CAN BIOMARKERS PLAY A ROLE IN THE DECISION ABOUT TREATMENT OF THE CLINICALLY NEGATIVE NECK IN PATIENTS WITH HEAD AND NECK CANCER?

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Abstract: For most patients with head and neck squamous cell cancer, a treatment decision concerning the neck is required. Since detection of small metastases in lymph nodes is difficult, many of the patients with no detectable metastases receive elective treatment of the neck. Additional information on the metastatic potential of the primary tumor before treatment may be useful to reduce the number of these elective procedures. Biomarkers may supply such information. Molecules involved in several pathways have been studied, but the complexity of the metastatic process makes it unlikely that a single marker for metastasis can be identified. Techniques allowing the study of many factors simultaneously seem to be the most promising ones. In recent years, microarray expression profiling and comparative genomic hybridization studies have yielded interesting results. If these results can be confirmed in larger studies, they may play a role in future clinical decision making on treatment of the clinically N0 neck.

Keywords: biomarkers; metastasis; neck dissection; head and neck cancer

One of the basic issues in the treatment of patients with head and neck squamous cell cancer (HNSCC) is the treatment of the clinically N0 neck. If nodal metastases are present, there is general agreement that the neck should be treated; on the other hand, in the absence of nodal metastasis, the decision to treat the neck is less clear-cut. The problem in clinical practice is that the accuracy of diagnostic (imaging) techniques to assess the neck is still limited.1,2 Therefore, uncertainty about the true status of the neck remains even after the use of the most advanced imaging techniques.
As a result of the limitations to accurate staging of the neck, many head and neck surgeons electively treat the neck of patients whose disease has been classified N0 when, based on T classification and site of the primary tumor, the likelihood of occult metastasis is estimated to be more than 15% to 20%. In most patients with an N0 neck who are treated surgically, a selective neck dissection will be performed.3,4

The alternative strategy for the N0 neck is to “wait and watch.”6 No study has convincingly demonstrated higher cure rates for patients undergoing elective neck dissection when compared to “watchful waiting.” However, in the latter group, much will depend on delay of treatment once the (previously occult) nodal metastases become apparent, and the intensity of follow-up is likely to influence outcome. Many (chiefly retrospective) studies have examined this subject with controversial results, and so the debate on the treatment of the N0 neck remains unresolved.

As a result of these conflicting strategies, some patients with head and neck cancer may receive suboptimal treatment. As many as 80% of patients with N0 disease will receive unnecessary treatment of the neck (along with its concomitant morbidity). Shoulder dysfunction in particular can be a disabling consequence of neck dissections, even when the spinal accessory nerve is preserved, with serious impact on the quality of life.6–8 Moreover, selective neck dissection is often considered a less appropriate treatment than a comprehensive (modified) radical neck dissection in patients with nodal metastasis, which had been mistakenly classified as N0 before treatment. On the other hand, a watchful waiting strategy harbors the risk of progression of occult metastasis to potentially incurable disease. Moreover, extracapsular spread, a known prognostic factor,9–11 is more common once metastasis becomes clinically apparent compared with clinically occult metastasis.12

If diagnostic techniques to assess the clinically negative neck before treatment could be improved, then metastases that were previously occult would be detected more readily. Such patients would then be treated therapeutically instead of electively, and this would reduce the risk of occult regional metastases in the remaining patients with N0 disease. If the incidence of occult regional metastasis could significantly be reduced to <20%, then the number of elective neck treatments could be decreased.

Most commonly used imaging modalities for assessment of nodal metastases are limited by the simple fact that metastases need to reach a certain size before they are detected. Up to one third of nodal metastases in laryngeal and hypopharyngeal cancer are micrometastases (<3 mm),13 which is at best about the detection threshold of advanced imaging techniques. This limitation has led to the search for additional more sensitive parameters or markers for nodal metastasis.

The choice of treatment and the prediction of tumor behavior is currently based mainly on the TNM classification. This in turn is based on anatomical considerations. However, tumor behavior may differ widely within these T categories. Biomarkers reflecting molecular alterations of the primary tumor and surrounding stroma may someday yield information about the metastatic potential of the tumor. Suitable biomarkers might eventually enable pathologists and clinicians to estimate the chance of nodal metastases in the individual patient, irrespective of the actual size of the tumor. Such advances would permit more appropriate therapeutic selection and limit elective treatment of the neck to patients who are likely to develop nodal metastases based on the molecular profile of the primary tumor.

The processes of carcinogenesis and metastasis in particular are bewilderingly complex. To metastasize, cells have to go through a series of alterations and modifications.14,15 This means that it is very likely that more than 1 marker will be required to assess an individual patient’s risk of nodal metastasis. The prevailing concept is that, in the initial phases of tumor progression, tumor cells undergo genetic changes, providing some kind of proliferative advantage. These advantages may be the ability to resist growth-inhibiting signals, the avoidance of programmed cell death (apoptosis), and the induction of blood-vessel growth (angiogenesis). To be capable to metastasize, tumor cells must acquire other additional capacities like degradation of the basal membrane, loss of cell adhesion and migration, lymphangiogenesis, and the ability to survive in the environment of the metastatic site. Whether these metastatic capacities are acquired later or initially in tumor progression remains unclear, although it seems likely that, in contrast to what has been commonly presumed, these changes may occur early.15 Moreover, many of these capacities may vary in time. For example, cell adhesion must decrease to allow cells to migrate and metastasize, but cell adhesion is again needed to settle at the metastatic site.

Many biomarkers have been studied for correlations with the presence of nodal metastasis in
HNSCC with widely varying results. Some of the particularly relevant markers are discussed in the following sections and are summarized in Table 1. The expression of most of them has been studied by immunohistochemistry.

**HISTOPATHOLOGICAL FEATURES**

Histopathological features like differentiation and tumor-associated inflammatory reaction were among the first markers to be studied in relation to the likelihood of development of lymph node metastasis in HNSCC.

The degree of inflammatory reaction surrounding a tumor has been proposed as a significant indicator of likelihood of progression of tumor growth and metastasis. A correlation between the presence of an inflammatory reaction and the absence of lymph node metastasis has been reported in the literature. 16

Some authors have described a relationship between the development of lymph node metastases and grade of differentiation. 16,17 Poorly differentiated tumors should, intuitively, be more likely to behave aggressively (and carry with them a greater metastatic potential) compared with well-differentiated tumors. However in many studies, different types of grading systems were used, making the evaluation of the results of these studies difficult, and in many studies, a correlation with the presence of nodal metastases could not be established.

The growth pattern of tumors has also been studied in relation to tumor behavior. A more invasive growth pattern is often presumed to be an indicator of more aggressive tumor behavior associated with a greater likelihood of recurrence and metastasis. In early-stage cancer of the oral tongue, growth pattern and depth of invasion have indeed been reported to correlate with both the presence 18 and delayed development 19–22 of nodal metastasis.

**GENES INVOLVED IN CELL CYCLE REGULATION, CELL PROLIFERATION, AND APOPTOSIS**

Although genes and proteins involved in these basic processes of tumor growth may not seem, on initial examination, to be directly involved in the process of metastasis, they are nevertheless important markers. For example, many tumor cells undergo apoptosis after intravasation. 23 Therefore, if apoptosis is inhibited, the metastatic potential might increase. Moreover, the tumor cells that arrive at a secondary site do not necessarily proliferate immediately. They may remain dormant until conditions for growth become favorable. 24

Several studies reported that amplification of the 11q13 region correlates with a variety of clinicopathological factors, including the presence of lymph node metastasis. 16,25–28 Although various genes are consistently present on the amplified 11q13 region, only CCND1 and EMS1 are likely to be the key genes within this region, because in addition to being frequently coamplified, they were found to be overexpressed in all tumors with 11q13 amplification. Cyclin D1 expression, studied with immunohistochemistry, has been found in about 45% to 50% of HNSCC 29 and appears to have prognostic significance. 30 A relation between overexpression of the cyclin D1 protein and lymph node metastasis has been described in HNSCC. 31,32 Other authors, however, did not find such a correlation of cyclin D1 expression with N classification. 29,33

Cortactin, the less frequently studied product of the other 11q13 gene EMS1, is a component of invadopodia. These structures are associated with

<table>
<thead>
<tr>
<th>Table 1. Steps in the process of metastasis and their relevant molecules.</th>
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<tr>
<td><strong>Cell-cycle regulation, cell proliferation, and apoptosis</strong></td>
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<td>Cyclins</td>
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<td>Syndecan-1</td>
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<td>E-selectin/sialyl Lewis-X and -A</td>
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<td>E-cadherin</td>
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<td>Proteolysis (degradation of the basal membrane and extracellular matrix components)</td>
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<td>Matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinases (TIMPs)</td>
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<td>Urokinase type plasminogen activators (uPA)/plasminogen activator inhibitors (PAI)</td>
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<td>Cortactin</td>
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<td>Microenvironmental factors: angiogenesis, lymphangiogenesis, and hypoxia</td>
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<td>Carbonic anhydrases (CA)</td>
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<td>Vascular endothelial growth factor (VEGF)</td>
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<td>Chemokines (direction of tumor cells)</td>
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<td>CXCL12 (stromal cell-derived factor-1, SDF-1) and its receptor CXCR4</td>
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Abbreviations: EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase.
extracellular matrix degradation, a topic that will be discussed later in this article. It may explain the association between cortactin overexpression and invasive and aggressive tumor behavior. 

Therefore, although amplification of the 11q13 genes seems to be correlated with the presence of nodal metastasis, this correlation has not conclusively been established for the expression of these genes.

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that regulates cell growth and invasive behavior. Expression of EGFR is found in a high number of patients with HNSCC. Overexpression of EGFR (measured by immunohistochemistry) has been associated with enhanced invasive and metastatic capacity, by way of stimulation of matrix metalloproteinases (MMPs). A correlation between overexpression (measured by immunohistochemistry or reverse transcriptase-polymerase chain reaction [RT-PCR]) of EGFR and nodal metastasis or high tumor stage at initial diagnosis has been described by some authors, although others have failed to find such a relation in immunohistochemical studies. However, as the expression rate of EGFR in HNSCC is high, the receptor is a target for many immunotargeting treatment studies, and molecular blockade of EGFR may ultimately prove its ability to influence the invasive and metastatic capacity in HNSCC.

One of the most frequently studied markers in HNSCC is p53. Point mutations of p53 are one of the most frequent genetic alterations in HNSCC. Such mutations lead to nuclear accumulation of the protein. Clinicopathological studies of alterations in p53 in HNSCC have shown varying results. A correlation with nodal metastasis was found in some predominantly immunohistochemical studies but was not established in most other studies. It appears at this juncture that p53 is not a significant factor in tumor behavior once invasion has occurred.

The p21 gene located at 6p21 is transcribed by p53 and produces a cyclin-dependent kinase inhibitor that induces cell cycle arrest. A correlation between decreased expression and nodal metastasis was described in some immunohistochemical studies, while others failed to find a correlation.

Survivin is a member of the inhibitor of apoptosis protein family that has been implicated in both control of cell division and inhibition of apoptosis. Specifically, its antiapoptotic function seems to be related to the ability to directly or indirectly inactivate caspases. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation. In addition, survivin overexpression has been associated with nodal metastasis in laryngeal and oropharyngeal squamous cell carcinoma in immunohistochemical studies.

**CELL ADHESION**

Changes in cellular adhesion are one of the key processes in metastasis; weakening of cell–cell and cell–extracellular matrix adhesion is obviously imperative for tumor cells to metastasize. Several molecules involved in cell adhesion have been studied, including cadherins, integrins, selectins, and CD44 isoforms.

Cell–cell adhesion in epithelial tumors is largely modulated by cadherins, and E-cadherin in particular. Cadherins are transmembrane glycoproteins responsible for Ca-dependent cell adhesion. Reduced expression can be caused by loss of E-cadherin expression, redistribution to other sites within the cell, and shedding and competition from other proteins. Such reduced expression may lead to invasion and metastasis. It has been demonstrated that downregulation of the E-cadherin gene is associated with poorly differentiated tumors, and invasion and metastasis in a variety of different types of cancer. In several, predominantly immunohistochemical, studies of HNSCC, a correlation was found between loss of expression of E-cadherin in the primary tumor and development of nodal metastases. However, other studies of HNSCC patients failed to find such a statistically significant relation.

As transmembrane glycoproteins, CD44 isoforms mediate contact between the extracellular matrix and the cytoskeleton. Their expression has been studied by immunohistochemistry by several groups. Decreased expression of CD44v6 in HNSCC and of CD44h in laryngeal cancer and cancer of the oral tongue has been associated with a higher metastatic tendency. Other authors, however, found no such a relation for CD44 in oral cancer, and CD44v6 in supraglottic laryngeal cancer and oropharyngeal cancer, and CD44h in cancer of the larynx and hypopharynx.

Syndecan-1 is a member of the syndecan family of cell surface heparan sulfate proteoglycans. High expression of this molecule is found on the entire surface of cells of stratified squamous epithelia, suggesting a role in cell adhesion and inter-
action with the extracellular matrix. The expression of syndecan-1 is reduced in cancers, including HNSCC. Differences in expression of syndecan-1 have been found to be related to prognosis, clinical stage of the disease, and the presence or absence of lymph node metastasis in laryngeal cancer, and other HNSCC, although this correlation (with metastasis in particular) was not consistently found in all similar immunohistochemical studies.²¹

To metastasize through lymph or blood vessels, cancer cells need to bind to endothelial cells. These endothelial cells express several adhesion molecules. E-selectin, in particular, is such an adhesion molecule that seems to contribute to binding of cancer cells. It is an inducible cell adhesion molecule found on vascular endothelium at sites of inflammation. Sialyl Lewis-X and -A (sLx and sLa) are its ligands. It is not completely clear whether expression of sLx and sLa by tumor cells or expression of E-selectin at the site of the metastasis is an important factor in the metastatic process.²²,²³

Expression of sLx and sLa on tumor cells has been correlated with advanced tumor stage, survival, and metastatic potential. In patients with skin cancer, expression of sLx and sLa was not found in normal skin and basal cell cancer, but it was found in all investigated squamous cell cancers, perhaps explaining the relatively low metastatic potential of basal cell cancers. Expression of sLa, but not sLx, was found to correlate with nodal metastasis in oral cancer.²⁴ Other studies found a trend toward higher expression of sLx at the site of metastasis, but no correlation with disease status.²²

In addition to reduced cell–cell adhesiveness, loss of adhesion of the epithelial cells to the extracellular matrix is also one of the fundamental pathways that promote tumor cell migration, invasion, and metastasis. A key factor involved in the control of cellular–extracellular matrix interactions is the focal adhesion kinase (FAK), an intracellular tyrosine kinase protein that is localized to cellular focal contact sites.²⁴ FAK protein overexpression has been demonstrated in different tumor types, and in some of them it has been correlated with the invasive potential of a tumor and poor patient prognosis.²⁵ FAK has been reported to be overexpressed in HNSCC, and strong FAK expression has been associated with nodal metastasis.²⁶ Moreover, the combination of E-cadherin and FAK expression resulted in a superior accuracy to assess the likelihood of nodal metastasis.²⁷

Since invasion and metastasis are very complicated multistep processes, it is not surprising that in this study, by using 2 markers, the ability to predict the presence of nodal metastasis was increased when compared with studies that analyzed only E-cadherin or FAK expression.

**PROTEOLYSIS**

Tumors need enzymes to degrade the basement membrane and extracellular matrix and to invade surrounding tissues. Loss of basement membrane components, in particular, has been shown to correlate with invasive and metastatic potential.²⁸,²⁹

MMPs are enzymes with proteolytic activity against extracellular matrix components like collagen, elastin, fibronectin, and gelatin. Both tumor cells and stromal cells can secrete MMPs. They belong to a family of 21 members. MMP-2 and MMP-9, in particular, are capable of degrading collagen type IV, a major component of the basement membrane. Increased MMP levels have been detected in HNSCC and play an important role in the invasion and metastasis of squamous cell carcinoma.³⁵,³⁸–³⁹ Tissue inhibitors of metalloproteinases (TIMPs) inhibit the action of these MMPs. Thus far, 4 different TIMPs have been identified. The balance between MMPs and TIMPs seems to be a key mechanism in the process of invasion and metastasis. For example, expression of MMP-2 and TIMP-2 have been found to correlate with nodal status in SCC of the oral tongue, and expression of MMP-1, -2, -3, and -9 and TIMP-1 was found to correlate with nodal metastasis in another immunohistochemical study on oral cancer.³¹ However, the precise interactions of MMPs and TIMPs in tumor invasion and metastasis need clarification.

It has been proposed that binding of urokinase type plasminogen activators (uPA) to its receptor is a mediator of cancer invasion and metastasis through degradation of the extracellular matrix by activating MMPs. Inhibitors like plasminogen activator inhibitors (PAIs) regulate this process. Indications that the uPA system plays a significant role in the invasive and metastatic potential of HNSCC were uncovered in several investigations.³⁴,³⁵ Expression of uPA correlated with mode of invasion and secondary development of regional metastasis after successful initial treatment for the primary tumor in a study on oral cancer.³⁵ However, expression of PAI-1 and -2 showed no correlation with clinicopathological parameters in the same study.
TUMOR MICROENVIRONMENT

**Angiogenesis and Hypoxia.** Several studies have demonstrated that microvascular density and hypoxia play a role in the process of tumor progression and metastasis.

Small tumor deposits (up to 1–2 mm in diameter) can receive nutrition by diffusion. For further growth, angiogenesis is necessary. Induction of angiogenesis is mediated by molecules released by both tumor and host cells. Neovascularization is important not only for the growth of the primary tumor but also for the access to blood vessels by tumor cells that are destined to metastasize. Metastatic tumor cells that arrive at a secondary site do not necessarily proliferate immediately. They may remain dormant until conditions for growth become favorable by, for example, angiogenesis. Tumor vascularization is often assessed by microvessel density measurements, using antibodies against CD-31 or factor VIII related endothelial antigen.

The relationship between angiogenesis and tumor growth and hematogenic metastasis may seem more immediately apparent than the relationship of angiogenesis with lymph node metastasis. However, a correlation between angiogenic activity and the nodal metastasis has been suggested by several studies in different types of cancers. Immunohistochemical studies in HNSCC have yielded conflicting results. Some did reveal such a direct correlation while others did not (in smaller, T1 and T2, oral cancers in particular). Tumor hypoxia is an adverse prognostic factor, as it influences tumor progression and response to treatment. Many genes involved in controlling tumor biology, including metastatic behavior, are regulated by oxygen. The most important known oxygen-regulated genes include hypoxia inducible factor-1 (HIF-1), carbonic anhydrases (CA) (of which CA-9 is the most relevant), genes involved in apoptosis (p53), and vascular endothelial growth factor (VEGF). Hypoxia induces increased genomic instability in tumors and may induce expression of gene products involved in the metastatic process.

Of the hypoxic markers, HIF-1 is considered to be a major regulator of physiological responses to hypoxia. It activates the transcription of genes that are involved in important aspects of tumor biology, including energy metabolism, angiogenesis (VEGF), and invasion (MMP). Many studies on tumor hypoxia and prognosis have been performed in patients treated with radiotherapy, and a correlation with poor outcome has been found. In studies on surgically treated patients using HIF-1 antibodies, a trend toward a higher incidence of metastasis was found but was not significant. In a study using lactate concentration as a parameter for hypoxia, it was found to be associated with development of nodal and distant metastasis.

In other tumor types, hypoxic tumors appear to have a higher propensity for nodal metastasis. Specifically, in HNSCC, it has been suggested that hypoxia, measured by CA-9 expression, is associated with higher rates of (distant) metastasis. It is interesting in this regard that overexpression of CA-9 was found to reduce the E-cadherin–mediated cell–cell adhesion. It may be that either hypoxia or lowered pH is responsible for these effects.

VEGF is probably the most important angiogenic growth factor in human tumors. It induces endothelial cell proliferation and is therefore important in wound healing, tumor growth, and metastasis. Its expression is increased in hypoxia. Its clinical significance is not yet clear. In HNSCC, its expression has not to date been found to be of prognostic value. However, in recent studies, VEGF has been shown to be a valuable preoperative biomarker to predict cervical metastasis in patients with early oral cancer. Yet, these results were not confirmed in hypopharyngeal cancer. Therefore, this biomarker is potentially valuable to guide the treatment of the N0 neck in early oral cancer. In addition, VEGF expression also correlated with survival in oral cancer. Microvessel density was also assessed by the expression of CD105 (endoglin) in these studies, and it was consistently associated with lymph node metastasis and poor prognosis.

**Lymphangiogenesis.** The process of tumor cell dissemination via the blood with the aid of angiogenesis seems to be better understood than the process of tumor cell spread through the lymphatics. As is the case with the process of angiogenesis, it has become apparent that tumors possess a similar ability to induce lymphangiogenesis.

Proliferation of lymphatic vessels is regulated by members of the VEGF family, specifically by VEGF-C, together with its major receptor VEGFR-3. However, as mentioned previously, VEGF is also expressed on blood vessels. LYVE-1 is a recently identified lymphatic marker, expressed on lymphatic endothelium. High lymph vessel density was found to correlate with the presence of metastasis.
Expression of other markers for lymphangiogenesis, such as D2-40, was also associated with an increased risk for lymph node metastasis in patients with an accompanying high degree of lymphangiogenesis. Other new and promising endothelial markers for lymphatic spread may be MR and CLEVER-1.

**CHEMOKINES AND CHEMOKINE RECEPTORS**

Chemokines are recognized as important factors involved in tumorigenesis and metastasis in several types of tumor including HNSCC. The chemokine CXCL12 (stromal cell-derived factor-1) and its receptor CXCR4 play important roles in processes such as inflammation (acting as a chemoattractant for leukocytes). Similarly, tumor cells can respond to chemoattractants produced by certain tissues. CXCR4 has recently been characterized in several types of cancer and is thought to play a role in directing the metastasizing cells to CXCL12-rich tissues. Expression of CXCR4, measured by RT-PCR and immunohistochemistry, in primary tumors with lymph node metastasis was significantly higher than in those tumors without metastasis.

In HNSCC cells lines arising from the oral cavity, CXCR4 was shown to be expressed, while CXCL12 was found to induce migration of these cells and to stimulate pathways important in cell motility. Furthermore, CXCL12 seems to stimulate the adhesion of HNSCC cells to fibronectin and collagen and to promote the activation of MMP-9 secreted from HNSCC cells. It so contributes to the degradation of the basal cell membrane.

Downregulation of chemokine receptor CCR6 and upregulation of CCR7 was consistently found in metastatic HNSCC and seem to have an important role in the direction of metastatic cells toward lymphoid tissues. It may be a promising target for therapy. Correlation of expression of CCR7 with nodal metastasis has not yet been studied, to our knowledge.

**OTHER MARKERS**

Several other putative markers for metastasis have been studied. eIF-4E may play a role in the survival of metastatic cells by supporting growth factor autonomy. It enhances the translation of proteins involved in the promotion of several aspects of tumor progression like cell survival and proliferation (c-myc, cyclin D1), angiogenesis (VEGF), and invasion and metastasis (MMP-9), and may therefore contribute to metastasis. Indeed, increased expression can be found in metastasizing tumor cells.

Squamous cell carcinoma related oncogene (SCCRO) is a gene located on the chromosome 3q26.3 that is frequently amplified in HNSCC. SCCRO-transfected cells were found to be capable of creating invasive tumors and developing regional lymph node metastasis in nude mice. Amplification and overexpression of the SCCRO gene correlates with nodal metastasis and outcome.

Amplification of cyclin L1, located on chromosome 3q and involved in G0 to G1 transition, correlated with the presence of nodal metastasis in a large series of HNSCC.

**DNA PLOIDY**

DNA ploidy reflects the amount of genetic material in the cell nucleus. Most cells are in the resting (G0), diploid phase in normal tissue. Tumor cells usually show genetic instability, and so the DNA content of tumor cells can differ significantly from the normal diploid state. In reports on the DNA ploidy status in HNSCC, correlations have been constructed with clinical parameters such as stage and metastasis. In studies of HNSCC of different sites examined together, correlation with stage or nodal metastasis was found in some series, but in others such a correlation could not be established. A correlation or some relation with lymph node metastasis in cancer of the oral cavity has been reported.

**HIGH THROUGHPUT MARKER STUDIES FOR METASTASIS IN HNSCC**

If markers are to be used for clinical purposes, the techniques to detect them should ideally be readily available and relatively easy for the clinical laboratory to perform. Most of the aforementioned markers can be studied on a protein level by immunohistochemistry. These techniques are relatively easy to execute and are relatively inexpensive. However, they are less practical when testing large panels of markers. Since a combination of multiple markers will probably be a better predictor of behavior than single markers alone, techniques allowing study of numerous markers simultaneously would be very attractive.

More recently, high throughput techniques have been developed. These techniques include gene-expression profiling and comparative genomic
hybridization (CGH) with microarray technology, which permit the study of (ten-) thousands of genes simultaneously. The resulting genetic signatures may be useful as predictors of the likelihood of exhibition of metastatic behavior, and may offer insight into the underlying processes and molecules involved in these processes.

A number of microarray gene expression studies of metastasis in HNSCC have been performed in recent years. Many of these studies comprised only a limited number of cases and should therefore be considered no more than pilot studies. However, Chung et al. studied 60 HNSCC using cDNA microarrays and used a supervised method of analysis to predict the N classification of primary tumors. They were able to accurately predict the presence of nodal metastasis in 80% of patients. Roepman et al. analyzed 82 primary tumors located in the oropharynx or oral cavity with oligonucleotide arrays and found a set of 102 genes that was able to predict the presence of metastasis in 74% of patients, with improved results with more recently stored samples. Furthermore, in this work, the predictor has a predictive accuracy for N0 status of 100%. No false-negative predictions were made, which is most important for the goal of achieving clinical relevance. Genes in this classifying set included epithelial marker genes, genes encoding extracellular matrix components, genes involved in cell adhesion, cell death, cell growth, and extracellular matrix remodeling. The initial set of 102 predictive genes is a subset of a larger group of 825 genes with predictive power. In a further analysis of the composition of the metastatic signature, the authors found that many of the predictive genes were interchangeable because of a similar expression pattern across the tumor samples, and raising the number of genes included in the signature could compensate for exclusion of the strongest predictive genes. Multiple accurate predictive signatures can be designed using various subsets of predictive genes. Including more genes with lower predictive power can compensate for the absence of genes with strong predictive power. This suggests that there are more predictive genes than required to design an accurate predictor.

A few CGH studies studying differences in DNA copy numbers between N0 and N+ primary tumors have been performed. Bockmuhl et al. using conventional CGH, studied differences between primary tumors and their corresponding metastases, as well as between metastasizing and nonmetastasizing primary HNSCC. They found that deletion of regions 5q34-q35, 8p12-p22, 10p12, 10q21-qter, 11p14-p15, 11q14, 11q23-qter, and 14q21-qter, and overrepresentation of 1q21-q22, 3q24-qter, 6q, 7q11.2, 12q12-q14, and 18p11.2, were significantly associated with metastasizing tumors. Chen et al. used microarray CGH to perform a genome-wide screening for chromosomal aberrations in 60 oral squamous cell carcinomas. When comparing these oral cancers with and without nodal involvement, they identified 12 targets that appeared to be associated with the presence of lymph node metastasis: loss of 18q tel, COX2 at 1q31.1 and INSR at 19p13.2, and gain of PRKCZ at region 1p36.33, region 6p tel, region 7p tel, EGFR at 7p12.1-3, CCND1, EMS1 and FGF4/FGF3 at 11q13, THRA at 17q11.2, and AIB1 at 20q12.

**COMMENTS ON THE USE OF BIOMARKERS FOR CLINICAL PURPOSES**

Although the use of biomarkers may seem very promising, there are several critical factors yet to consider. If markers are to be used in decision making with regard to treatment of the neck in patients with head and neck carcinoma, the only available material to study before treatment would be biopsy material. This biopsy material may not be representative of the tumor in its entirety as a consequence of the heterogeneity of tumors. Moreover, some traditional light microscopic parameters like depth of invasion may be more difficult to assess in biopsy material than in the resection specimen. Markers reflecting the interaction between tumor and surrounding stroma may also be more difficult to study on biopsy material. Some of these objections may be circumvented by a 2-stage surgical procedure with first resection and examination of the primary tumor, and subsequently a decision on the treatment of the neck based on the examination of the features of the resected specimen. Another factor related to the heterogeneity of tumors is the fact that changes in genes and protein expression may vary from 1 part of a given tumor to another part of the same tumor. In the course of tumor progression, some cells of a clone may acquire additional or different chromosomal alterations from their neoplastic neighbors, resulting in a subclone with different properties. The question then is which percentage of cells showing expression of a certain marker should determine a positive scoring result and which percentage a negative one. As could be predicted, different cutoff points have been...
selected in different studies. More uniformity is required in scoring categories, if markers are to be used for clinical purposes.

Instead of trying to characterize the entire tumor, it may be even more important to detect small subclones in a tumor with genetic changes, which are, presumably, relevant for metastasis. In theory, only a small portion of the tumor can metastasize. However, some authors have found that the metastatic potential of tumors is encoded in the bulk of a primary tumor, thus challenging the previously noted belief that metastases arise from rare cells with a capacity for metastasizing, within a larger primary tumor.137

Another factor to take into consideration is the possibility that different techniques can be used to study the same features of a tumor. Protein expression, for example, is studied in different ways by different authors. Techniques like immunofluorescence, immunohistochemistry, and Western blotting have been used for this purpose, making comparison of results of different studies difficult. Some authors use fresh-frozen material, whereas others use paraffin-embedded material. Moreover, many different antibodies directed against the same protein of interest are often available, and different staining protocols are followed. This may also contribute to some variation in results.

No distinction is made between primary tumor subsites in many studies of HNSCC. It may be that results derived from tumors arising in 1 locale are not immediately applicable to tumors arising in other locales. There are indeed indications of differences in expression of several genetic markers between tumors arising in the different subsites of the head and neck.138–140 These differences are probably reflected in the well-known variation of biological behavior of tumors arising in different areas of the head and neck. Since some studies are restricted to particular locales of the head and neck and other studies consider all these locales together, the results of these series are not comparable. This leads to an inconsistency of results and will therefore impede the acceptance of the use of biomarkers for clinical purposes.

There may be problems with the interpretation of the results of marker expression; but the same may be said with regard to the parameter to which this marker expression is related, ie, the N status. That is to say, the means by which this N status was determined is not always precisely defined. Histopathological examination of the neck dissection specimen is the most precise means of determining the status of the neck. However, this gold standard for the nodal status very much depends on the way the neck dissection specimen is examined.141

In most studies, the nodal status is based on (routine) histopathological examination of the neck dissection specimen. However, small metastatic deposits or micrometastases may still remain undetected.142 If the lymph nodes are examined more meticulously, small metastases may be detected, which may not yet be detected by conventional light microscopic examination. Special immunohistochemical investigations, which enhance the detection of particularly small deposits of malignant epithelial cells, have found additional micrometastases in 5% to 50% of the patients who had no evidence of metastases at the time of routine pathologic examinations, with a mean incidence rate of 15.2%.143 The use of molecular techniques increases this number yet further, to around 20%, in some studies.144–147 Apart from the clinical significance of finding these very small tumor deposits, it seems clear that the choice of reference standard for assessment of the cervical nodal status will influence the outcome of studies of the identification of markers correlated with nodal metastasis.

Finally, 1 of the most important reasons that markers are not yet used for the evaluation of the nodal status of patients may be that, until the results of microarray studies were reported, most studies focused only on single markers. Since the process of metastasis is very complex and many factors are involved, it is unlikely that a single marker is sufficient to predict the metastatic behavior of a tumor. The complexity of the metastatic process suggests that multiple parameters or markers will be needed.14 For the purpose of studying multiple genetic factors, microarrays are ideal. However, some points should again be considered concerning microarray studies.

mRNA transcript levels are measured in expression array studies; while this may be a good representation of the transcriptional activity of a gene, it does not measure the amount of active protein in the tissue, as it does not take into account posttranscriptional regulation such as activation of a protein or the rate at which the protein is broken down. Thus, the outcome will probably have to be validated on other platforms that measure the actual protein, to determine whether the transcriptional activity of a gene translates an actual protein activity in the tumor tissue.
Second, expression arrays are performed on snap-frozen tissue. RNA is fragile and easily broken down, and the time from taking the biopsy to freezing it is critical as is the time from storage of the tissue until analysis.\(^\text{132}\) Moreover, the isolated mRNA has to be labeled and amplified before it can be hybridized to the microarray. During these procedures, the RNA is also sensitive to breakdown. Furthermore, not all mRNA fragments may be amplified at the same rate, introducing another potential source of bias in the end result.

Third, the enormous amount of data generated by a microarray experiment poses a statistical challenge that requires new and sophisticated analysis methods. The choice of the analysis technique is a crucial element in any microarray study, for it can dramatically affect the outcome.

Finally, critics point out that the profiles identified in different studies do not always match up with one another.\(^\text{148}\) However, many genes with similar functions may be interchangeable, and so predictive profiles for the same tumor entity may be composed of different gene sets.\(^\text{133,149}\)

The results are very promising, but now need to be validated on larger numbers of patients. Currently multicenter studies are underway through the American College of Surgery Oncology Group and NWHHT (Dutch Head and Neck Cooperative Group).

**PERSPECTIVES**

Although it is unlikely that it will be possible to be 100% certain about the true status of the neck, continuing improvements in imaging techniques will contribute to improvement of pretreatment staging. The number of patients with occult metastasis will further decrease in this way. Biomarkers may provide additional and complementary information and may be able to identify patients with a low chance of having occult metastasis. This group may be suitable for a wait-and-see policy for the neck. Of the available markers and techniques currently available, microarray expression profiling and CGH seem the most promising. If the results of the first emerging studies can be confirmed in sufficiently large series of patients, these arrays may be introduced into clinical practice.

**REFERENCES**


Role of Biomarkers in the Decision about Treatment of the Neck


