Review of emerging biomarkers in head and neck squamous cell carcinoma in the era of immunotherapy and targeted therapy

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Abstract
Background: Biomarkers in head and neck squamous cell carcinoma (HNSCC) emerge rapidly in recent years, especially for new targeted therapies and immunotherapies.

Methods: Recent, relevant peer-reviewed evidence were critically reviewed and summarized.

Results: This review article briefly introduces essential biomarker concepts, including purposes and classifications (predictive, prognostic, and diagnostic markers), and the phases of biomarker development. We summarize current biomarkers in order of clinical utility; p16 and human papillomavirus status remain the most important and validated biomarkers in HNSCC. The rationale for biomarker study design continues to evolve with technological advances, especially whole-exome or whole-genomic sequencing. Noninvasive body fluid and liquid biopsy biomarkers appear to hold strong potential for development as tools for early cancer detection, cancer diagnosis, monitoring of disease recurrence, and outcome prediction. In light of discrepancies among different technologies, standardized approaches are needed.

Conclusion: Biomarkers from cancer tissue or blood in HNSCC could direct new anticancer therapies.

KEYWORDS
biomarker, cisplatin, head and neck cancer, immunotherapy, liquid biopsy, targeted therapy
1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCCC) is a heterogeneous disease characterized by malignant and uncontrolled growth of cells in various sites within the head and neck areas, such as the oral cavity, larynx, oropharynx, hypopharynx, paranasal sinuses, and nasal cavity. For the purposes of this review, discussion on nonsquamous cell cancers originating in the head and neck, including nasopharyngeal carcinoma, differentiated or undifferentiated thyroid cancer, and salivary gland cancer, are outside the scope of this work. While these cancers are conventionally defined as members of the family of head and neck cancers, they are generally thought of as different entities from HNSCC. The main reasons for this distinction are due to their different behaviors with regard to tumor development and progression, patterns of relapse, sensitivity to chemotherapy and radiotherapy, and patient outcomes.

According to the National Cancer Institute (NCI), a biomarker is defined as “a biological molecule found in blood, other body fluids, or tissues, that is a sign of a normal or abnormal process, or a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition.” In the field of cancer treatment, biomarkers have at least 4 key roles in clinical application, including (a) assisting in the diagnosis of cancer; (b) indicating likely clinical outcomes (prognostic role); (c) aiding in patient selection for a specific treatment, based on which patients are most likely to respond (predictive role), and (d) deciding at what dosage the drug might be most effective (pharmacodynamic role). The latter 3 roles are generally applicable for newer chemotherapies, targeted therapies, and immunotherapy, including antiprogrammed death (PD)-1 or anti-PD-ligand 1 (L1) agents. While the use of many biomarkers is not yet routinely available in current clinical practice, biomarkers can provide critically useful and cost-effective information.

In this review, we highlight the clinical evidence and utility of established HNSCC biomarkers in clinical practice, along with several emerging biomarkers under development. Note that cell line investigations were not included in this review.

2 | OPPORTUNITIES AND CHALLENGES IN BIOMARKER DEVELOPMENT

Before a biomarker can be adopted into routine practice to aid in clinical decision making, a series of strict processes must be undertaken during bench-to-bedside development (Figure 1). First, a biomarker target is identified (discovery phase) and preliminarily confirmed through larger-scale, repeated laboratory experiments (confirmation phase). In subsequent clinical trials (validation and refinement phase), researchers attempt to set appropriate endpoints to validate preclinical findings in an independent patient cohort, before routine use of the biomarker is adopted (adoption phase).

However, a number of challenges exist in the biomarker development process (Figure 1). First, an appropriate target must be identified. In this rapidly evolving era of high-throughput omics technology, thousands of candidate molecules can be investigated easily without an a priori hypothesis. A more specific, targeted approach is a post hoc, data-driven investigational study design, rather than conventional pathway rationale-driven or hypothesis-driven designs. Setting a relevant, reproducible biomarker cutoff value to guide subsequent clinical measurement and validation can also be a difficult hurdle in biomarker test planning.

Furthermore, results of biomarker analyses can be notoriously inconsistent, making it difficult to draw robust conclusions. Some studies might show negative or discrepant results, while the same biomarker might clearly demonstrate positive associations in other studies—for example, CCND1, cMET, p16, EGFR, and ERCC1. Plausible reasons for such discrepancies might include (a) small sample sizes with inadequate controls; (b) differing study
populations with true clinical variability; (c) differing treatment modalities; (d) variations in the biomarker assay, for example, different technological platforms used for detection and measurement; (e) differences in the biomarker source, for example, tissue vs liquid biopsy, or fresh vs fixed tissue; (f) varied antibody specificities and binding affinities among different batches or vendors; (g) biomarker instability, with a risk of false positivity or false negativity; (h) differing statistical testing methods; and (i) other methodological differences between studies, for example, evaluation of mRNA vs protein expression.

It is important that test protocols mitigate the effects of such confounders in biomarker testing. For example, the use of standardized materials and methods should be used where possible. If the sample size is inadequate, investigators may need to apply cross-validation-based methods. Preselection of target populations can also be of key importance in biomarker study success, as nonpreselected populations could lead to a high trial cost and risk of failure.

Finally, many factors can impact the adoption of a biomarker among clinicians, including the high cost of routine testing, low power of evidence, accessibility of information, and lack of avenues for clinical feedback with regard to biomarker-directed therapies. Unfortunately, the high number of challenges around biomarker development means that very few markers (as low as 0.1%) achieve a substantial clinical role, limiting the use of biomarkers in routine practice.

3 PROGNOSTIC AND PREDICTIVE BIOMARKERS

In most studies, baseline biomarkers are evaluated for their prognostic role for disease outcomes (regardless of treatment), reflected by critical clinical trial endpoints. Key endpoints include overall survival (OS), progression-free survival (PFS), disease-free survival (DFS), loco-regional (LR) control, and distant metastasis-free survival.

Some biomarkers have shown predictive value for outcomes with standard anticancer therapies, including platinum agents, cetuximab-based chemotherapy, panitumumab-based chemotherapy, afatinib, radiotherapy, concurrent chemoradiotherapy (CCRT), and immunotherapy. Such biomarkers are particularly important for clinical trial design, as they can aid in patient selection or stratification at randomization, serve as treatment-monitoring tools, and help predict which specific patient groups are likely to derive the greatest benefit from a particular treatment.

4 BIOMARKERS IN HNSCC TUMOR TISSUES

Several important tumor tissue markers with adequate clinical confirmation and/or validation have been identified as potentially robust biomarkers in HNSCC. To date, over 70 markers have been evaluated and reported. Table 1 summarizes the key biomarkers, their biological type (protein, messenger RNA [mRNA], mutations of DNA, single nucleotide polymorphisms [SNPs], microRNA, or epigenetic pathway and targets), and their clinical significance.

Among the key prognostic markers for survival, epidermal growth factor receptor (EGFR), cyclin D1(CCND1), ERCC1, p16, human papillomavirus (HPV), and B-cell lymphoma-extra large (Bcl-xL)/Bcl-2 as well as the amplification of genes including EMS1, FGFR1, and CCND1 have demonstrated some evidence in clinical trials. Others are slower to advance in clinical development because of unconvincing data and few published studies; for example, acetylcholinesterase (AchE), SNPs of specific genes, glutathione S-transferase (GST), CD44, amplification of specific genes, hypoxic markers, KLK-6, and MDA-7/IL-24; although, this does not preclude them from future investigations and clinical utility.

4.1 p16: an important prognostic and predictive tissue biomarker in HNSCC

Of the many biomarkers with a prognostic role in HNSCC, the most well established and validated is p16, which plays an essential role in HNSCC. Approximately one third of HNSCCs express p16. p16 is a widely used clinical biomarker for HPV—a well-known cause of HNSCC—and HPV status, in turn, is an important prognostic marker used for patient stratification in HNSCC. Notably, in the most recent American Joint Committee on Cancer (AJCC) cancer staging book (2017), a distinct staging system for HNSCC patients with positive p16 expression was recommended. The p16 protein is generally evaluated by immunohistochemistry (IHC), and HPV infection by DNA/mRNA polymerase chain reaction (PCR). HPV has been shown to be a significant diagnostic and prognostic biomarker in particular in the oropharynx and cancers with unknown primary (CUP) presenting with neck node squamous cell carcinoma. p16 as a surrogate for HPV demonstrated strong prognostic value in patients with oropharyngeal squamous cell carcinoma (OPSCC) in a phase III registration trial evaluating radiotherapy alone or in combination with cetuximab. One study has suggested that p16 status is an important prognosticator in both OPSCC and non-OPSCC, and that the p16 positive/HPV16
| Biomarkers | Type | Case | Role | Therapy | Significance | Reference |
|-----------|------|------|------|---------|--------------|-----------|
| AChE      | mRNA (PCR) | 47   | Prognostic |          | Low AChE activity in HNSCC can be used to predict survival | Castillo-Gonzalez, A. C. et al.64 |
| ATM       | DNA SNPs (PCR) | 210 | Predictive (ORR) | RT | ATM IVS62 + 60G > A, TGFβ29C > T, TGFβ-509C > T, and BCL2-938C > A can function as biomarkers of tumor radiosensitivity | Agostini, L. P. et al.19 |
| Bcl-2     | DNA SNPs (PCR) | 210 | Predictive (ORR) | RT | ATM IVS62 + 60G > A, TGFβ29C > T, TGFβ-509C > T, and BCL2-938C > A can function as biomarkers of tumor radiosensitivity | Agostini, L. P. et al.19 |
| Protein (tissue microarray) | 196 | Prognostic | CCRT | Bcl-2 (HR: 2.6; P = .08) for distant metastasis | Rasmussen, G. B. et al.51 |
| β-tubulin-1 | Protein (tissue microarray) | 196 | Prognostic | CCRT | β-tubulin-1 (HR: 1.8; P = .08) for locoregional failure | Rasmussen, G. B. et al.51 |
| Protein (tissue microarray) | 196 | Prognostic | CCRT | β-tubulin-2 (HR: 0.49; P = .06) for locoregional failure | Rasmussen, G. B. et al.51 |
| C4.4A     | RNA (tissue microarry) | 43   | Prognostic |          | C4.4A was a marker for poor prognosis of HNSCC and participated in the EMT program | Liu, J. F. et al.20 |
| CCND1     | Mixed | 1929a | Prognostic | Mixed | Cyclin D1 overexpression was significantly associated with lymph node metastasis (OR: 2.25; 95% CI: 1.76-2.87) and worse DFS (OR: 3.06; 95% CI 2.42-3.87) | Gioacchini, F. M. et al.48 |
| Protein (IHC) | 53   | Prognostic |          |         | The prognostic significance of cyclin D1 expression was confirmed using a proportional hazard regression model | Higuchi, E. et al. (2007)65 |
| Protein (IHC) | 116  | Prognostic |          |         | Multivariate Cox, proportional hazards testing, indicated that the hazard ratio of cyclin D1-positive margins for local recurrence was 4.58 (95% CI 1.14-21.69, P = .03) | Sakashita, T. et al.21 |
| mRNA      |      | 104  | No role |         | CCND1 amplification was not prognostic | Rodrigo, J. P. et al.9 |
| Targeted NGS | 122  | Prognostic |          |         | Genomic alterations involving the cell cycle (TP53, CCND1, CDKN2A) are prognostic biomarkers | Dubot, C. et al.22 |
| CD44      | Protein (IHC) | 165 | Predictive (ORR) | RT | The negative effect of CD44 and EGFR and the positive effect of p16 on radiotherapy results were observed | Slavik, M. et al.64 |
| CDKN2A    | Targeted NGS | 122 | Prognostic |          | Genomic alterations involving the cell cycle (TP53, CCND1, CDKN2A) are prognostic biomarkers | Dubot, C. et al.22 |
| CIAP1     | Protein (IHC) | 129 | Prognostic |          | Coexpression of XIAP and CIAP1 prompted a worse prognosis | Yang, X. H. et al.45 |

(Continues)
| Biomarkers | Type | Case N | Role | Therapy | Significance | Reference |
|------------|------|--------|------|---------|--------------|-----------|
| c-MET      | Protein (IHC) | 112 | No role | | Expression of cMET and p16 revealed no impact on OS or PFS | da Costa, A. et al⁹ |
| CXCR4      | Protein (IFC) | 141 | Prognostic | CCRT | SDF-1 and CXCR4 expression for LR control and OS | De-Colle, C. et al (2017)²⁴ |
| EGFR       | DNA SNPs (PCR) | 110 | Predictive (AE) | Cetuximab | Genetic variation of EGFR (rs2227983), KRAS (rs61764370) and FCGR2A (rs180127) as useful biomarkers for predicting reduced skin toxicity | Fernandez-Mateos, J. et al²⁵ |
|            | DNA SNPs (PCR) | 129 | Association | | The frequency of mutations was significantly associated with an advanced stage | Nagalakshmi, K. et al (2014)⁶⁶ |
|            | DNA(FISH) | 75 | Prognostic | | High copy number of EGFR gene is a poor prognostic indicator | Chung, C. H. et al²⁶ |
|            | DNA(FISH) | 204 | Prognostic | | EGFR is predictive of outcome in univariate analyses | Young, R. J. et al³⁸ |
| Mixed      | 6781ᵃ | Prognostic | | | Increase of EGFR expression and gene copy number could predict poor survival | Zhu, X. et al²⁷ |
| Protein (IHC) | 130 | No role | | | No evident association was observed between EGFR expression and DFS (HR:0.90, 95% CI 0.68-1.19) | Lundberg, M. et al¹² |
| Protein (IHC) | 165 | Predictive (ORR) | RT | | The negative effect of CD44 and EGFR and the positive effect of p16 on radiotherapy results were observed | Slavik, M. et al⁶¹ |
| Protein (IHC) | 268 | Prognostic | | | EGFR was a strong independent prognostic indicator for OS and DFS and a predictor for LR relapse but not for DM | Ang, K. K. et al²⁹ |
| Protein (IHC) | 102 | Prognostic | | | EGFR protein levels assessed by AQUA strongly predict for patient outcome, whereas EGFR FISH status does not | Pectasides, E. et al (2011)⁶⁷ |
| RNA        | 110c | Predictive (ORR) | Afatinib | | p16-negative and EGFR amplified HNSCC, p16-negative and cetuximab naive HNSCC patients did better with afatinib | Galot, R. et al⁵⁸ |
| EMS1       | RNA | 104 | Prognostic | | EMS1 amplification is an independent predictor of cancer death (P = .003) | Rodrigo, J. P. et al⁸ |
| ERCC1      | DNA SNPs (PCR) | 122 | Predictive (AE) | Platinum and RT | ERCC1 SNPs predicts RT-related toxicity | Borchelliini, D. et al²² |
|            | DNA SNPs (PCR) | 2055ᵃ | Prognostic | | ERCC1 rs3212986 polymorphism in Asians may predict OS and rs11615 polymorphism may OS | Ding, Y. W. et al²⁸ |
|            | Protein (IHC) | 48 | Prognostic | Platinum | In the adjuvant setting, ERCC1 expression + high-risk category were the best predictors for relapse. ERCC1 expression was the only unfavorable independent determinant for OS | Ciaparrone, M. et al²⁹ |

(Continues)
| Biomarkers | Type | Case N | Role | Therapy | Significance | Reference |
|------------|------|--------|------|---------|--------------|-----------|
| Protein (IHC) | 48 | No role | ERCC1 expression failed to have a predictive value in head and neck carcinoma patients treated with RT | Bisof, V. et al<sup>68</sup> |
| Protein (IHC) | 453 | Prognostic | OS in OCSCC ($P = .01$)<sup>b</sup> | Prochnow, S. et al<sup>30</sup> |
| Protein (IHC) | 1288<sup>a</sup> | Prognostic | Platinum | ERCC1 expression: unfavorable OS (HR: 1.95), PFS (HR: 2.39) and ORR (OR: 0.48); ERCC1 expression: OS in NPC (HR: 2.72) | Bisof, V. et al<sup>13</sup> |
| Protein (IHC) | 90 (Ph II) | Prognostic | Platinum | ERCC1-XPF protein expression by the specific FL297 and 4F9 antibodies is prognostic in patients undergoing CCRT | Bauman, J. E. et al<sup>31</sup> |
| RNA (RT-PCR) | 44 | Predictive (ORR) | Platinum | Predict poor response to induction platinum-based therapy | Ameri, A. et al<sup>53</sup> |
| RNA (tissue microarray) | 176 | Prognostic | Oropharyngeal cancer and ERCC1 expression may have better outcomes despite HPV status | Patel, M. R. et al<sup>69</sup> |
| FGFR1 | Protein (IHC) and FISH | 492 | Prognostic | High expression of FGFR1 as a candidate prognostic biomarker in HPV-negative HNSCC | Koole, K. et al<sup>32</sup> |
| RNA (Targeted NGS) | 122 | Prognostic | Genomic alterations involving the cell cycle (TP53, CCND1, CDKN2A), as well as FGFR1 amplification and tumor genomic alterations burden are prognostic biomarkers | Dubot, C. et al<sup>22</sup> |
| GST | Protein (tissue microarray) | 56 | Predictive (ORR) | Platinum | GST expression correlates well with response to platinum-based chemotherapy | Nishimura, T. et al<sup>24</sup> |
| Heregulin | qRT-PCR | 750 | Diagnostic | Herregulin expression levels define a biologically distinct subset of HNSCC patients | Shames, D. S. et al (2013)<sup>70</sup> |
| HPV | Mixed | 1149<sup>a</sup> | Prognostic | Mixed | Prevalence of HPV among SCC of unknown primary in the head and neck patients were lower than in oropharyngeal SCC. The survival benefit of HPV-positive tumor was conferred | Ren, J. et al<sup>33</sup> |
| mRNA+DNA (PCR) | 109 | Prognostic | HPV 16 infection showed a positive prognostic values. Methodology discussed | Bussu, F. et al<sup>34</sup> |
| Hypoxia markers | Protein (IHC) | 2656<sup>a</sup> | Prognostic | Mixed | Endogenous markers of hypoxia (HIF-1a, CA-IX, GLUT-1, and OPN) expression was negatively influenced prognosis | Swartz, J. E. et al<sup>35</sup> |
| KLK-6 | Protein (IHC) | 162 | Predictive (ORR) | RT | Low KLK6 expression in primary tumors represents a promising tool to stratify HNSCC patients with high risk for treatment failure | Schrader, C. H. et al<sup>40</sup> |

(Continues)
| Biomarkers | Type | Case N | Role          | Therapy | Significance                                                                                                                                                                                                 | Reference |
|------------|------|--------|---------------|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| LOC541471  | lncRNA (RNA-Seq) | 487 (dataset) | Prognostic | Mixed | A negative association was revealed between lncRNA LOC541471 expression and overall survival in all subtypes of HNSCC                                                                                             | Wu, H. et al (2019)71 |
| MDA-7/IL-24 | Protein (IHC) | 131 | Prognostic | | MDA-7/IL-24 can be a prognostic biomarker and an indicator of second primary malignancies (SPM) in HNSCC                                                                                                     | Wang, L. et al72 |
| mHsp70     | mRNA (PCR) | 21 | Association  | | Soluble Hsp70 levels were significantly higher in HNSCC patients compared to healthy human volunteers and high mHsp70 expression levels on tumor cells were associated with high sHsp70 levels in the serum of patients | Gehrmann, M. et al (2014)73 |
| miR-375    | miRNA (PCR) | 1340* | Prognostic | Mixed | The downexpression of miR-375 was correlated significantly with poor OS                                                                                                                                       | Wang, P. et al36 |
| miRNA      | miRNA (PCR) | 492c | Prognostic | Mixed | Six miRNA panel predicts poor outcomes. Multivariate Cox regression analysis, patients with high-risk factors had shorter OS (HR, 2.380, 95%CI, 1.361-4.303) than patients with low-risk scores in the total dataset | Shi, H. et al37 |
| MRP2       | Protein (IHC) | 91 | Prognostic | | MRP2 and RB both were shown to be independently associated with poor local control in patients treated with CCRT                                                                                         | Van Den Broek, G. B. et al55 |
| p16        | Protein (IHC) | 64 | Predictive (ORR) | CCRT | High expression of p16 predicts a better response to chemoradiation in patients with stage IVa/b                                                                                                             | Chen, Y. J. et al62 |
| p16        | Protein (IHC) | 73 | Prognostic | | Among patients with CUP, p16-positive status is an independent predictor of DFS but not OS                                                                                                                   | Dixon, P. R. et al50 |
|           | Protein (IHC) | 112 | no role | | cMET and p16 expression showed no impact on OS or PFS                                                                                                                                                       | da Costa, A. et al9 |
|           | Protein (IHC) | 130 | Prognostic | | p16 overexpression was associated with an improved DFS (HR:0.39, 95% CI 0.19-0.78)                                                                                                                         | Lundberg, M. et al12 |
|           | Protein (IHC) | 165 | Predictive (ORR) | RT | The negative effect of CD44 and EGFR and the positive effect of p16 on radiotherapy results were observed                                                                                                                                                       | Slavik, M. et al61 |
|           | Protein (IHC) | 166 | no role | | p16 expression is not associated with better survival                                                                                                                                                     | Satgunaseelan, L. et al10 |
|           | Protein (IHC) | 204 | Prognostic | | p16(INK4A) remains independently predictive                                                                                                                                                                 | Young, R. J. et al38 |

(Continues)
| Biomarkers | Type | Case N | Role | Therapy | Significance | Reference |
|-----------|------|--------|------|---------|--------------|-----------|
| Protein (IHC) | 568 | Prognostic | Similar prognostication as the newly adopted 8th edition of the UICC staging in the p16-positive patient cohort was independently confirmed | Rasmussen, J. H. et al39 |
| Protein (IHC) | 1448 | Prognostic | p16 has a similar prognostic role in both nonoropharyngeal and oropharyngeal cancer | Bryant, A. K. et al40 |
| Protein (IHC) | 110c | Predictive | Afatinib | p16-negative and EGFR amplified HNSCC, p16-negative and cetuximab naive HNSCC patients did better with afatinib | Galot, R. et al58 |
| Protein (IHC) | 182 (ph III) | Prognostic | Mixed | p16 status was strongly prognostic for patients with OPC | Rosenthal, D. I. et al41 |
| Protein (IHC) | 1929a | Predictive (PFS) | EGFRi plus chemo | A significant PFS benefit (HR: 0.58; P < .001) of adding an EGFR inhibitor to chemotherapy versus chemotherapy for p16-neg cancer while no PFS or OS benefit for p16-pos cancer | Su, Y. et al46 |
| Protein (IHC) | 657 (ph III) | Predictive / prognostic | Panitumumab + PF | p16 status could be a prognostic and predictive marker in patients treated with panitumumab and chemotherapy | Vermorken, J. B. et al74 |
| Protein (IHC)/ genotyping | 52 | No role | p16/HPV16-positive patients with LA-HNSCC treated with RT + EGFR inhibitors showed better survival | Pajares, B. et al11 |
| p53 | Protein(tissue microarray) | 196 | Prognostic | CCRT | A high p53 expression has opposite prognostic effects for increased risk of LR failure, but decreasing the risk of DM | Rasmussen, G. B. et al51 |
| PI3K pathway mutations | Gene profiling sequencing | 48 (Ph II) | Predictive (ORR) | Dacomitinib | PI3K pathway mutation and inflammatory cytokine expression help identify patients possibly gain benefit from dacomitinib | Kim, H. S. et al (2015)75 |
| PITX2 | DNA methylation | 528c | Prognostic | Mixed | Multivariate Cox analysis showed PITX2 promoter methylation was confirmed as a prognostic factor | Sailer, V. et al42 |
| PTEN | Protein (IHC) | 112 | Prognostic | Cetuximab, PF | A negative prognostic effect of PTEN loss was observed in the patients treated with cetuximab+ chemotherapy | da Costa, A. et al9 |
| RB | Genomic profiling | 1 + 279d | Prognostic | RB1 alterations have prognostic implications, particularly in high p16 expression | Beck, T. N. et al43 |
| Protein (IHC) | 91 | Prognostic | Platinum-CCRT | MRP2 and RB both were shown to be independently associated with poor local control in patients treated with CCRT | Van Den Broek, G. B. et al55 |
| SDF-1 | Protein (IFC) | 141 | Prognostic | CCRT | SDF-1 and CXCR4 expression for LR control and OS | De-Colle, C. et al (2018)76 |

(Continues)
negative group is likely a distinct and important subgroup for future trials.\textsuperscript{100, 101}

p16 also appears to be an important prognostic marker in cutaneous HNSCC, which commonly presents as cervical metastases secondary to CUP. High p16 expression has been shown to be indicative of primary HNSCC with better survivals\textsuperscript{10}; however, p16 expression was not associated with improved survival in this specific subgroup of HNSCC.\textsuperscript{10} In theory, p16 positivity would not be exclusively associated with HPV infection, considering p53 and retinoblastoma (RB) gene involvement.\textsuperscript{102, 103} The virus contains two oncoproteins, E6 and E7, which, when expressed, inactivate p53 and RB, respectively. p53 is frequently inactivated in HNSCC, and dysregulation of the RB gene is increased by the expression of p16.\textsuperscript{97} When testing for p16 and HPV, the two tests are not always concordant with each other.
p16 also has predictive value with regard to HNSCC outcomes with various specific treatments.\textsuperscript{57,62} Patients positive for p16 have shown a greater response to, and improved overall outcomes with radiotherapy,\textsuperscript{61} as well as improved outcomes from CTC count during treatment (CCRT),\textsuperscript{62} vs those with p16 nonexpression. In addition, some studies indicate a predictive role for p16 status in patients receiving EGFR-targeted therapy for HNSCC, including cetuximab plus chemotherapy,\textsuperscript{46} panitumumab plus chemotherapy (vs chemotherapy alone\textsuperscript{57}), and afatinib.\textsuperscript{58,59}

As excellent outcomes, in general, can be achieved in HPV-positive HNSCC, the reasonable next step is to de-intensify therapy, especially radiotherapy, to minimize treatment-related toxicities and improve quality of life without compromising survival.\textsuperscript{110,111} One thing to be noticed is that the de-intensification needs to happen carefully and only within the confines of a clinical trial. Given the distinctive pathobiology of HPV-positive HNSCC, innovative approaches targeting viral oncogenes and the immune system, integrated with the use of both established and novel biomarkers, are warranted.\textsuperscript{3}

### 4.2 Predictive tissue markers for platinum-based therapy

As early as 2000, the use of cisplatin or carboplatin in combination with radiotherapy has become a standard treatment approach for HNSCC.\textsuperscript{110,112} Several tissue biomarkers have shown a potential role in predicting response to platinum-based therapy. For example, ERCC1 protein expression may be a valuable marker for platinum chemoresistance,\textsuperscript{14,30,32,54,106} radiotherapy toxicity,\textsuperscript{52} and response\textsuperscript{113} in HNSCC. However, one study in 48 HNSCC patients\textsuperscript{68} showed no apparent role for mRNA amplification of the ERCC1 gene in terms of predicting platinum resistance, suggesting that such resistance might be attributable to non-ERCC1 pathways.\textsuperscript{114} This may be a unique phenomenon in HNSCC; in a prospective phase III trial in nonsmall lung cancer, ERCC1 mRNA expression was able to predict acquired resistance to platinum treatment.\textsuperscript{115}

Bcl-2 is another example; evidence suggests that this marker may contribute to distant failure in HNSCC patients receiving platinum-based CCRT.\textsuperscript{19,51} In addition, GSTs— that appear to play an essential role in the cell's defense against toxic substances—may predict platinum resistance in HNSCC.\textsuperscript{54} MRP2 protein expression and RB protein expression were also found to be independently associated with reduced local control in patients who received CCRT.\textsuperscript{55} Another study evaluated stromal cell-derived factor 1 (SDF-1)/CXCR4 and demonstrated predictive ability with respect to locoregional control and survival in 141 patients who underwent surgery and adjuvant CCRT.\textsuperscript{24} It should be noted, however, that some of these studies failed to discriminate between resistance to RT vs chemoresistance.

### 4.3 Predictive tissue markers for EGFR-targeted therapy

The family of human epidermal growth factor receptor (HER/EGFR/ErbB) contains 4 subtypes of EGFR members (ErbB1 to 4/EGFR, HER2-4), which play essential roles in cancer cell proliferation, vessel angiogenesis, and dissemination through downstream oncogenic signaling pathways. EGFR is overexpressed in more than 90% of HNSCC, but the loci of mutations are not in common hotspots.\textsuperscript{49,116,117} Currently, there are two main types of EGFR-inhibition-mediated therapeutic agents, including (i) EGFR monoclonal antibodies (mAbs, that is, cetuximab and panitumumab) that target extracellular ligand binding domains; and (ii) EGFR-tyrosine kinase inhibitors (TKIs, that is, gefitinib and afatinib) that target intracellular ATP-binding pockets in tyrosine kinase domains.\textsuperscript{118,119} In 2008, addition of cetuximab to platinum-fluorouracil chemotherapy significantly improved OS compared with platinum-based chemotherapy alone as first-line treatment in patients with recurrent or metastatic HNSCC.\textsuperscript{57,120} Cetuximab alone has also shown efficacy in platinum-refractory cases th HNSCC.\textsuperscript{54} Interest in establishing accurate predictive markers of response to EGFR inhibitors continues to grow.

Increased EGFR gene copy number appears to be largely restricted to p16\textsuperscript{INK4A}−negative oropharyngeal cancer.\textsuperscript{38} Biomarker studies evaluating the role of EGFR protein expression have shown inconsistent results.\textsuperscript{38} For example, as briefly described above, p16 status appears to influence response to EGFR-targeted therapies. Patients with p16-positive tumors responded well to cetuximab-based therapy,\textsuperscript{41,46} those with p16-negative tumors responded better to panitumumab-based therapy\textsuperscript{57,74} and afatinib.\textsuperscript{58,59} In the phase III LUX-Head&Neck 1 (LUX-H&N1) trial, 2nd line afatinib significantly improved PFS vs methotrexate in patients with recurrent/metastatic HNSCC.\textsuperscript{57} In subgroup analysis, patients who have benefited from afatinib were identified in those with p16\textsuperscript{neg}, EGFR\textsuperscript{amplified}, HER\textsuperscript{3}low, PTEN\textsuperscript{high} status.\textsuperscript{59} That indicates that p16, HER3, and PTEN might serve as predictive markers in afatinib treatment. Also, in another study, high heregulin mRNA and high HER3 protein levels independently correlated with poor OS in oropharyngeal cancer patients, which indicates targeting HER3 as one of the potential treatment targets.\textsuperscript{121}
It is reasonable to assume that biomarker signatures made up of combinations of established markers such as EGFR, RB, p53, CDK2, p16, p21, and HPV E6/E7 levels, may offer a more feasible approach to response prediction in HPV-positive HNSCC.

4.4 Predictive tissue markers for PD-1/PD-L1 inhibitors

Table 2 summarizes the available evidence for prognostic and predictive biomarkers in the era of immunotherapy, focusing on PD-1 and PD-L1 inhibitors. PD-1 and PD-L1 expression currently remain the most significant tissue biomarkers. PD-L1 expression has been associated with post-chemotherapy (docetaxel/platinum/5-fluorouracil regimen) status, co-occurrence with p16INK4 expression, and poorer OS (but improved RFS and OS). Among these studies, associations between high PD-L1 expression and favorable OS were all demonstrated in post-surgery HNSCC patients. It is possible that PD-L1 expression may differentially impact resectable and unresectable patients, which requires further investigation. In patients with HNSCC who underwent pulmonary metastasectomy, higher PD-L1 expression predicted poorer outcomes after palliative surgery.

Regarding studies of predictive markers for newer immunotherapies, PD-L1 expression was associated with a higher ORR and longer OS after nivolumab therapy, a favorable response to radiation (radiosensitivity), and an improved response to durvalumab. In addition to PD-L1 expression, microsatellite instability (MSI) predicted response to PD-L1 inhibitors in HNSCC. HPV status was predictive of improved response to durvalumab. Furthermore, a higher number of some subtypes of tumor-infiltrating lymphocytes (TILs), such as PD-1+TIM-3+CD8+TILs and PD-1+LAG-3+CD8+TILs, and higher tumor mutation burden (TMB) and CD8+TILs, all predicted improved response to anti-PD-1 or anti-PD-L1 therapies.

Several recent conference presentations have also highlighted novel data regarding predictive biomarkers in the era of immunotherapy (Table 2). For example, PD-1+ CD8+ effector T cells and PD-1+ Treg cells in tumor tissue predicted response to nivolumab, whereas mutational load and IFN-γ gene expression profile (GEP) predicted response to pembrolizumab. However, data on these and other emerging predictors of immunotherapy response remain inconclusive. An ongoing prospective trial, PRECISION-01 (NCT03917537; www.ClinicalTrials.gov), aims to resolve some of this uncertainty by evaluating biomarker signatures in cancer tissue via whole-genome/exome sequencing, in patients with platinum-refractory HNSCC receiving nivolumab monotherapy.

Clinical application uses current evidence of validated cutoff values of PD-L1 expression (Table 3). In brief, each clone of PD-L1 for an individual immunotherapy drug has its validated cutoffs, although these may vary between studies. To more fully elucidate the predictive role of PD-L1 expression, a study comparing HNSCC patient populations identified by different PD-L1 assays is now underway. However, PD-L1 expression on cancer tissue currently still has no bearing on the management of patients with HNSCC.

5 IMAGING BIOMARKERS IN HNSCC

In recent years, advances in diagnostic imaging technologies have not only aided cancer diagnosis and staging, but have also become an important tool in predicting disease outcomes, relapse patterns, and treatment outcomes. The most commonly utilized tool is functional magnetic resonance imaging (MRI), using diffusion-weighted imaging (DWI), blood oxygen level-dependent (BOLD), and dynamic contrast-enhanced (DCE) sequences.

Some studies have established multidimensional prognostic or predictive signatures by combining more than one type of biomarker. For example, one or more imaging parameters (eg, maximal standard uptake value, metabolic tumor volume, apparent diffusion coefficient, or gross tumor volume) may be combined with tissue protein expression markers, or liquid biopsy markers.

6 BIOMARKERS FROM LIQUID BIOPSIES

Some biomarkers can be isolated from body fluids, such as blood, urine, and saliva, and objectively measured. Important benefits of liquid biomarkers include their non-invasive nature, and convenience in terms of taking serial measurements. However, a number of factors have the potential to impact the dynamics and reliability of liquid markers, limiting their clinical utility. These may include the timing of collection, fluid viscosity, nutritional factors, inflammatory conditions, secretory gland injury, or other environmental factors. Table 4 summarizes some examples of liquid biopsy markers in HNSCC; however, it should be noted that some data lack proper validation.

Blood is a relatively stable body fluid; several serum protein markers have traditionally been used to help predict outcomes in HNSCC patients, including CDK4, midkines, tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), IL-2R, and VEGF-A. However, some are not always cancer-specific, giving rise to problems in
**TABLE 2**  Immunotherapy-associated tissue biomarkers

| Biomarkers-clone | Type (method, cutoff) | Case N | Role | Therapy | Significance | Reference |
|------------------|-----------------------|--------|------|---------|--------------|-----------|
| Published data   |                       |        |      |         |              |           |
| PD-L1-SP263      | Protein (IHC, ≥25%)   | 111    | Prognostic/Predictive | Durvalumab | Durvalumab demonstrated antitumor activity with acceptable safety in PD-L1-high patients with rmHNSCC | Zandberg, D. P. et al122 |
|                  | Protein (IHC, ≥50%)   | 26     | Predictive (surgery) | Pulmonary metastasectomy | High PD-L1 expression in pulmonary metastases could be an independent predictor of poor outcome in HNSCC patients undergoing pulmonary metastasectomy | Okada, S. et al (2018)123 |
| PD-L1-SP142      | Protein (IHC)         | 203    | Prognostic | | PD-L1 expression (≥ 50%) was an independent prognostic factor for poor OS in anti-PD1/PD-L1 untreated HNSCC patients | Ngamphaiboon, N. et al124 |
|                  | Protein (IHC, ≥5%)    | 402    | Prognostic | | High PD-L1 expression on IC, but not TC, and high abundance of PD-1(+) T cells and Foxp3(+) Tregs are favorable prognostic factors | Kim, H. R. et al125 |
| PD-L1-EPR1161    | Protein (IHC)         | 293    | Prognostic | | Strong correlation between PD-L1 expression and reduced OS | Muller, T. et al126 |
| PD-L1-E1L3N      | Protein (IHC, ≥5%)    | 313    | Association | TPF chemo | TPF induction chemotherapy in advanced HNSCC increases PD-L1 positivity on TIL ICs, as well as CD8+ lymphocytes density | Leduc, C. et al127 |
| PD-L1-288        | Protein (IHC, ≥1%)    | 361 (CM-141) | Predictive (ORR, OS) | Nivolumab | OS rate (16.9%) vs IC (6.0%), and | Ferris, R. L. et al63 |

(Continues)
| Biomarkers-clone | Type (method, cutoff) | Case N | Role          | Therapy | Significance | Reference |
|------------------|----------------------|--------|---------------|---------|--------------|-----------|
| PD-L1/PD-L2      | mRNA (tissue microarray) | 33     | Prognostic    |         | OS was significantly worse in patients with higher levels of PD-L1 and PD-L2 score ($P < .05$) | Moratin, J. et al\textsuperscript{128} |
| PD-L1            | Protein (IHC)        | 112    | Prognostic    |         | PD-L1 expression in tumor cells or TILs predicts longer DFS | Roper, E. et al\textsuperscript{129} |
| PD-L1            | Protein (IHC)        | 161    | Prognostic    |         | PD-L1 expression constitutes an independent prognostic marker in patients received adjuvant CCRT | Balermpas, P. et al\textsuperscript{130} |
| PD-L1            | Protein (tissue array) | 106    | Association   |         | Expression of PD-L1 was associated with $p16^{INK4A}$ expression ($P < .01$) | Chen, S. C. et al\textsuperscript{131} |
| PD-1/PD-L1       | DNA Gene signature   | 517    | Predictive (ORR) | RT      | High expression of PD-1/PD-L1 was strongly related to radio-sensitivity | Lyu, X. et al\textsuperscript{132} |
| TMB              | WES                  | 126    | Predictive (ORR) | anti-PD-1/PD-L1 Tx | Higher TMB and CD8+ T cell infiltrates predicted anti–PD-1/L1 benefit ($P < .01$, $P < .01$, respectively) among virus-negative tumors | Hanna, G. J. et al\textsuperscript{133} |
| PD-1$^+$TIM-3$^+$CD8$^+$TILs | Protein (IHC)        | 126    | Predictive (ORR) | anti-PD-1/PD-L1 Tx | TIM-3/LAG-3 coexpression with PD-1 was higher on T cells among nonresponders ($P = .03$ and .02, respectively) | Hanna, G. J. et al\textsuperscript{133} |
| PD-1$^+$LAG-3$^+$CD8$^+$TILs | Protein (IHC)        | 126    | Predictive (ORR) | anti-PD-1/PD-L1 Tx | TIM-3/LAG-3 coexpression with PD-1 was higher on | Hanna, G. J. et al\textsuperscript{133} |

(Continues)
| Biomarkers-clone | Type (method, cutoff) | Case N | Role | Therapy | Significance | Reference |
|------------------|----------------------|--------|------|---------|--------------|-----------|
| MSI              | DNA (PCR)            | NA     | Predictive (durable CR) | PD-L1 inhibitor | MSI associated with durable complete response to PD-L1 inhibitor | Tardy, M. P. et al<sup>130</sup> |
| HPV              | Protein (IHC, positive) | Prognostic/predictive | Durvalumab | HPV-positive patients had a numerically higher response rate and survival than HPV-negative patients | Zandberg, D. P. et al<sup>122</sup> |
| Microbiota       | NGS                  | NA     | Predictive (ORR) | Ipilimumab | B. fragilis was associated with the efficacy of CTLA-4 blockade | Vetizou, M. et al<sup>134</sup> |

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| Biomarkers-clone | Type (method, cutoff) | Case N | Role | Therapy | Significance | Reference |
|------------------|----------------------|--------|------|---------|--------------|-----------|
| MDM2/MDM4        | amplification (NGS)  | 5      | Predictive (HPD) | anti-PD-1/PD-L1 Tx | The incidence of hyper-progression after checkpoint blockade in patients with MDM2/MDM4 amplification was as high as 66% (2/3) | Singavi, A. et al (2017)<sup>135</sup> |
| **Effector T cells** | Flow cytometry | 62 (CM-141) | Predictive (ORR) | Nivolumab | The percentage of PD-1+ CD8+ effector T cells was significantly lower in responders and TBP pts vs NTBP pts | Haddad, R. et al<sup>136</sup> |
| **PD-1+ Treg**   | Flow cytometry | 62 (CM-141) | Predictive (ORR) | Nivolumab | At D1, TBP pts, similar to responders, had a significantly lower PD-1+ Treg percentage vs NTBP pts | Haddad, R. et al<sup>136</sup> |
| **Mutation Loads (ML)** | WES | 107 (KN-012) | Predictive (ORR) | Pembrolizumab | ML and GEP are independently predictive of response to pembrolizumab in HPV−/EBV− patients with HNSCC | Haddad, R. I. et al<sup>137</sup> |

(Continues)
More recently, markers from liquid biopsies that are directly related to cancer cells or cancer-produced molecules have been employed, including circulating tumor cells (CTCs),

cell-free DNA (cfDNA)/circulating tumor DNA (ctDNA),
cell-free RNA (cfRNA), and exosomes. It is generally accepted that CTCs may play an important role in cancer metastasis. Data suggest that an elevated baseline CTC count is associated with advanced stage of HNSCC, risk of relapse, and a poor prognosis, while a declining CCRT could indicate an improved prognosis and treatment response. In some patients, an increased CTC count has been observed after HNSCC treatment by surgery and radiotherapy, which is thought to possibly reflect stimulation of tumor cell dissemination and a poorer prognosis; although, the exact reasons for this observation remain unclear. A subgroup of CTCs express podoplanin, a known prognostic factor for HNSCC. Evidence suggests that an elevated podoplanin:EpCAM ratio in CTCs may be associated with a poor prognosis and treatment failure.

In addition, PD-L1-positive CTCs may play a role in predicting response to immunotherapy. With regard to cfDNA, mitochondrial cfDNA content appears to be strongly associated with HNSCC in patients with lifestyle risk factors for the disease. Various other liquid biomarkers may provide additional diagnostic and prognostic information in HNSCC. Examples include elevated serum miR-21, circulating PD-L1+...
| Biomarkers | Type       | Case N | Role                  | Therapy  | Significance                                                                                                                                                                                                 | Author (Year)     |
|------------|------------|--------|-----------------------|----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| CDK4       | SPR + western | 100    | Diagnostic/prognostic |          | Elevated serum CDK4 levels were observed in HNSCC patients compared with controls. Higher CDK4 levels in HNSCC patients were associated with poorer survival outcomes                                                   | Banerjee, J. et al 154 |
| cfdNA      | DNA        | 50     | Diagnostic            |          | Mitochondrial cfDNA content is strongly associated with HNSCC in patients with lifestyle risk factors (eg, smoke and smokeless tobacco, betel quid chewing, and alcohol)                                             | Kumar, M. et al 155 |
| CRP + ARG  | ELIZA      | 80     | Diagnostic            |          | Serum ARG and CRP together were diagnostic for HNSCC                                                                                                                                                       | Choudhury, B. et al (2014) 156 |
| CRP + TNFα | ELIZA      | 100    | Diagnostic/prognostic |          | Significantly elevated levels of CRP and TNFα were found in HNSCC patients. Combination of upregulated CRP and TNFα in plasma was significantly associated with shorter survival                                         | Andersson, B. A. et al (2014) 157 |
| CTC        | CellSearch | 110    | Prognostic            |          | DTCs and CTCs are independent prognostic markers for disease relapse. Positive correlation to T, N stages                                                                                                 | Gröbe, A. et al 158 |
| CellSearch | 53         |        | Prognostic            |          | CTCs were significantly associated with patient characteristics and poor prognosis                                                                                                                        | Grisanti, S. et al 159 |
| CellSearch | 73         |        | Prognostic            |          | CTC absence after treatment associated with improved response and survival                                                                                                                               | Buglione, M. et al 160 |
| CellSearch | 15         |        | Prognostic            |          | CTCs associated with lung nodules and disease progression                                                                                                                                                  | Nichols, A. C. et al 161 |
| CellSearch | 40         |        | Prognostic            |          | Detection of CK20 mRNA in peripheral blood is prognostic in OSCC                                                                                                                                         | Toyoshima, T. et al 162 |
| CellSearch | 100 (ongoing) |        | Prognostic            |          | Combining CTC and structural information from MRI may provide more information than either modality alone                                                                                                  | Ng, S. P. et al 147 |
| Flow cytometry | 144   |        | Predictive (ORR)      | Surgery + RT | Detection of CTC was useful to predict patients likely to benefit from therapy                                                                                                                                 | Tinhofer, I. et al 163 |
| Negative selection | 47     |        | Prognostic            | CCRT     | CTC decline status was an independent prognostic factor in PFS ($P = .03$) and OS ($P = .05$) in multivariate analyses                                                                                       | Wang, H. M. et al 164 |
| Negative selection | 53     |        | Prognostic            |          | The CTC ratio of podoplanin-positive/EpCAM-positive is a prognostic factor                                                                                                                                  | Hsieh, J. C. et al 165 |

(Continues)
| Biomarkers                        | Type                      | Case N | Role       | Therapy | Significance                                                                 | Author (Year)                      |
|----------------------------------|---------------------------|--------|------------|---------|-----------------------------------------------------------------------------|-----------------------------------|
| Negative selection               | 48                        | Prognostic |            |         | Lack of CTCs associate with significantly longer DFS                        | Jatana, K. R. et al^166            |
| Negative selection               | 36                        | Prognostic |            |         | Detection of CTCs pre- or intraoperatively indicates a high risk of local and distant recurrence and reduced survival | Partridge, M. et al^167            |
| SERS                             | 82                        | Prognostic |            |         | A liquid biopsy identified patients at higher risk of disease progression     | Morgan, T. M. et al^168            |
| ClearCell FX system              | 56                        | Prognostic |            |         | CTCs predicted of poorer outcomes                                            | Kulasinghe, A. et al (2018)^169    |
| Mixed                            | 429^a                     | Prognostic |            |         | Presence of CTCs was not prognostic for OS, although it could reflect outcomes of loco-regional disease | Cho, J. K. et al^170               |
| Mixed                            | 909^a                     | Prognostic |            |         | CTCs used as a monitoring tool for early detection of tumor recurrence/progression, advanced disease, and nodal metastasis | Sun, T. et al^171                  |
| Negative selection               | 38                        | Association | Surgery |         | A statistically significant was found to increase in CTCs after surgery ($P = .02$) | Jatana, K. R. et al^153            |
| Laser scanning cytometry         | 40 (ph II, TISOC-1)       | Prognostic |            |         | Baseline CTCs and maximal CTCs during therapy both were strong prognostic markers for OSCC treated by chemotherapy, surgery, and CCRT | Inhestern, J. et al^172            |
| Flow cytometry                   | 31                        | Association | RT       |         | Definitive radiotherapy regimens of locally advanced HNSCC can increase the number of CTCs | Tinhofer, I. et al^173             |
| CellSearch                       | 15                        | Prognostic |            |         | CTC count was significantly associated with lung nodules $>1$ cm. Improved survival was noted in CTC-negative patients | Nichols, A. C. et al^161           |
| Flow cytometry                   | 42                        | Association |          |         | Detection of CTCs correlated with regional metastasis in inoperable HNSCC    | Hristozova, T. et al (2011)^174    |
| Negative selection               | 48                        | Prognostic |            |         | CTCs per mL was negatively associated with DFS                               | Jatana, K. R. et al^166            |
| DNA methylation patterns         | Methylation (PCR)         | 46      | Diagnostic |         | Methylation levels for the two identified CpG clusters were significantly different between healthy and HNSCC individuals | Ovchinnikov, D. A. et al (2014)^175 |

(Continues)
| Biomarkers          | Type         | Case N | Role                      | Therapy | Significance                                                                 | Author (Year)             |
|---------------------|-------------|--------|---------------------------|---------|------------------------------------------------------------------------------|---------------------------|
| Gycoprotein L-fucose | ELIZA       | 50     | Diagnostic                |         | Serum glycoprotein L-fucose levels can be used as a useful indicator in conjunction with clinical diagnostic procedures | Shetty, R. K. et al (2013)<sup>176</sup> |
| LBC-RTF             | Protein (ICC)| 68     | Prognostic                | Surgery | LBC-RTF significantly improved the diagnostic accuracy of traditional intraoperative diagnosis | Kinoshita, Y. et al (2018)<sup>177</sup> |
| midkine             | ELIZA       | 103    | Predictive (chemosensitivity) | Chemotherapy | Serum midkine levels in patients with HNSCC were associated with malignancy, chemosensitivity, and prognosis | Yamashita, T. et al<sup>178</sup> |
| miR-21              | mRNA (PCR)  | 15     | Prognostic                |         | miR-21 expression might be an essential tool for treatment planning and a prognostic tool for HNSCC patients undergoing organ preservation protocols | Arantes, L. M. et al<sup>179</sup> |
| NK cells            | Flow cytometry | 70     | Association               |         | The population of circulating immunoregulatory CD56(bright) NK cells is lower in the peripheral blood of patients with HNSCC compared with that in healthy donors | Wulff, S. et al (2009)<sup>180</sup> |
| PD-L1               | Plasma exosome (PCR) | 40     | Prognostic                |         | Circulating PD-L1(high) exosomes in plasma, but not soluble PD-L1 levels, were associated with disease progression | Theodoraki, M. N. et al<sup>181</sup> |
| PD-L1+CTC           | Negative selection | 30     | Prognostic                |         | CTCs can reflect the treatment effects of the use of immune checkpoint inhibitors | Tinhofer, I. et al<sup>182</sup> |
| mRNA(RT-qPCR)       | 113         |        | Prognostic/Predictive (CR)| Curative treatments | CTCs overexpressing PD-L1 may provide important prognostic information, and adjuvant PD-1 inhibitors may be useful in patients in whom PD-L1(+) CTCs are detected after curative treatment | Strati, A. et al<sup>183</sup> |
| RAS                 | Mutation (NGS) | 46     | Association               | Failure of EGFR-Tx | RAS mutations associated with acquired resistance to EGFR-targeted therapy in a substantial proportion of HNSCC patients | Braig, F. et al<sup>184</sup> |
| RRM1                | cfRNA (PCR) | 60     | Predictive (AE)           | CCRT    | RRM1 gene expression in cfRNA allows for estimation of the risk of severe oral mucositis in patients subjected to radiotherapy | Mikl, R. et al<sup>185</sup> |
| Saliva CD44         | ELIZA       | 26     | Diagnostic                |         | Salivary solCD44 was effectively detected in HNSCC at all stages | Franzmann, E. J. et al (2005)<sup>185</sup> |
| Saliva miRNA        | RT-qPCR    | 56     | Diagnostic                |         | Saliva-derived miRNAs miR-9, miR-134 and miR-191 may serve as novel biomarkers to reliably detect HNSCC | Salazar, C. et al<sup>186</sup> |
Several important advances have been achieved in recent years, that have improved the potential of liquid biopsy markers as robust clinical tools. Such advances include (a) estimation of tumor mutational burden through evaluation of plasma cfDNA; (b) more therapy-directed applications of CTCs, for example, PD-L1 expression in CTCs; (c) use of CTCs as a longitudinal monitoring tool to detect minimal residual disease after curative surgery; (d) CTC-derived xenografts used as surrogates to study tumor biology; and (e) three-dimensional CTC cultures and CTC-derived organoids to aid in individualized precision medicine.

7 | FUTURE PERSPECTIVES

The rapid evolution of the background understanding of cancer physiology and biology has affected every aspect of disease management and patient care. In this evolving era of precision medicine, there is an ever-building need, including in HNSCC, for novel prognostic and predictive biomarkers with robust clinical application.

Ideally, biomarker trials should be designed based on an actual clinical need; as such, peer review panels evaluating biomarker research proposals now pay close attention to the potential clinical utility of biomarker tests. Given the challenges of biomarker development discussed above, concerted efforts also need to be made to harmonize assays, methodologies, and cutoffs, to ensure consistency of results and allow accurate extrapolation of trial data to the clinical setting.

Identification of driver mutations relevant to specific targeted therapies remains an ongoing area of research. A number of genetic and histological markers under development may prove integral to patient selection for the testing of novel targeted therapies—for example, those being evaluated in the ongoing National Cancer Institute Molecular Analysis for Therapy CHoice (NCI-MATCH) Precision Medicine Clinical Trial. It is hoped that more purposeful patient selection will enable inhibition of specific aspects of oncogenic pathways, and optimize the applicability of trial data, with the goal of stabilizing disease and improving survival in the greatest number of patients possible.

Finally, future development of artificial intelligence technology may help predict clinical outcomes more precisely than current technology and traditional statistical analysis.

8 | CONCLUSION

This article critically reviewed more than 100 biomarkers reported in the literature, which were investigated and
expected to predict disease or treatment outcomes in patients with HNSCC. According to the level of current evidence, tissue p16 and HPV status remain the most robust biomarkers in HNSCC where the others still require large-scale, validation trials. Although some investigations on biomarker are still ongoing, it is becoming clear that liquid biopsies might have promising potential for clinical development as noninvasive data-gathering tools for HNSCC patient selection, prediction of disease course and treatment outcomes, and clinical decision making. In particular, targets such as PD-L1 expression on CTCs may provide important information to help predict outcomes with immunotherapy. In the near future, there is an urgent unmet need to further progress biomarker research through the critical phases of development and validation, to continually improve the care of patients with HNSCC.

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