MOLECULAR MARKERS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA: PROMISING SIGNS IN NEED OF PROSPECTIVE EVALUATION

Phillipe Lothaire, MD,1 Evandro de Azambuja, MD, MSC,2 Didier Dequanter, MD,1 Yassine Lalami, MD,2 Christos Sotiriou, MD, PhD,2 Guy Andry, MD,1 Gilberto Castro Jr, MD, MSC,2 Ahmad Awada, MD, PhD2

1 Department of Surgery, Institut Jules Bordet, Brussels, Belgium
2 Translational Unit of Medical Oncology Clinic, Institut Jules Bordet, Boulevard de Waterloo, 125, 1000 Brussels, Belgium. E-mail: ahmad.awada@bordet.be

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Abstract: Background. The aim of this article is to review recent developments in the biological understanding of head and neck squamous cell carcinomas.

Methods and Results. We describe the markers according to their function and their prognostic or predictive roles. Some associations can be found between molecular markers and invasiveness, aggressiveness, degree of differentiation, and tumor stage, but only a few clinical studies have shown an impact on prognosis. In addition, despite an increasing number of articles relating to this topic, the small number of patients included in the studies reported reduces the clinical implications of these results. Few studies applied a more comprehensive molecular analysis approach, such as DNA microarrays or differential expression profiling by polymerase chain reaction, to identify a combination of markers that could be more informative than a single molecular marker.

Conclusion. Some progress has been made with respect to molecular markers and head and neck cancers. Translational and prospective, hypothesis-driven research must proceed with sufficient rigor to facilitate the clinical applicability of such results.

Keywords: head and neck cancer; molecular markers; proliferative markers; squamous cell carcinoma; EGFR

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide with a global yearly incidence of 780,000 new cases.1 Despite aggressive and multidisciplinary treatment approaches, including preoperative or postoperative chemotherapy and/or radiotherapy with reconstructive surgery, there has been no significant improvement in 5-year survival over the past 20 years. Treatment failures still occur in the form of locoregional recurrence, distant metastases, and/or second primary tumors.2–4

Currently, treatment strategies rely on clinical, radiologic, and histopathologic parameters to determine the stage of the disease. It is widely accepted that the presence of lymph node metastases...
tases is the most adverse independent prognostic factor in HNSCC. Furthermore, tumors might differ according to their primary site, because the behavior of an oral cancer is quite different from that of a laryngeal one, for example.

Wide heterogeneity in terms of clinical outcome and response to treatment exists even in patients who are assigned to the same risk group. Thus, much work is currently focused on the identification of better biological and clinical factors that may serve as prognostic and predictive markers. The recent advances in basic research and genomics have improved our understanding of the biological basis of tumor development and progression. As a result, a variety of molecular tumor markers characterized in the laboratory have been studied in HNSCC for their potential to predict disease outcome or response to therapy in patients.

With these developments, we hope in the near future to be able to either predict patients at risk for disease progression after standard therapy or, for example, identify those who may benefit from postoperative radiotherapy as well as those with radioresistant tumors.5

In this article, we reviewed the literature on the use of molecular biomarkers in HNSCC as a potential prognostic and predictive tool using PubMed to find the articles. Our search was limited to articles published after 1990 and written in English. Only studies with multivariate analysis of clinical outcome were reported.

Molecular tumor markers were allocated into four groups according to their function: (1) tumor growth; (2) tumor suppression; (3) immune response; and (4) angiogenesis, tumor invasion, and metastatic potential.

**MOLECULAR MARKERS INVOLVED IN TUMOR GROWTH, PROLIFERATION, AND APOPTOSIS**

**Epidermal Growth Factor Receptor Family (Epidermal Growth Factor Receptor, Her-2/neu, c-erbB 3-4).** The epithelial growth factor receptor is a transmembrane receptor that binds epidermal growth factor (EGF), transforming growth factor (TGF)-alpha, and other regulating proteins. Epidermal growth factor receptor (EGFR)–mediated signals result in a complex cascade of signaling pathways that influence normal cellular proliferation and differentiation and lead to strong mitogenic activity.5 The EGFR family includes EGFR, HER-2/neu (c-erbB-2), HER-3 (c-erbB-3), and HER-4 (c-erbB-4).6 Overexpression of EGFR is observed in 42% to 80% of studied HNSCCs.7

Until now, seven of eight trials showed in multivariate analysis a poorer outcome for patients with EGFR overexpression, accounting for 756 patients (Table 1), than for those patients without. Most of the trials used immunohistochemistry (IHC) as a method to analyze EGFR, and two of them used polymerase chain reaction (PCR). The trials reported different primary tumor sites, numbers of patients (47–140), and follow-up periods (18–70 months).6,8–14 Five trials report worst overall survival (OS). Conversely, only Wen et al10 found no association between EGFR and outcome in 68 patients with laryngeal carcinoma treated with radiation ther-

<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Median follow-up</th>
<th>Method of detection</th>
<th>Prognostic value*</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity carcinoma (n = 47)</td>
<td>Surgery ± RT ± Chemotherapy</td>
<td>NR</td>
<td>IHC</td>
<td>OS (6)</td>
<td></td>
</tr>
<tr>
<td>Primary HNSCC (n = 109)</td>
<td>Chemotherapy</td>
<td>18 mo</td>
<td>IHC</td>
<td>OS and RFS (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Surgery RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laryngeal carcinoma (n = 140)</td>
<td>Surgery</td>
<td>49 mo</td>
<td>IHC</td>
<td>OS and RFS (9)</td>
<td></td>
</tr>
<tr>
<td>Laryngeal carcinoma (n = 68)</td>
<td>RT</td>
<td>70 mo (mean)</td>
<td>IHC</td>
<td>OS (10)</td>
<td></td>
</tr>
<tr>
<td>Primary HNSCC (n = 91)</td>
<td>Surgery ± RT ± Chemotherapy</td>
<td>49 mo</td>
<td>IHC</td>
<td>DFS (11)</td>
<td></td>
</tr>
<tr>
<td>Oral cavity carcinoma (n = 110)</td>
<td>Surgery ± RT</td>
<td>NR</td>
<td>DNA PCR for erbB-1/2</td>
<td>PFS (12)</td>
<td></td>
</tr>
<tr>
<td>Primary HNSCC (n = 59)</td>
<td>Surgery ± RT or Chemotherapy + RT</td>
<td>NR</td>
<td>ELISA</td>
<td>OS (2-y) (13)</td>
<td></td>
</tr>
<tr>
<td>Primary HNSCC (n = 54)</td>
<td>Surgery (adjuvant therapy in some of them)</td>
<td>26 mo</td>
<td>RT-PCR</td>
<td>OCSS (14)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RT, radiotherapy; NR, not reported; IHC, immunohistochemistry; OS, overall survival; HNSCC, head and neck squamous cell carcinoma; RFS, relapse-free survival; DFS, disease-free survival; PCR, polymerase chain reaction; PFS, progression-free survival; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-PCR; OCSS, overall cancer specific survival.

*Multivariate analysis: The expression of the marker predicts worse outcome.

Only univariate analysis was performed.
apy (RT). This study had the longest follow-up period (mean, 70 months), and only a univariate analysis was performed.

In patients treated with cisplatin-based chemotherapy for recurrent head and neck cancer, positive staining for c-erB-2 was correlated with shorter progression-free survival and OS ($p = .023$). Importantly, worse OS was also seen in patients with c-erB-2+ who were treated with cisplatin and paclitaxel ($p = .027$), suggesting that this overexpression may be associated with chemotherapy resistance in these patients.

EGFR seems to be strongly correlated with worse prognosis in HNSCC in patients treated with either chemotherapy or RT. A better understanding of its signaling pathways may improve the treatment results of patients in this setting.

**Cyclin D1.** Cyclins are very important during the cell cycle and are divided into four types: A, B, D, and E. Cyclin D1 (also known as PRAD1) is a protooncogene that responds to extracellular mitogens and is a controller of G1 phase progression through the cell cycle. The most commonly reported alteration of cyclin D1 is gene amplification (11q13). This results in the expression of a structurally normal protein, but at abnormally high levels, which leads the cell to a state of uncontrolled proliferation.

Cyclin D1 may be amplified in 30% to 50% of laryngeal carcinomas, and its deregulation may increase the aggressiveness of certain cancers. Capaccio et al. found in 32 HNSCCs that cyclin D1 expression is significantly correlated with tumor extension and advanced clinical stage ($p = .002$ and $p = .001$, respectively).

Five of seven studies analyzed cyclin D1 using the IHC method, and Southern blot and DNA amplification were used in other two (Table 2). The shortest follow-up period was at least 18 months and the longest was 60 months. All patients ($N = 812$) were treated with surgery with or without RT.

The prognostic significance of cyclin D1 remains controversial, although current data suggest a relationship between cyclin D1 overexpression/amplification and poor outcome. Five of seven trials showed either worse disease-free survival (DFS) or OS in patients with cyclin D1 overexpression/amplification than in patients without this characteristic. Three of these trials studied only patients with laryngeal carcinoma, and one included only patients with anterior tongue carcinomas. The largest trial with 282 patients failed to find a relationship between cyclin D1 gene amplification and DFS or OS. Nevertheless, the evidence from five trials suggests a poor prognosis for patients with HNSCC with cyclin D1 overexpression.

**Ki67 (MIB-1) Antigen.** Ki67 is a monoclonal antibody that binds to a protein that is expressed during the G1, S, and G2 phases of the cell cycle. There is a correlation between the percentage of Ki-67–labeled cells at the time of the surgery and the TNM stage. Several studies have also revealed that cell proliferation in invasive tumors measured by Ki-67 highly correlated with histologic grading in human oral squamous cell carcinoma (OSCC). In addition, a high percentage of immunolabeled cancer cells for Ki-67 has been significantly associated with neck metastases and inversely correlated with the degree of cancer cell differentiation.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Median follow-up</th>
<th>Method of detection</th>
<th>Prognostic value*</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal carcinoma ($n = 51$)</td>
<td>Surgery</td>
<td>29 mo</td>
<td>Southern blot analysis</td>
<td>OS</td>
<td>(17)</td>
</tr>
<tr>
<td>Primary HNSCC ($n = 282$)</td>
<td>Surgery ± RT</td>
<td>28 mo</td>
<td>DNA amplification of 11q13</td>
<td>No</td>
<td>(18)</td>
</tr>
<tr>
<td>Primary HNSCC ($n = 75$)</td>
<td>Surgery ± RT ± Chemotherapy</td>
<td>At least 18 mo</td>
<td>IHC</td>
<td>OS</td>
<td>(19)</td>
</tr>
<tr>
<td>Primary HNSCC ($n = 45$)</td>
<td>Surgery</td>
<td>44.5 mo</td>
<td>IHC and PCR</td>
<td>5-y survival</td>
<td>(20)</td>
</tr>
<tr>
<td>Laryngeal carcinoma ($n = 149$)</td>
<td>Surgery</td>
<td>60 mo</td>
<td>IHC</td>
<td>DFS</td>
<td>(21)</td>
</tr>
<tr>
<td>Anterior tongue carcinoma ($n = 148$)</td>
<td>Surgery ± RT</td>
<td>57 mo (mean)</td>
<td>IHC</td>
<td>OS</td>
<td>(22)</td>
</tr>
<tr>
<td>Laryngeal carcinoma ($n = 62$)</td>
<td>Surgery ± RT</td>
<td>More than 3 y</td>
<td>IHC</td>
<td>No</td>
<td>(23)</td>
</tr>
</tbody>
</table>

Abbreviations: OS, overall survival; HNSCC, head and neck squamous cell carcinoma; RT, radiotherapy; IHC, immunohistochemistry; PCR, polymerase chain reaction; DFS, disease-free survival.

*Multivariate analysis: the expression of the marker predicts worse outcome.
Four studies had used IHC for Ki-67 evaluation, with a total of 621 patients, a minimum follow-up of 31 months, and with controversial results (Table 3).29–32

Few studies investigated the role of Ki-67 in either DFS or OS. One of these studies showed no prognostic significance in multivariate analysis.30 It seems that patients with high levels of Ki-67 have better local control than those patients with low levels.29,32 Curiously, Ki-67 positivity predicted a worse OS in 105 primary HNSCC.31 Because of the scarce and conflicting data, no clear conclusions can be made for the use of Ki-67 in clinical practice.

Furthermore, in terms of prediction, two recent studies showed that highly proliferating tumors (Ki-67 >20%) might respond better to radiotherapy than slowly proliferative ones.29,32 These results are consistent with clinical experience, whereby rapidly proliferating tumors are more sensitive to radiation therapy than those growing slowly.24

**Bcl-2.** Bcl-2 is an antiapoptotic protein, being part of the regulatory system that controls the cell cycle and the induction of apoptosis. Alterations in apoptosis-related genes may play an important role in tumor progression and in the efficacy of cytotoxic therapy and radiotherapy.33 However, no significant correlation exists between the expression level of the bcl-2 protein with histologic grade and the presence of neck metastases in tongue carcinomas.34

Bcl-2 overexpression may be associated with poor prognosis in some types of cancer (breast, colon, and lung), favorable prognosis in others (cervix, melanoma, bladder, and prostate), or no significance in yet others (head and neck, endometrium, gastric, thyroid, and ovary).33

Two of six studies using IHC did not find any association between Bcl-2 overexpression and outcome (Table 4).35,36 Four of them found a better local control rate in bcl-2–positive patients, but an association with better OS was reported in only one study, including 400 patients with primary HNSCC enrolled in a randomized trial with continuous hyperfractionated accelerated radiation treatment or conventional RT.30,33,37,38 Conversely, Gallo et al37 reported that bcl-2 positivity was associated with worst OS and DFS in 71 primary HNSCCs. Currently, the relationship between bcl-2 and outcome is not well known, and

<table>
<thead>
<tr>
<th>Patients</th>
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<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary HNSCC (n = 111)</td>
<td>Chemotherapy + RT</td>
<td>46.9 mo</td>
<td>IHC</td>
<td>Better LC</td>
<td>(35)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 400)</td>
<td>CHART or RT</td>
<td>At least 4 y</td>
<td>IHC</td>
<td>Better LC and OS</td>
<td>(33)</td>
</tr>
<tr>
<td>Advanced HNSCC (n = 79)</td>
<td>Chemotherapy + RT</td>
<td>72 mo</td>
<td>IHC</td>
<td>No</td>
<td>(36)</td>
</tr>
<tr>
<td>Laryngeal carcinoma (n = 83)</td>
<td>Pre- or postoperative therapy</td>
<td>At least 10 y</td>
<td>IHC</td>
<td>No</td>
<td>(36)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 71)</td>
<td>RT</td>
<td>At least 5 y</td>
<td>IHC</td>
<td>Worse OS and DFS</td>
<td>(37)</td>
</tr>
<tr>
<td>Primary or recurrent HNSCC (n = 93)</td>
<td>CHART (3 times day)</td>
<td>19 mo</td>
<td>IHC</td>
<td>Better LC</td>
<td>(38)</td>
</tr>
</tbody>
</table>

Abbreviations: HNSCC, head and neck squamous cell carcinoma; RT, radiotherapy; IHC, immunohistochemistry; LC, local control; OS, overall survival; CHART, continuous hyperfractionated accelerated radiation treatment; DFS, disease-free survival.

*Multivariate analysis: High Bcl-2 tumors predict either better LC or worse outcome.
no standardization of this method in these patients has been done.

Interestingly, in terms of prediction, a recent study identified bcl-2 protein expression as an independent marker to tumor response to induction platinum–fluorouracil-based chemotherapy using two different cutoffs (5% and 10%) in 141 patients with HNSCC. However, the inclusion of one bcl-2+ patient who did not respond to the treatment resulted in a very high and imprecise 95% confidence interval upper limit (95% confidence interval, 2.3–170).39

Fas/FasL. Fas (or Apo-1/CD95) and its ligand FasL are important mediators of apoptosis. The Fas antigen is a cell surface protein that triggers apoptosis on binding to the Fas ligand, largely through activation of caspase 3, a downstream protein of the apoptotic signal transduction cascade.40 Fas antigen is a member of the receptor family of cell surface proteins, whereas FasL belongs to the tumor necrosis factor family.41

Upregulation of functional FasL was reported in human tumors such as the esophagus, lung, brain, colon, and pancreas.41 Chen et al41 showed downregulation of Fas and upregulation of FasL expression in 24 OSCC. Sundelin et al42 observed Fas expression in all of 24 moderately or well-differentiated squamous cell carcinomas (SCCs) of the tongue. Lora et al43 demonstrated that FasL expression was negatively correlated with the degree of differentiation and apoptosis in 19 OSCCs. There is evidence that Fas protein is fully functional on the surface of HNSCC44 and that serum levels of FasL are lower in patients with HNSCC with active disease than in those patients with no active disease or without tumors ($p < .0183$).45 Low serum levels and high levels of FasL expression on the tumor suggest that the tumor exerts systemic suppressive effects on immune cells, which may be partially mediated by the Fas/FasL pathway.46 No Fas receptors were detected in poorly differentiated tumors, which might indicate a lack of activation of the apoptotic pathway.47

Although Fas is a very important mediator for apoptosis, its role in prognosis in patients with HNSCC still needs to be defined, and more studies with large numbers of patients are needed to reach a clear conclusion.

The lessons learned from all these molecular markers involved in tumor growth, proliferation, and apoptosis show us that they might have a role on patient prognosis, in particular EGFR and cyclin D1.

MOLECULAR MARKERS INVOLVED IN TUMOR SUPPRESSION

p27. p27 (Kip1) is a cyclin-dependent kinase inhibitor (CDI), a member of the Cip/Kip group, which negatively regulates the G1 phase progression of the cell cycle by binding to the cyclinE/cyclin–dependent kinase-2 (CDK2) complex.48 p27 is activated in response to extracellular signals such as serum deprivation, contact inhibition, TFG-β,49 cyclic-AMP, and a growth inhibitory drug (rapamycin).50 p27 is not a frequent target of mutations predisposed to cancer and is considered a tumor suppressor gene.50

Overexpression of p27 using an adenovirus vector system in several HNSCC cell lines has shown a marked decrease in the proportion of cells in the S phase, an increase in the G1 phase, and a marked decrease in clonogenicity.51 In contrast, high p27 degradation activity has been found in highly invasive carcinoma cell lines (MSCC-1).52

Reduced expression of p27 has been associated with progression in precancerous lesions of the larynx (leukoplasia), indicating the role of p27 as a potent tumor suppressor gene.53 When 17 patients with oral epithelial dysplasia were followed until progression to carcinoma, the high p27 overexpression observed in the precancerous lesions (88%) was replaced by low p27 overexpression in carcinomas (82%), and this reduction may be involved in abnormal cell proliferation.54 Similar results are reported in 22 epithelial dysplasia specimens in 70 OSCC cases. Moreover, patients with metastases had lower levels of p27 compared with patients with tumors and no metastases.55

In 31 untreated patients with HNSCC, a significant correlation has also been found between low p27 (Kip1) expression and high-grade tumors, advanced T stages, and positive cervical nodal status. Furthermore, multivariate analysis indicated that p27 (Kip1) expression was the most significant predictor of OS among the different variables assessed ($p = .03$).48 Similarly, in 94 patients with oral tongue squamous cell carcinoma, those with low p27 levels had shorter 5-year survival rates than patients with high levels ($p = .024$).50 In another study, no difference in survival was observed in 93 patients with laryngeal and oral cavity carcinoma.49
Clearly, p27 plays a very important role in the cell cycle and carcinogenesis. Furthermore, low expression of p27 confers a worse prognosis in patients with HNSCC. However, it is not yet an acceptable marker for clinical decision making.

p53. In the late 1970s, p53 was discovered and was supposed to be a critical modulator of the cellular response to exogenous and endogenous stress. As inactivation of one or more components of the p53 network is an extremely common event in human neoplasia. In HNSCC, disabling p53 occurs in a high proportion of cases by mutation in the p53 gene. However, other mechanisms of inactivation, such as the presence of human papillomavirus (HPV) and molecular abnormalities in other components of this pathway, have also been reported. In patients with HNSCC who have HPV, this may occur by means of the interaction of p53 with the E6 protein encoded by so-called oncogenic HPV types, mainly HPV16 and HPV18. Another mechanism involves mouse double minute protein 2 (MDM2), which binds to p53 and promotes the ubiquitination of the C-terminus of p53 and its subsequent degradation.

In 69 patients with OSCC, expression levels of MDM2 combined with p53 assessed by IHC have been associated with tumor proliferation. In addition, combined expression levels of p53/p21 and p53/mdm2/p21 have been reported as significantly correlated with lymph node metastases. Similarly, p53 expression was positively associated with nodal involvement in 53 patients with laryngeal and tongue SCC. Conversely, p53 was not an independent predictor of metastases in 70 patients with advanced HNSCC.

The frequent changes occurring in the p53 pathway in HNSCC imply that molecular genetic and immunohistochemical analysis of this critical tumor suppressor network may be of diagnostic and prognostic use in the clinical management of HNSCC. However, the prognostic value of p53 remains controversial. Teppo et al. failed to find any statistically significant association between prognosis and p53, cell proliferation status, and angiogenesis in a cohort of 100 patients with laryngeal carcinoma.

In patients with HNSCC, many studies have been conducted to evaluate p53 as a prognostic factor. These studies had different numbers of patients (from 43–304), follow-up periods (39–120 months), and treatments (surgery, RT, surgery + RT, chemotherapy, chemotherapy + RT) (Table 5). Multivariate analysis showed no prognostic value in five of 17 studies using the IHC method to detect p53 mutation. Four of them had used PCR as a method to detect this. A significantly worse OS in patients with p53 overexpression was found in nine trials. Some authors failed to find a significant difference in OS, but they found either a worse DFS or local recurrence in patients overexpressing p53 than in those not overexpressing p53. In laryngeal carcinoma, worse OS was found in 62 patients, and worse DFS was reported in 83 patients. The former had more than 3 years of follow-up, and the latter had at least a 10-year follow-up. In patients with hypopharyngeal SCC, no prognostic value was found in 43 patients. In brief, our review revealed that 15 of 20 studies showed worse prognosis for those patients with a p53 mutation than those without, and five of the studies were unable to demonstrate this negative effect of the p53 mutation. In the era of evidence-based medicine, a meta-analysis, which is level 1 evidence-based, should be undertaken to help us to determine the real role of p53 mutations in patients with HNSCC.

With regard to p53 status and response to chemotherapy in HNSCC, Bradford et al. studied the relationship of p53 mutations and sensitivity to cisplatin in 23 HNSCC cell lines in vitro. They showed that HNSCC cell lines harboring p53 mutations were more sensitive to cisplatin than those cell lines with wild-type p53, suggesting the role of the p53 mutation as a potential predictive marker for response to cisplatin-based chemotherapy, although this difference was not significant (p = 0.4). In patients with HNSCC treated with cisplatin and 5-fluorouracil in a neoadjuvant setting, those whose disease was unresponsive to treatment had a higher prevalence of p53 mutations than responders had (p < .04). Similarly, p53 expression was a strong predictor of poor response in 236 patients with HNSCC treated with induction chemotherapy containing cisplatin and 5-fluorouracil. Concerning radiotherapy response, Kokoska et al. studied 121 patients with glottal SCC treated with exclusive radiotherapy, and no p53 predictive value was found.

A recent report has shown the utility of p53R2 mRNA expression, a new p53 target, as a marker to predict tumor development and sensitivity to radiochemotherapy in OSCC. p53R2 expression was detected by in situ hybridization and was more frequently expressed in dysplasias and SCC.
Interestingly, p53R2 was significantly associated with tumor size, lymph node metastases, and histologic differentiation, as well as with complete pathologic response to radiochemotherapy. The discovery of p53 autoantibodies (AAB) has created a new serologic method to detect these cells in HNSCC. Of note, 30% of patients who were initially seen with p53 AAB did not correlate with any histologic parameter, but a correlation with the clinical course of the disease was found in nine of 32 patients. Five of these nine are still alive without any sign of tumor, and they show constantly declining p53 AAB levels after primary therapy. Three of these nine cancer patients died during follow-up because of distant metastases, one died without known cause, and all had a p53 AAB increase before they died.

**Table 5. Trials of p53 in head and neck squamous cell carcinoma.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Median follow-up</th>
<th>Method of detection</th>
<th>Prognostic value*</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal carcinoma (n = 62)</td>
<td>Surgery ± RT</td>
<td>&gt;3 y</td>
<td>IHC</td>
<td>OS</td>
<td>(23)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 101)</td>
<td>RT</td>
<td>31 mo</td>
<td>IHC</td>
<td>LC</td>
<td>(29)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 105)</td>
<td>Chemotherapy + RT</td>
<td>At 4 y</td>
<td>IHC</td>
<td>RFS and DSS</td>
<td>(31)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 304)</td>
<td>RT</td>
<td>48 mo</td>
<td>IHC</td>
<td>LC</td>
<td>(32)</td>
</tr>
<tr>
<td>Advanced HNSCC (n = 79)</td>
<td>Chemotherapy + RT</td>
<td>72 mo</td>
<td>IHC</td>
<td>No</td>
<td>(35)</td>
</tr>
<tr>
<td>Laryngeal carcinoma (n = 83)</td>
<td>Preoperative or postoperative therapy</td>
<td>At least 10 y</td>
<td>IHC</td>
<td>DFS</td>
<td>(36)</td>
</tr>
<tr>
<td>Hypopharyngeal SCC (n = 43)</td>
<td>RT → Surgery</td>
<td>43 mo</td>
<td>IHC</td>
<td>No</td>
<td>(61)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 73)</td>
<td>Chemotherapy + RT</td>
<td>NR</td>
<td>IHC</td>
<td>DFS</td>
<td>(62)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 69)</td>
<td>Surgery</td>
<td>NR</td>
<td>IHC</td>
<td>Time to SPM and TTF</td>
<td>(63)</td>
</tr>
<tr>
<td>Oral or oropharyngeal SCC (n = 69)</td>
<td>Surgery</td>
<td>At least 5 y</td>
<td>IHC</td>
<td>OS</td>
<td>(64)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 115)</td>
<td>RT</td>
<td>12–55 mo</td>
<td>PCR</td>
<td>LDDFS</td>
<td>(66)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 39)</td>
<td>RT or chemotherapy</td>
<td>2–54 mo</td>
<td>PCR</td>
<td>DFS and OS</td>
<td>(69)</td>
</tr>
<tr>
<td>Primary HNSCC (n = 68)</td>
<td>Neoadjuvant chemotherapy</td>
<td>At 24 mo</td>
<td>IHC</td>
<td>OS</td>
<td>(70)</td>
</tr>
<tr>
<td>Advanced HNSCC (n = 111)</td>
<td>Chemotherapy + RT</td>
<td>NR</td>
<td>IHC</td>
<td>DFS and OS</td>
<td>(71)</td>
</tr>
<tr>
<td>Laryngeal, pharyngeal, and oral cavity SCC (n = 114)</td>
<td>RT or surgery</td>
<td>After 60 mo</td>
<td>DGGE and PCR</td>
<td>LC, DFS, DSS, and OS</td>
<td>(72)</td>
</tr>
<tr>
<td>Invasive HNSCC (n = 190)</td>
<td>Surgery</td>
<td>30 mo</td>
<td>IHC and PCR</td>
<td>OS and DSM after 550 days</td>
<td>(73)</td>
</tr>
<tr>
<td>Advanced laryngeal or pharyngeal SCC (n = 71)</td>
<td>Chemotherapy ± RT</td>
<td>67.4 mo</td>
<td>IHC</td>
<td>OS</td>
<td>(74)</td>
</tr>
</tbody>
</table>

Abbreviations: RT, radiotherapy; IHC, immunohistochemistry; OS, overall survival; HNSCC, head and neck squamous cell carcinoma; LC, local control; RFS, relapse-free survival; DSS, disease-specific survival; DFS, disease-free survival; NR, not reported; SPM, second primary malignancy; TTF, time to failure; SCC, squamous cell carcinoma; PCR, polymerase chain reaction; LDDFS, locoregional DFS; DGGE, denaturing gradient gel electrophoresis. *Multivariate analysis: the expression of the marker predicts worse outcome.

p53 and p27 are the two most studied molecular markers involved in tumor suppression in HNSCC. For p27, its low expression has been related to carcinogenesis, and it is associated with a worse outcome. p53 was linked to poor prognosis in most studies and has been implicated in outcome for different tumors. Either a meta-analysis using individual patient data or randomized prospective trials should be conducted to better clarify its prognostic role in HNSCC.

**MOLECULAR MARKERS INVOLVED IN TUMOR ANGIOGENESIS**

Angiogenesis is the growth of new microvessels, and this process depends on the motility, proliferation, and tube formation of endothelial...
cells. Tumor vascularization is thus one of the rate-limiting steps for tumor growth, invasion, and metastases.

The vascular endothelial growth factor (VEGF) family contains specific and highly potent angiogenic proteins that act to increase vessel permeability, endothelial cell growth, proliferation, migration, and differentiation. At least six members of the VEGF family have been identified so far, including VEGF-A/vascular permeability factor, placenta growth factor, VEGF-B/VEGF–related factor, VGFRC/VEGF–related protein, VEGF-D/c-fos–induced growth factor, and VEGF-E. VEGF and basic fibroblast growth factor (bFGF) are mitogenic and chemotactic for endothelial cells and are known to accumulate at the site of angiogenesis in situ.

Shang et al, in a small study of 31 patients with OSCC, reported the circulating level of VEGF and its relationship with clinicopathologic features and prognosis. They showed that high levels of serum VEGF correlated with node metastases (p = .011) and clinical stage (p = .024). Enhanced expression of VEGF-A (isoforms 121 and 165) and VEGF-C may also predict the presence of cervical lymph nodes metastases. Correlation between the intensity of VEGF expression and lymph node metastases was reported in 44 patients with primary oral SCC.

Similarly, Teknos et al assessed serum VEGF levels in a cohort of 183 patients with HNSCC, as well as in normal, healthy controls. They found that serum VEGF levels were significantly elevated in patients with laryngeal carcinoma compared with healthy controls. In addition, they showed that high levels of VEGF were associated with a supraglottal location of the tumor and with node-positive disease. Finally, elevated pretreatment serum VEGF levels tended to indicate an aggressive disease state and a short OS.

Neuchrist et al have investigated the expression of VEGFR3 and its ligand VEGF-C by semiquantitative reverse transcriptase–PCR (RT-PCR) in four HNSCC cell lines and six HNSCC specimens and by IHC in another 18 HNSCC tumor specimens from 18 patients. They failed to find a correlation between the expression of VEGF-C and clinical parameters. In addition, Ninck et al assessed the expression of several angiogenic cytokines, including VEGF, bFGF, platelet-derived growth factor (PDGF)-AB, PDGF-BB, granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) in HNSCC in vivo. They showed that tumors producing at least three cytokines revealed a significantly poorer patient prognosis, with a worse OS in univariate analysis, than tumors that did not produce this. Moreover, they suggested that VEGF and PDGF-AB might play a key role in HNSCC and that the additional secretion of G-CSF or GM-CSF might contribute to a poor prognosis.

In 45 patients with oral SCC, those who were VEGF positive had poorer OS than those who were not. High levels of VEGF correlated with more recurrence and a short disease-free interval. Similar results were found in patients with high levels of VEGF and interleukin-8 (IL-8). Increased levels of b-FGF showed a significant correlation with short time of locoregional control in 26 patients with advanced HNSCC.

In multivariate analysis, Salven et al showed no association between VEGF and OS in 156 patients with HNSCC treated with surgery and postoperative radiotherapy. Conversely, in 77 patients with oral or oropharyngeal SCC treated with surgery and postoperative radiotherapy, VEGF was the most significant predictor of poor DFS (relative risk [RR], 2.75; 95% confidence interval [CI], 1.30–5.79) and OS (RR, 3.53; 95% CI, 1.75–7.13). In 60 patients with HNSCC, the 2-year DFS of patients with high VEGF levels was significantly lower than that of patients with low VEGF levels (p = .01).

In brief, VEGF has been highly correlated with nodal invasion and worse prognosis in patients with HNSCC. New biological agents targeting tumor angiogenesis have been studied alone or in combination with chemotherapy to achieve better results in this patient population, and the results of this research will be available soon.

Hypoxia-inducible factors (HIF1α and HIF2α) are key proteins for response to hypoxic stimulus. The normal head and neck mucosa does not show any reactivity for these two factors. In human oral cancer cell lines, hypoxia is a mechanism that upregulates VEGF and HIF2α. In HNSCC, HIF1α and HIF2α overexpression are significantly associated with high microvessel density, VEGF expression, incomplete response to chemotherapy, and a poor local relapse-free survival (RFS) and OS.

Tumor angiogenesis can be quantified by counting vessels per high-powered microscopic fields. CD-31, the technique of choice to detect microvessel density (MVD), failed to show any correlation between tumor aggressiveness and tumor angiogenesis in 19 patients with T1 oral
cavity SCC. Using the IHC technique, no relationship between mean MVD and DFS or OS was seen in 39 patients with HNSCC. Conversely, in 20 of 332 patients with laryngeal SCC enrolled in the prospective trial “The Department of Veterans Affairs Cooperative Studies Laryngeal Cancer Study #268”, the mean vessel count was significantly lower in patients who had had chemoresponsive tumors \( (p \leq .008) \). The most vascular tumors, those greater than 1 SD above the mean, had a poor OS \( (p = .0345) \).

Tumor microvessels are important for tumor growth and survival, and the blockage of the tumor vascular bed seems to be an attractive approach in cancer treatment. Studies using biological agents, alone or in combination with chemotherapy, have already entered the clinic, and it is hoped that the results will confirm the exciting preclinical data.

**MOLECULAR MARKERS INVOLVED IN TUMOR INVASION AND METASTATIC POTENTIAL**

Matrix metalloproteinases (MMPs) are a family of zinc metalloenzymes that are involved in extracellular matrix remodeling. The family of MMPs is subdivided into collagenases, gelatinases, stromelysins, stromelysins-like MMP, membrane-type MMPs, and new MMPs. It has been shown that malignant cells are capable of using MMPs to help break down the basement membrane and degrade interstitial stroma, thus facilitating tumor invasion and/or metastases. The expression of MMPs in OSCC has been reported to be correlated with tumor stage. However, several published reports showed controversial results of MMPs and their tissue endogenous inhibitors (tissue inhibitors of metalloproteinases; TIMPs) in relation to tumor progression, as well as to the association between the plasma activity of these enzymes and the known clinical and pathologic parameters of patients with head and neck carcinoma.\(^4^,\(^1\)\)

MMP-2 expression was significantly correlated with nodal status and OS in 106 patients with advanced HNSCC. Molecular analysis of surgical margins was done in 52 patients treated with surgery with or without RT. Fourteen of 52 patients had recurrences, and 11 (79%) of these patients had MMP-9–positive margins. There was a significant difference in the recurrence rate between MMP-9–positive and MMP-9–negative margins \( (\text{chi-square} = 4.71; p = .03) \). A significant correlation between patients with node-positive disease and overexpression of MMP-9 was observed \( (p = .002) \).

Serum levels of MMP-2 and MMP-9 were collected in 86 patients with HNSCC and in 47 normal controls. Using a quantitative sandwich enzyme immunoassay technique, the MMP-9 concentration was significantly higher in patients with HNSCC than in the controls \( (p = .001) \) and was correlated with advanced-stage disease \( (p = .0449) \). No difference was found in MMP-2 serum levels. MMP-3, MMP-8, and MMP-9 serum concentrations had a statistically significant difference in 73 patients with HNSCC and 69 controls \( (p < .001; p = .04; p = .001) \), respectively. A significant correlation was found between MMP-8 serum concentration and T status \( (p = .02) \), N status \( (p < .001) \), and Union Internationale Contre le Cancer (UICC) staging \( (p = .03) \).

Although a correlation exists between MMP and cancer development in these studies, its use is not recommended outside clinical trials. More studies including a large number of patients are necessary to define the real prognostic role of MMP in HNSCC.

**OTHER MOLECULAR MARKERS**

The melanoma antigen (MAGE) genes encode certain tumor-associated antigens recognized by cytotoxic T lymphocytes. To identify immune response targets in HNSCC, Kienstra et al studied the presence or absence of three antigens identified to stimulate immune response, namely, NY-ESO-1, MAGE-1, and MAGE-3, in 45 HNSCC specimens by either RT-PCR or IHC. Interestingly, 27 of the 45 tumors assessed were positive by RT-PCR for at least one of the antigens, indicating a potential approach to immunotherapy for HNSCC. However, Kienstra et al were unable to find any correlation between antigen expression and patient demographics.

Finally, the binding of labeled galectin-3, an endogenous lectin that reacts with glycan epitopes of membrane and extracellular glycoproteins in primary HNSCC (from the tonsil, base of the tongue, and larynx), and lymph node metastases has been assessed. It has been demonstrated that galectin-3 has bound a nonproliferating pool of tumor cells and can be localized with desmosomal proteins, suggesting that galectin-3 might be a potential new tool for monitoring the degree of cell differentiation in HNSCC. Unfortunately, these markers are still not ready, and a better
understanding of their role as prognostic markers in HNSCC is necessary.

Recently, a combination of circumstances, including the advent of array-based technology and progress in the human genome initiative, has provided us with an ideal opportunity to begin efforts aimed at performing comprehensive molecular and genetic profiling of human cancers that will lead to a greater understanding of the basic biology of HNSCC. Microarray technology is a method used to interrogate 1000 to 40,000 genes simultaneously. This technique permits the assessment not of individual genes but of clusters of genes that are coordinately expressed to generate “fingerprints” of biological status. Comparative genomic hybridization is a technique for detecting segmental genomic alterations, in particular chromosomal aberrations, giving us the possibility to detect genomic changes in malignant cells in cases where aberrations are too complex to study or when chromosomes are not available at all.109,110

When comparing the gene expression profiles of HNSCC and normal tissue, in the former, altered expression levels of genes involved in the control of cell growth and differentiation, angiogenesis, apoptosis, cell cycle, and signaling may be seen.108

Following from this, Lemaire et al1 undertook a large-scale differential display comparison of hypopharyngeal SCC and histologically normal tissue in a small series of patients. They identified 70 genes that exhibit a striking difference in expression in tumor versus normal tissue. Thirty-six genes were detected with a greater than twofold higher expression in tumor tissue than in normal samples, and, inversely, 34 genes were detected with a greater than twofold higher expression in normal tissue than in tumor samples. At the genome level, a series of differentially expressed genes cluster at 12p12-13 and 1q21, two hotspots of genome disruption. The known genes share functional relationships in keratinocyte differentiation, angiogenesis, immunology, detoxification, and cell surface receptors. The differentially expressed genes that were identified are potential new markers and therapeutic targets.

Patients with hypopharyngeal SCC have six overexpressed genes (EIF4G1, DVL3, EPHB4, MCM7, BRMS1, and SART1) and, from the comparison of patients with nonaggressive and aggressive tumors (without or with clinical evidence of metastases 3 years after surgery), it was found that 164 different expressed genes are probably involved in the acquisition of metastatic potential.111 From a study of nine patients with HNSCC, gene expression changes were reproducibly observed in 227 genes representing previously identified chemokines, tumor suppressors, differentiation markers, matrix molecules, membrane receptors, and transcription factors that correlated with neoplasia, including 46 previously uncharacterized genes.112

An analysis of 45 primary HNSCC by comparative genomic hybridization revealed significant correlations between the gain of 3q25-27 and deletion of 22q with reduced disease-specific survival. In addition, the gain of 17q and 20q, deletion of 19p and 22q, and amplification of 11q13 were significantly associated with reduced disease-free survival. A Cox proportional hazard regression model identified the deletion of 22q as an independent prognostic marker.113 Belbin et al114 studied a set of 9216 sequence-verified human IMAGE cDNA clones in 17 patients with HNSCC treated with surgery. Two genotypic groups were identified, and a trend to better 2-year cause-specific survival in the second group was found (56% vs 100%; p = .057).

Smad2 protein was expressed in 99% of 170 HNSCC specimens. The activated form of Smad2, pSmad2, was expressed in 86% of these tumors, indicating their ability to survive and proliferate despite the presence of bioactive TGF-β within the tissue microenvironment.115

Another study suggested that the development of cisplatinum-resistant phenotypes in head and neck cancer is accompanied by alterations of gene expression, including a glycoprotein hormone and membrane proteins. These gene products might be new molecular markers for resistance to cisplatinum. They demonstrate an upregulation of GPHα in cisplatinum-resistant HNSCC lines and two downregulated genes, human folate receptor and L6 antigen.116

Using Atlas human cancer 1.2 cDNA array in 1187 tumor-related genes in either radioresistant or radiosensitive tumors, 60 tumor-related genes were selected as a predictors of radiation response of HNSCC.117 According to a very recent literature review, this is a powerful method that has an immense potential to help us understand the important genetic changes in HNSCC.118

These high-throughput techniques are promising; however, they are not ready to be used in the clinic. In the near future, tailored treatment may become an option for patients with HNSCC,
which would avoid excessive toxicities from over-treatment.

CONCLUSION

Head and neck cancers are heterogeneous in location as well as in their biological or clinico-pathologic behavior. After the enthusiasm surrounding p53, many biomarkers have been identified and are now available. However, despite advances in new techniques, those markers present limited interest for the clinician so far and are not able to provide firm conclusions about their value in screening and as prognostic and predictive factors.

The trials undertaken so far are also too heterogeneous to provide reliable data, as is the case with p53. Some studies have demonstrated an association between p53 abnormalities and poor outcome, but other large studies have failed to demonstrate such an association.

There is no doubt, however, that future developments will render these biological markers as useful diagnostic, prognostic, and predictive tools. Array technologies have made the examination of genetic events possible and for thousands of genes to be monitored simultaneously, making it possible to better understand the events that characterize the different stages of cancer development. Nevertheless, to achieve useful clinical goals, we need well-designed translational research programs and well-conducted prospective trials.

REFERENCES

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