate of approximately 75%; HPV-related HNSCC is rare in nonoropharyngeal sites (such as the oral cavity, larynx and hypopharynx).[15,16] Approximately 90% of HPV-associated oropharyngeal cancers can be attributed to high-risk HPV type 16 (HPV16).[17] Expression of the viral oncoproteins E6 and E7 results in the rapid degradation of two important tumour suppressor proteins, p53 and Rb,[18–20] and persistent expression of E6 and E7 is required for tumour maintenance (Figure 2).[21] Disruption of Rb function induces a compensatory increase in expression of $p16^{INK4A}$,[22] which has been used as a surrogate marker for HPV in oropharyngeal cancers.[15,23–26]

HPV-positive oropharyngeal cancer is associated with unique demographic characteristics, such as male gender, better performance status, and lower consumption of tobacco and/or alcohol compared with HPV-negative cancers.[15] The current standard of care for locally advanced oropharyngeal cancers is either

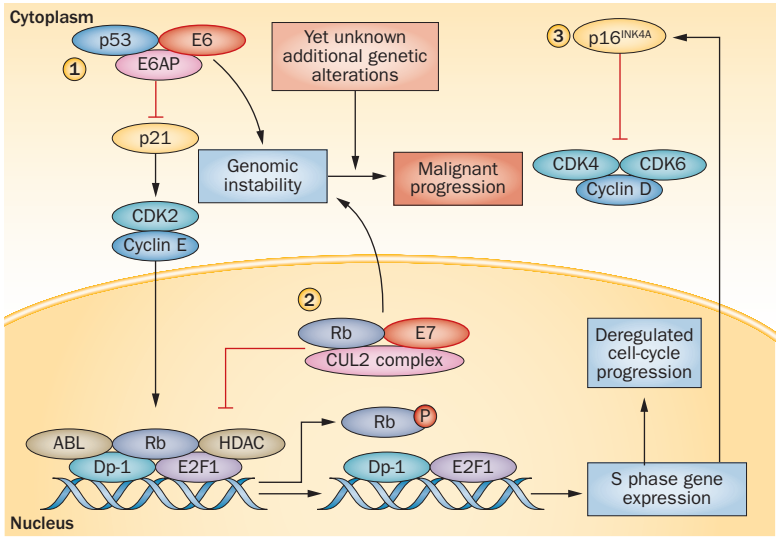

Figure 2 | Schematic diagram of molecular pathogenesis of HPV-related OPC. HPV can override cell-cycle checkpoints and result in genomic instability, and therefore promote malignant transformation. (1) Ubiquitination by viral E6 and ubiquitin ligase E6AP leads to p53 degradation.[19,182] (2) Ubiquitination by viral E7 and the CUL2 ubiquitin ligase complex leads to Rb degradation.[20,183–185] (3) Increased expression of $p16^{INK4A}$ as a consequence of increased S phase gene expression resulting from the absence of Rb function.[186] Abbreviations: CDK, cyclin-dependent kinase; CUL2, cullin-2; HDAC, histone deacetylase; HPV, human papillomavirus; OPC, oropharyngeal cancer; Rb, retinoblastoma protein.

surgery followed by adjuvant radiotherapy with or without concurrent chemotherapy, or definitive concurrent chemoradiotherapy.[27] As excellent outcomes are achieved in this population (Table 1), the rational next step is to de-intensify therapy to minimize treatment-related late toxicities for improved quality of life, without compromising survival.[28] One of the complications with de-intensification of therapy is that a small subset of HPV-positive patients have a less-favourable outcome, such as those with a smoking history. Tobacco smoking is a well-established independent risk factor for poor outcome in HNSCC, and strong evidence indicates that continued smoking during radiotherapy is associated with a worse outcome.[29,30] A recent secondary analysis of two large randomized trials showed that the risk of progression or death increased by 1% for each pack-year or 2% for each year that the patient has been a smoker.[31] This evidence points to a clear need for biomarkers to determine the risk of locoregional or distant disease failure in HPV-positive patients in order to avoid undertreatment during de-intensification of therapy. Given the distinctive biology of HPV-positive HNSCC, innovative approaches targeting viral oncogenes and/or the immune system, integrated with novel biomarkers, are also needed.

HNSCC unrelated to HPV

HPV-negative HNSCCs are typically associated with heavy use of tobacco and alcohol consumption.[32] Betel nut chewing is another independent risk factor for HNSCC of the oral cavity, especially in Southeast Asia.[33] Genetic factors have a role, as exemplified by the increased risk of HNSCC in patients with Fanconi anaemia.[34] Treatment for locally advanced HPV-negative disease is not any different from therapy for HPV-positive disease; however, patients with HPV-negative HNSCC have considerably worse outcomes compared with HPV-positive patients (Table 1).[15,35,36] Efforts to improve the outcome of disease in HPV-negative patients by intensifying treatment (through induction chemotherapy[37,38] or addition of the anti-EGFR antibody cetuximab[39] to concurrent chemoradiotherapy), have not resulted in a significant survival benefit. Identification of novel therapeutic targets and development of effective targeted agents, as well as predictive companion biomarkers, would greatly benefit this patient population.

Currently established biomarkers

Virus-related and virus-unrelated HNC differ substantially in epidemiology, molecular carcinogenesis, cancer genetics and clinical outcomes. The most-robust biomarkers in routine clinical use exploit these biological differences based on viral aetiology involving EBV for NPC and HPV for oropharyngeal cancers.

EBV as a prognostic biomarker in NPC

Circulating serum EBV DNA is an independent prognostic biomarker for patients with NPC. This assay uses real-time quantitative PCR (qPCR) to measure the concentration of naked DNA fragments, usually shorter than 180 base pairs, in cell-free plasma (Table 2). Pretreatment EBV DNA was found in 96% of patients with NPC in Hong Kong, and high levels of EBV DNA were associated with advanced-stage disease, distant relapse and worse survival.[40,41]

Elevated post-treatment EBV DNA is a robust negative prognostic factor in prospective studies of radiotherapy alone, concurrent chemoradiotherapy, and induction chemotherapy followed by radiotherapy with or without concurrent chemotherapy.[42–44] In a phase II trial of induction chemotherapy followed by concurrent chemoradiotherapy, post-treatment EBV DNA concentration was substantially elevated in eight of nine patients with persistent or recurrent disease versus none with tumour control.[44] In a study of 99 patients receiving induction chemotherapy followed by radiotherapy, EBV DNA at 1 week post-treatment was elevated in 10 patients, seven of whom later developed recurrence.[43] Serum EBV DNA might be elevated 3–7 months before clinical recurrence, demonstrating its potential as a biomarker of subclinical disease.[42,43] An upcoming phase II–III trial (NRG-HN001; NCT02135042)[45] will incorporate serum EBV DNA as a biomarker to guide individualized treatment for patients with NPC. Patients will undergo concurrent chemoradiotherapy with EBV DNA testing before and after treatment; those patients with a complete biomarker response will be randomly assigned to observation or adjuvant cisplatin and 5-fluorouracil (5-FU), and those with an incomplete response will be randomized to adjuvant cisplatin and 5-FU or gemcitabine and paclitaxel. Importantly, four international laboratories have undergone EBV DNA qPCR standardization and harmonization for this trial.[46]

Table 1 | Impact of HPV status on HNSCC outcomes in phase II–III trials

Study	Site	Detection method	Number of patients	PFS rate			OS rate		
				HPV^+	HPV^-	HR	HPV^+	HPV^-	HR
Fakhry *et al.* (2008)[35]	OP; L	DNA ISH	96	86% at 2 years	53% at 2 years	3.57 (1.33–9.09)	95% at 2 years	62% at 2 years	2.86 (1.25–6.67)
Ang *et al.* (2010)[15]	OP	DNA ISH	323	73.7% at 3 years	43.4% at 3 years	2.50 (1.75–3.45)	82.4% at 3 years	57.1% at 3 years	2.63 (1.82–3.85)
Rischin *et al.* (2010)[51]	OP	$p16^{INK4A}$ IHC	185	87% at 2 years	72% at 2 years	2.56 (1.35–5.00)	91% at 2 years	74% at 2 years	2.78 (1.35–5.88)
Posner *et al.* (2011)[36]	OP	DNA PCR for E6/E7	111	78% at 5 years	28% at 5 years	NA	82% at 5 years	35% at 5 years	5.00 (2.63–10.00)
Lassen *et al.* (2011)[52]	OP; OC; L; P	$p16^{INK4A}$ IHC	794	68% at 5 years	57% at 5 years	1.52 (1.14–2.04)	62% at 5 years	47% at 5 years	1.61 (1.28–2.04)

Abbreviations: HNSCC, head and neak squamous-cell carcinoma; HPV, human papillomavirus; HR, hazard ratio; ISH, *in situ* hybridization; IHC, immunohistochemistry; L, larynx; NA, not available; OC, oral cavity; OP, oropharynx; OS, overall survival; P, pharynx; PFS, progression-free survival.

Serum EBV antibody titres have also been investigated as potential biomarkers for NPC, but they are less sensitive than serum EBV DNA—although with comparable specificity (Table 2).[47] IgA anti-virus-capsid-antigen antibodies and IgG anti-early-antigen antibodies were detected in >80% of patients with nonkeratinizing NPC, and persistently elevated titres 1 year after treatment were significantly associated with risk of relapse (P <0.01).[48,49] These antibodies have been used for screening patients at high risk of NPC in southern China, with subsequent annual mirror examination identifying early NPC in 41 of 2,823 seropositive patients.[50] These antibodies have also been used for surveillance after treatment for NPC, with increased titres 1 year after radiotherapy demonstrated to be predictive of relapse.[49,50]

HPV as a prognostic biomarker in HNSCC

The presence of HPV is an established prognostic biomarker of favourable outcome in locally advanced oropharyngeal cancers and numerous studies have reported a considerably reduced risk of death (reduced by 40–80%) or relapse (reduced by 60–70%) in HPV-positive patients compared with HPV-negative patients, after adjusting for known clinical prognostic factors, when treated with standard multimodality treatments (Table 1).[15,35,36,51,52] A study by Fakhry *et al.*[53] showed that patients with $p16^{INK4A}$-positive oropharyngeal cancers also had significantly longer overall survival durations, even after development of recurrent and/or metastatic disease, than patients with $p16^{INK4A}$-negative oropharyngeal cancer (2-year overall survival 54.6% versus 27.6%; median overall survival 2.6 years versus 0.8 years; P <0.001). The prognostic value of $p16^{INK4A}$ status in multivariable analysis was independent of tumour stage at enrolment, progression type (distant versus locoregional), salvage surgery (yes versus no) or smoking history (pack-years >20 versus <20).[53] The independent $p16^{INK4A}$ prognostic value seemed greater in patients with locoregional progression compared with distant metastasis, or in patients who underwent salvage surgery compared with those not treated with salvage surgery, although the interaction did not reach statistical significance in both comparisons.[53]

The detection methods that should be used to determine HPV status remain the subject of debate. Current methods to detect the presence of HPV include HPV E6/E7-mRNA quantitative reverse transcription-PCR (qRT-PCR), HPV DNA *in situ* hybridization (ISH), and HPV-DNA PCR for E6/E7 viral oncogenes. In addition, $p16^{INK4A}$ immunohistochemistry is used widely as a surrogate marker of HPV status.[15,23–25] Although detection of HPV E6/E7-mRNA by qRT-PCR is considered to be the gold-standard methodology, HPV-DNA ISH and $p16^{INK4A}$ immunohistochemistry are the most widely used assays in the clinical setting because of their practicality and high sensitivity and specificity—at least for the oropharyngeal tumour subtype of HNSCC that is commonly attributable to HPV (Table 3).

The overexpression of $p16^{INK4A}$ in oropharyngeal cancers —which are commonly HPV-related HNSCCs—is an outstanding surrogate for HPV positivity. Expression of $p16^{INK4A}$ is upregulated in HPV-positive cancer and frequently lost in HPV-negative tumours.[15,23,24,51] Encoded by *CDKN2A*, $p16^{INK4A}$ is a tumour suppressor that regulates the cell cycle by inhibiting phosphorylation of CDK4 and CDK6, thus impeding Rb phosphorylation (Figure 2). Nonphosphorylated Rb binds to and inactivates the transcription factor E2F1, an essential component of cell-cycle progression.[54] In the normal mucosal epithelium, $p16^{INK4A}$ is usually undetectable or expressed in only 5–10% of the cells with a patchy staining pattern because cells in the epithelia do not generally undergo the cell cycle synchronously.[55,56] However, cells in HPV-positive tumours undergo the cell cycle without control due to loss of Rb caused by the HPV oncoprotein E7, and therefore $p16^{INK4A}$ is expressed in a diffuse pattern in the majority of the tumour tissue—often greater than 50–70% of the tumour.[57] Furthermore, HPV-positive tumours rarely have loss of $p16^{INK4A}$ function, whereas up to 90% of HPV-negative tumours have loss of $p16^{INK4A}$ as a result of *CDKN2A* mutation, promoter methylation, or deletion of the gene or chromosome 9p21 where *CDKN2A* is located.[58,59]

Overexpression of $p16^{INK4A}$ outside of the oropharynx, in locations at which HPV-related HNSCC is rare and the carcinogenic role of this virus is unclear, should be

Table 2 | Comparison of EBV-detection methods in NPC*

Study	Number of samples	Serum EBV-DNA PCR‡			Serum EBV serology§		
		Sensitivity (%)	Specificity (%)	Cutoff (copies per ml)‖	Sensitivity (%)	Specificity (%)	Cutoff titre
Lynn *et al.* (1985)[187]	271	NA	NA	NA	81	98	>1:40
Lo *et al.* (1999)[40]	100	96	93	5	NA	NA	NA
Chan *et al.* (2002)[42]	170	91	NA	0	NA	NA	NA
Lin *et al.* (2004)[43]	159	95	100	0	NA	NA	NA
Leung *et al.* (2004)[47]	317	95	98	60	81	96	>1:10

*Results are shown for pretreatment samples only compared with the gold standard of tumour histology confirmation of NPC. ‡PCR probe is for BamHI-W region of viral genome. §Serology for IgA anti-viral-capsid-antigen antibodies. ‖These low cutoff values are for EBV DNA as a diagnostic biomarker for NPC. When using EBV DNA as a prognostic biomarker, the authors recommend higher cutoff values to distinguish good versus poor prognosis (1,500–4,000 copies per ml for pretreatment; 0–500 copies per ml for post-treatment). Abbreviations: EBV, Epstein–Barr virus; NA, not applicable; NPC, nasopharyngeal carcinoma.

interpreted with caution. In one study, $p16^{INK4A}$ expression and high-risk HPV status were determined by immunohistochemistry and ISH, respectively, in nonoropharyngeal cancers (of the oral cavity, hypopharynx and larynx) from patients enrolled in three clinical trials. The concordance between $p16^{INK4A}$ expression and high-risk HPV status was modest ($n = 273$; phi coefficient 0.46, 95% CI 0.34–0.58).[16] In addition, the concordance varied depending on the anatomic sites, with greater concordant when the primary site was hypopharynx (phi coefficient 0.54, 95% CI 0.28–0.81) or larynx (phi coefficient 0.52, 95% CI 0.35–0.68) compared with the oral cavity (phi coefficient 0.35, 95% CI 0.13–0.57). Another study also confirmed the lack of concordance between $p16^{INK4A}$ expression and HPV status in oral cavity squamous-cell carcinoma, only 5.6% of which were HPV-positive.[26] Because of its low prevalence, the PPV of $p16^{INK4A}$ expression for HPV status was only 41.3% even though sensitivity and specificity of the test itself were excellent (79% and 93%, respectively).[26] An algorithm using $p16^{INK4A}$ as a screening assay and HPV ISH as a confirmatory assay has been proposed, but the optimal approach to HPV testing remains to be determined.[60]

Efforts to improve HPV detection are, therefore, ongoing, especially considering the development of treatments specific to HPV-positive patients and HPV-targeted agents. Even though detection of E6/E7-mRNA expression is generally regarded as the gold-standard for HPV detection, it requires complex tissue processing, which limits its routine use. E6/E7-mRNA ISH enables direct visualization of viral transcripts in routinely processed formalin-fixed paraffin-embedded tissues.[61] Although HPV-DNA ISH is highly specific but less sensitive (Table 3), mRNA ISH demonstrated 97% sensitivity and 94% specificity compared with qRT-PCR for confirmation of HPV status.[62] In addition, some of the commercially available ISH assays use cocktail probes detecting multiple high-risk HPV types to improve sensitivity and specificity, and report the result as "high-risk HPV-positive" rather than specifying a HPV type. The inability to identify a specific HPV type could limit our ability to define a population that might benefit from HPV-type-specific therapies, such as the HPV16-specific vaccine. Ongoing effort to develop HPV-targeting therapies necessitates improved HPV detection assays.

PET features as prognostic biomarkers in HNC

Functional imaging with ^{18}F-fluorodeoxyglucose PET (FDG-PET) improves the accuracy of HNC disease staging and treatment-response assessment in comparison with CT or MRI alone. Among the PET-derived quantitative parameters, the most reliable and widely studied HNC biomarker is the maximum standardized uptake value (SUV_{max}).[63] For pretreatment PET–CT, two meta-analyses demonstrated that high SUV_{max} of the primary tumour is associated with increased risk of progression, recurrence and death;[64,65] however, the proposed cutoff values for high-risk pretreatment SUV_{max} vary widely among studies due to a lack of standardization. Post-treatment FDG-PET is now commonly used for HNC-response assessment after definitive radiotherapy or chemoradiotherapy.[66,67] In retrospective studies of locoregionally advanced HNC, post-treatment FDG-PET has a remarkably high NPV of 95–100% for detection of residual neck disease (Table 4).[68–70] In patients with initially bulky nodal disease, this modality has been used to reduce the number of post-treatment neck dissections by up to 85%; however, the PPV in these studies was only 38–77%, as inflammation related to radiation can also cause FDG uptake.[68,70–72] These data seem to hold true for HPV-related oropharyngeal cancers, as studies show NPVs of 91–100% and PPVs of 11–84% in this population.[73–75] The PPV could potentially be improved with proper timing and more-consistent interpretation criteria; the rate of false positivity declines with time after treatment cessation, and studies have suggested an optimal time interval of 12 weeks.[67] However, even with optimal timing, absolute SUV_{max} cutoffs have not reliably distinguished residual cancer from inflammation.[68,76] A decrease in SUV_{max} of >50% compared with the pretreatment value has been associated with higher rates of complete response, locoregional control, and overall survival.[63] Various other interpretation criteria have also been suggested, including a combination of lymph-node size and intensity, grading of focal intensity, and/or comparison with background activity.[67,76,77]

Mid-treatment PET has also shown potential for early HNC response assessment, and this approach could be used during induction chemotherapy or during chemoradiotherapy to identify patients who would benefit

Table 3 | Comparison of HPV-detection methods in OSCC*

Study	Number of samples	HPV-DNA PCR (%)		ISH/FISH (%)		$p16^{INK4A}$ IHC (%)		$p16^{INK4A}$ IHC interpretation		
		Sens.	Spec.	Sens.	Spec.	Sens.	Spec.	Intensity	%	Pattern
Smeets *et al.* (2007)[188]	19	100	92	83	100	100	70	≥1+	>10	N or C
Shi *et al.* (2009)[189]	111	NA	NA	84	92	89	81	Strong	N/A	N and C
Schache *et al.* (2011)[190]	95	97	87	88	88	94	82	Strong	>70	N and C
Schlecht *et al.* (2011)[191]	21	NA	NA	38	100	90	100	≥2+	≥75	N and C
Rotnaglova *et al.* (2011)[192]	109	100	89	NA	NA	94	96	≥1+	>50	N or C
Jordan *et al.* (2012)[25]	235	99	63	88	95	97 92	84 90	≥2+ H score ≥60‡	>70	N and C

*Sensitivities and specificities are based on the gold standard of E6 mRNA qRT-PCR. ‡H score is derived from the cross-product of the intensity score (0–3) and from the percentage of tumour staining at the highest intensity (0–100%). Abbreviations: C, cytoplasmic; FISH, fluorescence *in situ* hybridization; HPV, human papillomavirus; IHC, immunohistochemistry; N, nuclear; NA, not applicable; OSCC, oropharyngeal squamous cell carcinoma; qRT-PCR, quantitative reverse transcription PCR; Sens., sensitivity; Spec., specificity.

Table 4 | Comparison of post-treatment PET methods for HNC nodal metastases*

Study	Technique	Number of patients	Timing	Criteria for positivity	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Wong *et al.* (2002)[193]	PET	143	7 months post-treatment	Not specified	92	95	77	99
Yao *et al.* (2005)[69]	PET	53	3 months post-treatment	SUV_{max} ≥2.9	100	94	43	100
Ong *et al.* (2008)[68]	PET–CT	65	3 months post-treatment	Focal FDG uptake	71	89	38	97
Yao *et al.* (2009)[76]	PET or PET–CT	188	3 months post-treatment	Not specified	86	97	71	99
Gupta *et al.* (2011)[66]	PET or PET–CT	2,335	Post-treatment	NA (meta-analysis)	73	88	52	95

*The gold standard method for comparison was histology or 6 months of clinical follow-up. Abbreviations: FDG, ^{18}F-fluourodeoxyglucose; HNC, head and neck cancer; NA, not applicable; NPV, negative predictive value; PPV, positive predictive value; SUV_{max}, maximum standard update value.

from treatment intensification or de-intensification. Brun *et al.*[78] showed that median SUV after one cycle of chemotherapy or 24 Gy of radiation was associated with locoregional control in 47 patients. A newer radiotracer ^{18}F-fluorothymidine (FLT), a marker of proliferation and thymidine kinase-1 activity, is particularly promising in detecting early HNC response to radiation in preliminary studies.[79] Other novel PET tracers are being studied for HNC imaging of EGFR (with ^{65}Cu-DOTA-cetuximab or anilinoquinazolines) and hypoxia (with ^{18}F-fluoromisonidazole [^{18}F-FMISO]).[67]

Emerging biomarkers

Although EBV, HPV, and PET imaging features are robust prognostic biomarkers, current prognostic biomarkers clearly do not serve as predictive biomarkers and, at this time, cannot provide information regarding how patients should be treated differently. For the use of biomarkers to make a marked impact on care, predictive biomarkers are being developed to enable selection of patients for a specific therapy in HNC, as seen in the treatment of cancers at numerous other sites (Table 5). This goal can only be achieved by a comprehensive understanding of the biology, and genetic and epigenetic alterations of HNC.

To date, genomic data from three large genome-wide sequencing studies and a cancer-gene-targeted sequencing study of HNSCCs are available.[12,13,59,80,81] The results from sequencing data have been disappointing from a therapeutic target standpoint because of the presence of more-frequent tumour suppressor gene (TSG) mutations than oncogene mutations (Table 6). In general, TSGs are poor therapeutic targets because restoring loss-of-function in these genes is more difficult than inhibiting increased activity resulting from gain-of-function in oncogenes. Also, mutations in TSGs are more difficult to detect due to lack of hotspot mutations, as seen in oncogenes.[82] Of the 15 most-frequent mutations in HNC (Table 6), only two genes are known oncogenes, *PIK3CA* and *HRAS*, and only *PIK3CA* is currently considered to be therapeutically targetable. As expected, the most-striking difference is again seen in the HPV-positive versus HPV-negative tumours, consistent with the clinical differences described earlier (Table 1); HPV-positive tumours have a lower average number of mutations per tumour, and rarely have *TP53* mutation and loss of $p16^{INK4A}$ function compared with HPV-negative tumours, reflecting the biological differences in these tumours.[12,13,59,80] In addition to whole-exome sequencing, The Cancer Genome Atlas (TCGA),[83] which is an effort driven by the NCI and the National Human Genome Research Institute to accelerate understanding of the molecular characteristics of cancer via the application of genome-analysis technologies, has provided rich data for biomarker development through comprehensive analysis of copy-number variations in HNSCC DNA, RNA sequencing, microRNA sequencing, and reverse phase protein arrays.

HPV as a predictive biomarker of EGFR response

Genomic studies suggest *EGFR* alteration is more common in HPV-negative tumours compared with HPV-positive tumours.[81,84] The levels of total and phosphorylated EGFR protein expression are also higher in

Table 5 | Predictive biomarkers in various cancers and HPV as a prognostic biomarker in OSCC

Study	Disease	Marker	Treatment	HR	95% CI
Heinrich *et al.* (2003)[194]	GIST	*KIT* mutation	Imatinib	25	8.33–100.00
Kantarjian *et al.* (2004)[195]	CML	Philadelphia chromosome (*BCR–ABL* translocation)	Imatinib	5.88	4.37–7.39
Rosell *et al.* (2009)[196]	Lung cancer	*EGFR* mutation (L858R)	Erlotinib	2.98	1.48–6.04
Shaw *et al.* (2011)[197]	Lung cancer	*EML4–ALK* translocation	Crizotinib	2.78	1.33–5.88
Chapman *et al.* (2011)[198]	Melanoma	*BRAF* mutation (V600E)	Vemurafenib	2.70	1.82–3.85
Ang *et al.* (2010)[15]	OSCC	HPV	NA	2.38	1.51–3.74
Karapetis *et al.* (2008)[199]	Colon cancer	*KRAS* wild type	Cetuximab	1.81	1.35–2.44
Coiffier *et al.* (2002)[200]	DLBCL	CD20	Rituximab	1.81	1.30–2.70
Schulz *et al.* (2007)[201]	Follicular lymphoma	CD20	Rituximab	1.59	1.27–1.96
Bang *et al.* (2010)[202]	Gastric cancer	HER2 overexpression	Trastuzumab	1.54	1.20–1.96
Slamon *et al.* (2001)[203]	Breast cancer	HER2 overexpression	Trastuzumab	1.25	1.00–1.56

Abbreviations: CML; chronic myeloid leukaemia; DLBCL, diffuse large-B-cell lymphoma; GIST, gastrointestinal stromal tumour; HPV, human papillomavirus; HR, hazard ratio; NA, not applicable; OSCC, oropharyngeal squamous-cell carcinoma; Philadelphia chromosome, chromosome 9 and 22 reciprocal translocation.

HPV-negative tumours versus HPV-positive tumours.[85] These findings suggests that the development of EGFR inhibitors could be more relevant in treating HPV-negative tumours. Although the role of HPV as a prognostic biomarker is firmly established, its role as a predictive biomarker of improved overall survival is controversial. A recent study suggested that $p16^{INK4A}$-negative patients might have a greater overall survival benefit from the anti-EGFR antibody panitumumab, when given with cisplatin and 5-FU (CF), compared with $p16^{INK4A}$-positive patients. For $p16^{INK4A}$-positive patients, the median overall survival was 11.0 months with panitumumab plus CF and 12.6 months with CF alone (HR 1.0 [0.62–1.61], $P = 0.998$), suggesting the addition of panitumuab did not provide any benefit.[86] For $p16^{INK4A}$-negative patients, the median overall survival was 11.7 months with panitumumab plus CF and 8.6 months with CF alone (HR 0.73 [0.58–0.93], $P = 0.0115$), demonstrating the benefit of adding panitumumab to the standard chemotherapy.[86] Another study evaluated tumours from patients who were treated with CF with/without cetuximab, but an interaction between $p16^{INK4A}$ status and cetuximab benefit was not observed.[87] However, both studies were retrospective, unplanned subset analyses with a small sample size of $p16^{INK4A}$-positive patients in each treatment arm.[86,87] In addition, the cutoff points of $p16^{INK4A}$ immunohistochemistry regarded as positive staining differed between the two studies (>10% in the panitumumab study versus >70% in the cetuximab study). Moreover, the use of $p16^{INK4A}$ immunohistochemistry as a surrogate marker of HPV status in non-oropharynx sites remains controversial. To clearly delineate the predictive value of HPV and/or $p16^{INK4A}$ in response to anti-EGFR inhibitors, a larger, prospective study is required.

Targeting oncogene alterations in HNSCC

The most-successful implementation of biomarkers in recent years has been based on functionally activating gene mutations (such as c-KIT activating mutations in gastrointestinal stromal tumour) and copy-number gain in oncogenes (such as *ERBB2*/*HER2* amplification in breast cancer; Table 5). On the basis of current genome-wide sequencing and copy number data, only a few oncogenes in HNC are immediately targetable with agents in clinical development, namely *EGFR, FGFR, MET, CCND1* and *PIK3CA,* and each gene alteration and their therapeutic potential are briefly discussed in the following sections.

EGFR

EGFR is a member of the HER family of cell-surface receptor tyrosine kinases. Ligand binding triggers homodimerization or heterodimerization of EGFR with members of the HER family and activates downstream effectors, thus promoting cell proliferation.[88] EGFR is commonly overexpressed in HNSCC and is associated with poor prognosis,[89–92] but can also be associated with clinical benefit of radiation treatment in HNSCC.[93] EGFR expression determined by immunohistochemistry has not been adopted widely as a biomarker due to wide variations in the sensitivity and specificity of anti-EGFR antibodies, staining protocols, and quality controls for the experiments. In addition, evaluation of *EGFR* alteration as a predictive biomarker has not been associated with response to EGFR-targeted therapies.[94] TCGA data have detected only 14% of HNSCC with *EGFR* alteration (37/243 [15%] of HPV-negative HNSCCs and 3/36 [8%] of HPV-positive HNSCCs),[80,84] suggesting that some of the previously published studies might have overestimated the presence of *EGFR* mutations and copy-number gain by gene amplification or 7p11 chromosomal gain.[59,95,96] Lack of correlation between *EGFR* alteration and EGFR inhibitor sensitivity might be, at least in part, due to inaccurate assays as well as the nature of increased gene copy number being a less predictive biomarker compared with activating mutations (Table 5). Enriching the treated population with a higher frequency of *EGFR* alteration might improve assay performance.

Table 6 | Comparison of commonly mutated genes in HNC sequencing studies

Mutated gene	Cancer gene class[82]	Percentage of tumours with gene mutation by study		
		Agrawal *et al.* (2011)[13] (*n* = 120)	Stransky *et al.* (2011)[12] (*n* = 74)	TCGA[59,80,84] (*n* = 279)
TP53	TSG	47	62	72
NOTCH1	TSG	15	14	19
CDKN2A	TSG	9	12	22
PIK3CA	Oncogene	6	8	21
FBXW7	TSG	5	NA	5
HRAS	Oncogene	4	5	4
SYNE1	NA	NA	20	18
FAT1	NA	NA	12	23
KMT2D (MLL2)	TSG	NA	11	18
CASP8	TSG	NA	8	9
PTEN	TSG	NA	7	2
NSD1	NA	NA	NA	10
KMT2C (MLL3)	TSG	NA	NA	8
EP300	TSG	NA	NA	7
AJUBA	NA	NA	NA	6

Abbreviations: HNC, head and neck cancer; NA, not applicable; TCGA, The Cancer Genome Atlas; TSG, tumour suppressor gene.

FGFR

FGFRs belong to a family of transmembrane tyrosine kinase receptors with four members: FGFR1–4.[97] A related receptor is also recognized, FGFR5 or FGFRL1, which does not have a tyrosine kinase domain.[98] These receptors are differentially activated by the 18 FGFs. Binding of these ligands to their receptors results in FGFR dimerization and activation of FGFR substrate 2 (FRS2), which triggers further downstream signalling that includes MAPK, PI3K, p38 MAPK, JNK, STAT and RSL2 pathways in a context-dependent manner.[97] The FGFRs have diverse functions, such as cellular differentiation, proliferation, migration, survival, angiogenesis and wound repair. Activating mutations, amplification and translocation resulting in fusion genes involving these receptors, as well as amplification of their ligands, have been reported in many cancers.[97] *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4* are amplified or mutated in 10%, 2%, 2% and 0.4% of HPV-negative HNSCCs, respectively.[84] In HPV-positive HNSCC, alterations in *FGFR1* and *FGFR2* are not seen, but 11% have *FGFR3* mutations or fusions, and 3% have *FGFR4* mutation.[84]

In a study in lung squamous-cell carcinoma, which shares important molecular characteristics with HPV-negative HNSCC,[99] *FGFR1*-amplified tumours were sensitive to a FGFR inhibitor only if they coexpressed MYC. This finding suggests that patients with *FGFR1*-amplified and MYC-overexpressing tumours could benefit the most from FGFR inhibitors.[100] In addition, evaluation of nine HNSCC cell lines showed FGF2 was widely expressed among other ligands, and FGFR2 and FGFR3 were also generally coexpressed in the majority of the cell lines, whereas only one cell line had a very high expression levels of FGFR1.[101] One preclinical study in HNSCC reported that inhibition of FGFR1 suppresses cell growth and invasion,[102] and another study reported no effect on proliferation in the tumour, but inhibition of fibroblast and endothelial cell proliferation in the stroma.[103] These studies were conducted in cell lines without characterization of the mutation or copy number status of *FGFR* and *FGF* gene family; thus, further studies are warranted.

MET

Hepatocyte growth factor receptor (MET; also known as c-Met) is a receptor tyrosine kinase associated with enhanced migration, invasion, and angiogenesis when overexpressed in cancer.[104] The prevalence of aberrations in MET and its ligand HGF, in terms of gene amplification and mutation, is relatively low, reported in 2–13% and 6% of HNSCC, respectively.[105] However, MET and/or HGF are overexpressed in ~80% of HNSCCs when assessed by immunohistochemistry.[106] In addition, although the *MET* activating mutation was seen in only 1% of the TCGA HNSCC data set,[84] it was detected in 14% of patients treated in a chemoradiotherapy trial for locally advanced HNSCC and was associated with decreased metastasis-free survival duration.[107] In addition, acquired *MET* amplification or upregulation is a well-established mechanism of resistance to EGFR inhibitors by compensating for the EGFR signalling inhibition at both the PI3K and MAPK nodes.[105,108] Although considerable evidence implicates the MET–HGF axis as a therapeutic target in HNSCC, appropriate assays to detect oncogenic addiction and subsequent patient selection using a biomarker are lacking and further investigation is warranted.

Cyclin D1

Cyclin D1 is a cell-cycle protein that regulates the key G1-to-S phase transition through formation of complexes with cyclin dependent kinases (CDKs), such as CDK4 and CDK6 (Figure 2). The cyclin D1–CDK4/6 complex phosphorylates Rb, which releases the break on cell-cycle progression and promotes expression of S-phase proteins. Cyclin D1 also has CDK independent function through interaction with the CCAAT-enhancer-binding protein α, with subsequent DNA binding resulting in cell proliferation.[109] In a TCGA study, 28% of HNSCCs had *CCND1* (the gene encoding cyclin D1) amplification (77/243 [32%] in HPV-negative and 2/36 [6%] of HPV-positive patient samples).[84] Overexpression of cyclin D1 and amplification of *CCND1* in HNSCC are associated with poor prognosis, cisplatin resistance and EGFR-inhibitor resistance.[59,111,112] Although direct targeting of cyclin D1 is not feasible currently, inhibition of its binding partners, CDK4 and/or CDK6, might have a role in patients with *CCND1* amplification.

PIK3CA

The only oncogene with frequent activating mutations and existing targeted agents is *PIK3CA* (Table 6). *PIK3CA* encodes p110α, a catalytic subunit of phosphoinositide 3-kinase (PI3K), which is a heterodimeric kinase with enzymatic activity on lipid and protein substrates;[113–115] however, only the lipid kinase activity is required for oncogenic

signalling.[116] Activated PI3K catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which activates PDK1 and AKT, triggering subsequent downstream effects on transcription, protein synthesis, metabolism, proliferation and apoptosis.[117,118] Data from the TCGA study[84] showed *PIK3CA* amplification and/or mutation in 37% of HNSCC (83/243 [34%] of HPV-negative and 20/36 [56%] of HPV-positive patients samples). When further evaluation of the mitogenic pathways relevant to HNSCC (the MAPK, JAK/STAT and PI3K pathways) were conducted in 151 tumours, the PI3K pathway was the most-frequently mutated pathway (30.5%).[119] When patient-derived HNSCC tumour xenografts were treated with a mTOR/PI3K inhibitor, tumours with *PIK3CA* mutations were shown to be more sensitive to the drug compared with those lacking *PIK3CA* mutations.[119]

Current data suggest there are differences in the *PIK3CA* mutation hotspots between HPV-positive and HPV-negative tumours. HPV-positive tumours have mutations in the helical domain, whereas HPV-negative tumours have mutations throughout the entire gene, although mutations in the helical (E542K and E545K) and kinase (H1047R) domains are more frequent.[84] The location of the mutations and resulting amino acid substitutions might cause functionally different mutant proteins, clinical outcomes, and response to PI3K-targeted agents that will have an important impact on the development of predictive biomarkers.[120,121] In addition, when the protein profiles of HPV-positive and HPV-negative HNSCC were compared, five pathways that regulate cell-cycle, DNA-repair, apoptosis, receptor-kinase signalling and the PI3K/AKT/mTOR pathway were shown to be differentially involved.[85] Interestingly, the downstream signalling between mutant and wild-type *PIK3CA* within oropharyngeal cancers differed according to activating mutations in genes encoding mTOR/S6 rather than AKT.[85] This finding indicates that PI3K/mTOR inhibitors might be preferable to AKT inhibitors for the treatments of patients with HPV-positive *PIK3CA*-mutant cancers. Currently, numerous clinical trials are evaluating PI3K inhibitors targeting the kinase domain in HNSCC, and *PIK3CA* mutations need to be further characterized and developed as a predictive biomarker in order to be integrated into the clinical trials.

Tumour suppressor alterations in HNSCC

Although therapeutic targeting of mutated TSGs is more difficult than targeting oncogene mutations, emerging data are shedding light on ways to exploit these genetic alterations.

TP53

The p53 protein is a short-lived transcription factor and tumour suppressor that has a vital role in the regulation of genes responsible for cell-cycle arrest, senescence and apoptosis.[122] *TP53* is the most commonly mutated gene in HNSCC, with an incidence of 47–86%;[12,13] furthermore, loss of p53 function is estimated to occur in more than 90% of HNSCC through loss of heterozygosity, interaction with HPV viral oncoprotein E6 or increased expression of MDM2 (which promotes the rapid degradation of the p53 protein and is amplified in approximately 5% of HNSCC).[12,13,22,123] The majority of *TP53* mutations are missense mutations, causing single amino-acid substitutions that lead to loss of DNA-binding capability.[124] Certain missense mutations can elicit a gain of function by modulating protein–protein interactions among components of the transcriptional regulation machinery.[125] Nonsense mutations create a stop codon resulting in a truncated protein. The p53 protein functions in a dominant-negative manner—that is, co-translation of the mutant and wild-type p53 tetramers cannot achieve a functional protein conformation, resulting in an inactive protein.[126,127]

The value of loss of p53 function as a prognostic marker has been debated; however, many of the early studies have been confounded by poor mutation-detection assays, small sample sizes with poor clinical characterization and/or indiscriminate analysis of functional and nonfunctional mutations.[128] Poeta *et al.*[129] reported a prospective study of 560 surgically treated patients with HNSCC, with 53.3% of patients reported to have *TP53* mutations. Any *TP53* mutation was associated with a worse overall survival compared with patients with wild-type *TP53* (HR 1.4, $P = 0.009$), and the association was much stronger with a functionally disruptive *TP53* mutation (HR 1.7, $P < 0.001$). This finding was confirmed in an independent cohort of 141 patients, which reported a *TP53* mutation rate of 62.4%.[130] In subgroup analyses, only nonsense mutations that produced a truncated protein were considered significant prognostic factors (HR 2.54, $P = 0.008$).[130]

TP53 mutation is relatively rare in HPV-positive HNSCC.[12,13,81] Nevertheless, it has been suggested that a *TP53* mutation correlates with a poorer prognosis, even among HPV-positive patients. In a retrospective review of 90 patients who had surgical treatment for oropharyngeal cancers, patients who were positive or negative for HPV with mutated *TP53* or HPV-negative patients with wild-type *TP53* had a poorer prognosis compared with HPV-positive patients with wild-type *TP53*;[131] no statistically significant difference in survival was observed between patients with HPV-positive or HPV-negative *TP53*-mutant tumours and patients with HPV-negative *TP53* wild-type tumours. Therefore, HPV-positive patients with functionally disruptive *TP53* mutations probably have a shorter time to disease progression and overall survival compared with patients with wild-type *TP53*, but this hypothesis needs to be formally addressed in a larger, prospective study.

When a recent high-throughput RNA interference functional genomic screen of the human kinome was performed in HNSCC cell lines, WEE1, CAM2KB and NEK4 were identified as the most promising candidate target kinases.[132] In particular, WEE1 (a G2–M cell-cycle regulator) can render synthetic lethality in *TP53*-mutant tumours because cells without functional p53 lack an effective G1 checkpoint and rely heavily on the G2 checkpoint regulators, such as WEE1, resulting in increased sensitivity of *TP53*-mutant cells to WEE1 inhibitors. Thus, *TP53* mutations with functional consequences need to be revisited as prognostic and predictive biomarkers in HNSCC.

Notch

The Notch pathway consists of four receptors (Notch1–4) and two families of ligands: the Jagged (JAG1 and JAG2) and the Delta-like (DLL1, DLL3 and DLL4) proteins.[133] Upon ligand binding, Notch receptors undergo a conformational change enabling cleavage and nuclear translocation of the intracellular domain to release the transcriptional repression of downstream target genes.[134] As the effects of Notch signalling vary between cell types, the tumour suppressive and oncogenic role of Notch in tumorigenesis is contextual. Two independent whole-exome sequencing studies reported *NOTCH1* mutations in 14% and 15% of HNSCC tumours, respectively.[12,13] Although these mutations remain to be characterized, both groups hypothesized *NOTCH1* to be a tumour suppressor in HNSCC on the basis of mutational characteristics, such as lack of mutational hotspots and high proportion of nonsense mutations.[12,13]

Emerging data have also suggested that a subset of HNSCC have activating *NOTCH1* mutations. Song *et al.*[135] reported that 43% (22/51) of oral squamous-cell carcinomas from a Chinese population harboured *NOTCH1* mutations that included novel mutations in the heterodimerization and *abruptex* domains predicted to result in gain of function. Furthermore, *NOTCH1* mutations were associated with lymph-node metastasis and worse clinical outcome.[135] Sun *et al.*[136] also reported overexpression of downstream Notch effectors in 32% of HNSCC (44 HNSCC tumours and 25 normal mucosal samples) evaluated for DNA-copy number, methylation and gene expression of the 47 Notch signalling pathway genes. Notably, overexpression of *JAG1* and *JAG2,* and *NOTCH3* were common , whereas only 10% of HNSCC had inactivating *NOTCH1* mutations, indicating the Notch pathway as a potential therapeutic target in a subset of HNSCC.

Classification by expression profiles

Beyond genetic alterations, HNSCC can also be classified based on their gene-expression profile. The classification reflects overall biological characteristics of the subgroups and some of these dominant characteristics can be used to generate hypotheses for novel therapeutic targeting and patient selection. Chung *et al.*[137] first reported four distinct molecular subtypes in HNSCC, designated as Groups 1–4. The presence of these subtypes has now been validated in two independent datasets of the University of North Carolina ($n = 138$) and TCGA ($n = 297$).[59,99] The Group 1–4 molecular subtypes of HNSCC were subsequently named to reflect their molecular characteristics as 'basal', 'mesenchymal', 'atypical' and 'classical', respectively. The atypical subtype included most of the HPV-positive HNSCCs. Various aspects of the HPV-negative HNSCC progression model could be associated with these four subtypes.[99] For example, gain of expression within the 7p chromosomal locus, which contains *EGFR*, was observed in all subtypes except atypical HNSCCs, consistent with current observations concerning less-common *EGFR* alteration and lower EGFR expression in HPV-positive HNSCC.[59,81,138] Loss of the 9p region, which contains *CDKN2A* ($p16^{INK4A}$), was associated with the basal and classical subtypes, whereas this feature was not observed in the atypical subtype, consistent with the absence of $p16^{INK4A}$ expression in most HPV-negative HNSCCs and overexpression in HPV-positive HNSCCs.[15] All three of these studies, however, suffer from limited representation of HPV-positive tumours.

Keck *et al.*[139] analysed HNSCCs, in a population enriched in HPV-positive tumours, and demonstrated that the HPV-positive tumours can be further divided into two different subtypes resulting in five molecular subtypes of HNSCC: basal, mesenchymal, classical, HPV-mesenchymal and HPV-classical. The HPV-positive tumours, which are distinct from HPV-negative tumours, harbour HPV-specific gene expression.[139] This observation is underscored by the findings that both mesenchymal and HPV-mesenchymal subtype tumours share an epithelial-to-mesenchymal transition (EMT)-related gene signature, but HPV-mesenchymal tumours demonstrate a unique upregulation of immune-response genes and cell-cycle-related genes.[139] Similarly, both classical and HPV-classical tumours have higher expression levels of cell-cycle-related genes and genes located in the chromosomal region 3q26–27, but only HPV-classical tumours demonstrate an upregulation of immune-response-related genes.[139] Consequently, the shared alterations in immune-response genes and cell-cycle-related genes unify the HPV-positive HNSCC subgroups. Some of these subtypes share similar prognostic features, however, these subtypes are based on molecular characteristics that might serve as better biomarkers in the future compared with histology or limited prognostic grouping based on HPV status.

In addition, De Cecco *et al.*[140] reported a 172-gene-expression signature generated from nine microarray gene-expression datasets that are available in the public domain ($n = 841$). The gene-expression signature was highly prognostic of relapse and poor outcome, independent of the HPV status.[140] Furthermore, microRNAs (miRNAs) are also being investigated for molecular characterization. miRNAs are short, noncoding RNAs involved in various cellular regulatory processes and capable of controlling hundreds of mRNA transcripts. Studies have shown that abnormal regulation of miRNAs is critically important in human cancer development as well as in defining response to radiation therapy, chemotherapy and patient outcome in HNSCC.[141–144] miRNAs can be easily detected through RT-PCR in body fluids, such as plasma and saliva, and have a potential to serve as a biomarker, and require further investigation.[145]

Hypoxia and radiation sensitivity

Tumour hypoxia is a well-established marker of poor prognosis in HNSCC.[146] Hypoxic stress results in increased angiogenesis and metastasis, decreased apoptosis, and resistance to radiation, largely mediated by hypoxia-inducible factor 1 (HIF-1).[147] Expression of HIF-1 and the downstream proteins lysyl oxidase, osteopontin, carbonic anhydrase IX (CAIX) and galectin-1

have shown potential as biomarkers for HNSCC, and have been validated in specimens from clinical trials.[148–150] For example, Overgaard *et al.*[150] showed that patients with high levels of plasma osteopontin benefited from treatment with the hypoxic-cell radiosensitizer nimorazole. HPV oncoprotein E6/E7 expression has been shown to enhance HIF-1 accumulation.[151] Contrary to what is expected, HPV status does not seem to influence tumour pO_2 or CAIX levels, and patients with HPV-related HNSCC did not benefit from treatment with nimorazole.[152,153] Of note, a microarray study identified a hypoxia expression signature of 99 genes that clustered with known hypoxia genes.[154] In an independent HNSCC dataset, this signature was validated as a prognostic factor for recurrence-free survival.[154] PET imaging with ^{18}F-fluoromisonidazole (FMISO), a bioreductive molecule that is trapped intracellularly under hypoxic conditions, has also been developed as a hypoxia biomarker. In a prospective trial testing the hypoxic-cell cytotoxin tirapazamine, FMISO-PET showed hypoxia in 32 patients, of whom eight patients had disease recurrence without tirapazamine treatment and only one patient had recurrence with tirapazamine.[155] Thus, hypoxia biomarkers have the potential to predict for response to hypoxic-cell radiosensitizers or cytotoxins.

In patients with HNSCC treated with radiotherapy, intrinsic radiosensitivity of the tumour and normal tissues influences the relative rate of tumour control versus late toxic effects. Successful biomarkers for radiosensitivity would enable personalized treatment selection and radiation-dose escalation or de-escalation. In this regard, an *in vitro* model of tumour radiosensitivity was used to create a 10-gene radiosensitivity index (RSI), and this index was validated as a clinical prognostic marker for locoregional control in patients with HNSCC and other cancers treated with concurrent chemoradiotherapy.[156]

Immune checkpoint-related biomarkers

HNC is an immunosuppressive disease; patients with HNC demonstrate low absolute lymphocyte counts, impaired natural killer (NK)-cell activity, and decreased antigen-presenting function.[157] HNC achieves immune evasion by several different mechanisms. The antigen-processing molecules, TAP1/2, and antigen-presentation molecule, MHC 1, are downregulated.[158] At the same time, co-inhibitory receptors, programmed death ligand-1 (PD-L1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4), which induce immune tolerance to HNC, are frequently expressed on tumours.[159,160] The cytokine milieu in the tumour microenvironment promotes tumorigenesis with excessive immunosuppressive cytokines such as TGF-β, VEGF, IL-6 and IL-10.[157,161]

The success of immune-checkpoint inhibitors in solid tumours, along with the increases in HPV-positive HNSCC incidence, has raised enthusiasm for novel immunotherapeutic approaches and development of corresponding biomarkers. HPV-positive HNSCC arises from deep crypts in lymphoid tissue of the tonsil and tongue base, and has characteristic tumour infiltrating lymphocytes (TILs) in the stroma and tumour nests.[162] Expression of PD-L1 occurs within deep tonsillar crypts as well as 70% of HPV-positive HNSCC.[163] As PD-L1 expression seems to correlate with the likelihood of response in early clinical studies with anti-PD1 and anti-PD-L1 antibodies,[164,165] the presence of TILs and expression of PD-L1 are good potential candidates as predictive biomarkers for benefit from immune-checkpoint inhibitors in HNSCC. Furthermore, when a T-cell inflamed phenotype (TCIP) defined by a 12-gene chemokine signature (CCL2, CLL3, CLL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11 and CXCL13), initially generated in melanomas,[166] was evaluated in a cohort of 134 HNSCC cases from the University of Chicago and a cohort of 424 HNSCC samples from TCGA, the presence of the TCIP was associated with infiltration of $CD8^+$ cells in a subset of HNSCCs;[167] 21% of HPV-negative tumours were TCIP-high and 51% of HPV-positive tumours were TCIP-high.[167] In addition, the TCIP-high phenotype was associated with mesenchymal subtype and higher prevalence of PD-L1 expression, suggesting that this phenotype could represent the sensitivity to anti-PD1/PD-L1 therapies.[167] A clinical trial of the anti-PD1 antibody, pembrolizumab (MK3475) produced encouraging responses and was well tolerated in patients with recurrent and/or metastatic HNSCC,[168] warranting further clinical development.

In addition to the co-inhibitory receptors, inducible and targetable immune co-stimulatory molecules are being evaluated. For example, cetuximab has shown to induce antibody-dependent cell-mediated cytotoxicity through the ability of NK cells to bind to the IgG1 Fc-region heavy chain of cetuximab via their Fc receptor (FcγRIII).[169] Therefore, augmenting the NK-cell activity by inducing a co-stimulatory signal would improve the cetuximab response. CD137 (4-1BB) is one of the co-stimulatory molecules expressed on activated NK cell and memory T cells, and cetuximab-bound tumour cells induce CD137 expression.[170] In murine xenograft models, a combination of cetuximab and agonistic CD137 monoclonal antibody resulted in synergistic antitumour effects and prolonged survival.[170] In patients treated with cetuximab, the percentage of CD137-expressing NK cells was markedly increased and CD137-expressing NK cells correlated with increased EGFR-specific $CD8^+$ T cell numbers.[170] A clinical trial evaluating the combination of cetuximab and urelumab (agonistic anti-CD137 antibody) is currently ongoing in patients with colorectal cancer or HNSCC.[171]

Genetic alterations: the liquid biopsy

In the clinical setting, obtaining tumour DNA can be challenging and time-consuming; therefore, the ability to determine gene aberrations in DNA obtained from blood can be beneficial (Figure 3). To detect rare mutant DNA fragments in the blood, highly sensitive and specific assays are required with a detection limit of 0.01% or lower.[172] With advancements in digital genomic technologies, such as digital PCR,[173] tagged-amplicon deep sequencing,[174] pyrophosphorolysis-activated

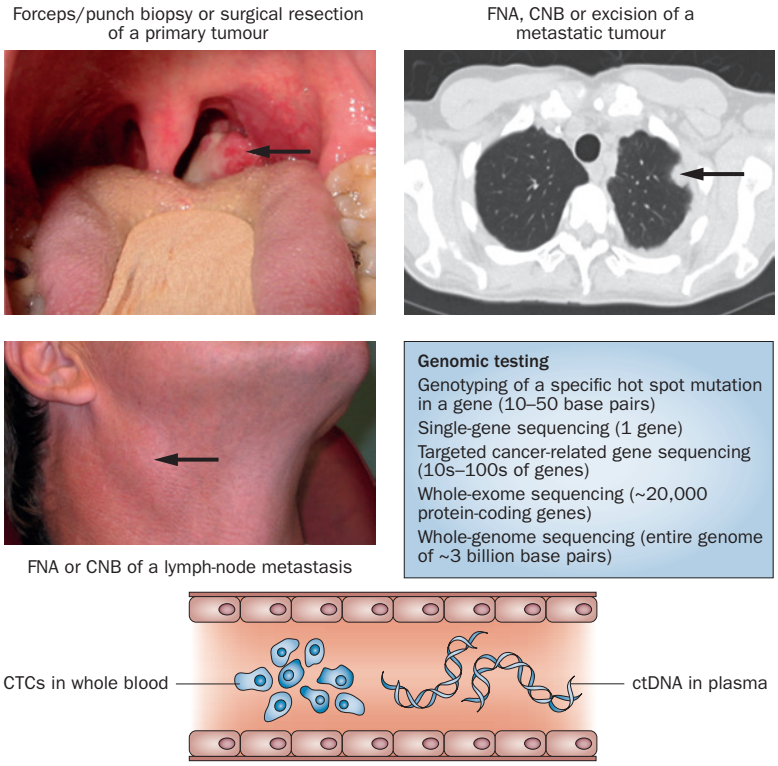

Figure 3 | Genomic testing using clinical specimens. DNA for genomic testing can be obtained by FNA, CNB, or forceps or punch biopsy, excisional biopsy and complete surgical resection of the tumour. There are advantages and disadvantages for each sample collection and testing approach. For example, samples obtained by FNA, and ctDNA and CTC might not yield enough tissue for comprehensive genomic studies.[172,178] However, such an approach is less invasive to patients, and it is easier to obtain serial samples using these methods. Use of next-generation sequencing platforms for whole-exome sequencing or genome sequencing provide additional information beyond point mutations and small insertions or deletions, including copy-number variations, translocations or detection of non-human sequences—such as the HPV genome—in one assay, but generally require a large amount of good quality DNA (>100 ng).[177] Abbreviations: CNB, core needle biopsy; CTC, circulating tumour cells; ctDNA, circulating tumour DNA; FNA, fine-needle aspiration; HPV, human papillomavirus.

polymerization,[175] and BEAMing,[176] reliable detection of circulating DNA from clinical specimens has become feasible. The DNA from blood can be obtained by two methods, either as circulating tumour DNA (ctDNA) or from circulating tumour cells (CTC). The technical aspects of genomic studies are reviewed elsewhere.[172,177]

A study determined ctDNA from 410 patients with localized and metastatic cancers from 14 different tissue types, including HNSCCs.[178] The patients with metastatic disease had a higher amount of ctDNA compared with those with local disease. HNSCC was one of the 14 tumours with reliably detectable ctDNA.[178] In addition to the detection of point mutations, ctDNA can also be used to detect gene-methylation changes, copy-number variations, or structural changes or rearrangements.[172] Although this particular study showed that analysis of ctDNA was more sensitive than evaluation of CTC for detecting mutations,[178] the technology to isolate rare CTC is improving owing to novel methods such as a negative depletion methodology through immunomagnetic depletion.[179,180] One distinct advantage of CTCs assessment over ctDNA is that CTCs can be used to evaluate cellular proteins, for example, monitoring EGFR expression during radiation therapy.[181] These novel assays can be applied in the early detection of cancer, disease monitoring and surveillance after treatment, tracking of resistance to targeted agents before any detectable imaging changes, and to gain insight in the molecular mechanisms of resistance without the risk of invasive tissue acquisition, especially in the metastatic setting.[172,178]

Conclusions

In the emerging era of biomarker-driven cancer therapy, we need to explore ways to incorporate the wealth of data into patient care. EBV, HPV and PET-imaging features provide robust prognostic biomarkers and are actively being incorporated into clinical trials. However, we need to move beyond prognostic biomarkers and focus on development of predictive biomarkers that will enable selection of patients for a specific therapy. For HPV-positive patients, the priority is in the development of treatment de-intensification strategies through less toxic, molecularly targeted agents and a robust biomarker to identify those with a poor outcome. For HPV-negative patients, the priority is to improve survival through identifying novel therapeutic agents with accompanying predictive biomarkers for patient selection, because the therapeutic window of opportunity for such patients with rapidly progressive disease is narrow. We need to develop strategies to prevent and control distant metastasis. We also need to focus on exploiting common TSGs, rather than limiting ourselves to the rare oncogenes, in drug development. Moving forward, it will be necessary for clinicians to educate themselves in order to convert laboratory data into meaningful clinically information. This will require an understanding of the basic technologies used in biomarker studies, as well as their limitations and basic biology. Each biomarker needs to be critically assessed and applied to patient care with comprehensive pretesting and post-testing counselling. Unfortunately, additional limitations exist beyond science and medicine. Although the technology and science are available, the clinical research, health-care policy, insurance policy and ethical considerations have not kept pace regarding implementation of emerging biomarkers. However, regardless of these seemingly impossible challenges, we are optimistic that the goal of delivering individualized cancer therapy for patients with HNSCC is within our reach.

Review criteria

We searched PubMed for English-language full-text manuscripts and abstracts published between 2000 and 2014. The search terms used, alone and in various combinations, were “head and neck cancer”, “squamous cell carcinoma”, “nasopharyngeal carcinoma”, “biomarker”, “genomics”, “imaging”, “HPV”, “EBV” and “hypoxia”. The reference lists of the articles identified were also searched for additional relevant publications.

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Author contributions

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