Abstract: Background. Hypoxia and tumor cell proliferation are important factors determining the treatment response of squamous cell carcinomas of the head and neck. Successful approaches have been developed to counteract these resistance mechanisms although usually at the cost of increased short- and long-term side effects. To provide the best attainable quality of life for individual patients and the head and neck cancer patient population as a whole, it is of increasing importance that tools be developed that allow a better selection of patients for these intensified treatments.

Methods. A literature review was performed with special focus on the predictive value and clinical relevance of endogenous hypoxia-related markers.

Results. New methods for qualitative and quantitative assessment of functional microenvironmental parameters such as hypoxia, proliferation, and vasculature have identified several candidate markers for future use in predictive assays. Hypoxia-related markers include hypoxia inducible factor (HIF)-1, carbonic anhydrase IX, glucose transporters, erythropoietin receptor, osteopontin, and others. Although several of these markers and combinations of markers are associated with treatment outcome, their clinical value as predictive factors remains to be established.

Conclusions: A number of markers and marker profiles have emerged that may have potential as a predictive assay. Validation of these candidate assays requires testing in prospective trials comparing standard treatment against experimental treatments targeting the related microregional constituent.

Keywords: head and neck squamous cell carcinoma; hypoxia; proliferation; endogenous markers; predictive assays

It has become increasingly evident that the tumor microenvironment plays a critical role in treatment resistance. Tumor cell proliferation and hypoxia are important factors determining the response of squamous cell carcinomas to surgery, chemotherapy, and radiation treatment. It was already shown in 1955 by Thomlinson and Gray that the radiocurability of human tumors is limited by hypoxia.\(^1\) This was based on pathologic observations on the relationship between necrosis and blood vessels.\(^1\) Since then, evidence has accumulated that hypoxic cells are responsible for failure to achieve tumor control. Clinically relevant hypoxia is detected in approximately 50% of all solid tumors irrespective of their size and histological features.\(^2\)
demonstrated that tumor hypoxia is associated with worse outcome in head and neck carcinomas, tumors of the uterine cervix, and breast carcinomas. Furthermore, in vitro and in vivo studies in a variety of tumor types have shown that the efficacy of some chemotherapeutic agents can also be decreased under hypoxic conditions.

Tumor hypoxia is known to play an important role in promoting genetic instability, tumor cell invasiveness, metastasis, and overall adverse clinical outcome. It may thus serve as a physiological selection pressure in tumors by promoting apoptosis in some cells but survival of those cells that have lost their apoptotic potential. The causes of hypoxia are multifactorial and include factors such as abnormal tumor vasculature, impaired blood perfusion, rate of oxygen consumption, and anemia.

Tumor cell proliferation is another important determinant of the efficacy of cancer treatment. The prognostic relevance of proliferation for tumor control in head and neck carcinomas has been studied extensively both in experimental and clinical tumors. Proliferation is affected by several factors such as differentiation status, cell cycle gene regulation, and microenvironmental factors including oxygen and nutrient availability. Hypoxia delays progression of the tumor cell through the cell cycle, which may allow rapid repopulation under better conditions. Cells that retain proliferative capacity under hypoxic conditions may therefore represent an important clonogenic subpopulation of tumor cells responsible for treatment failure.

Several approaches have been considered to overcome tumor hypoxia and repopulation. For these treatment modalities to be successful and for proper selection of patients, it is important to understand the complexity and the dynamics of the tumor microenvironment. The introduction of new methods for qualitative and quantitative assessment of functional microenvironmental parameters such as hypoxia, cell proliferation, and vasculature may provide important information for optimization of treatment strategies and better selection of patients by pretreatment testing.

This article provides an overview of the literature concerning the analysis of microenvironmental tumor parameters such as hypoxia and proliferation in head and neck squamous cell carcinomas. The main focus will be on endogenous markers of tumor hypoxia and their role in patient selection based on treatment outcome and survival.

PATHOPHYSIOLOGY OF TUMOR HYPOXIA

The presence of hypoxic areas is a characteristic pathophysiological property of many solid tumors and has been found in a wide range of human malignancies. Hypoxia arises as a result of an imbalance between the supply and consumption of oxygen. In contrast to normal tissues in which oxygen supply meets metabolic requirements, the oxygen consumption rate of tumor cells may outweigh an insufficient oxygen supply resulting in the development of tissue areas with very low oxygen levels. Tumor hypoxia may be a consequence of several major pathogenetic mechanisms such as abnormal tumor vasculature, limited tissue perfusion, and tumor-associated or therapy-associated anemia leading to a reduced oxygen transport capacity of the blood.

Two types of tumor hypoxia can be distinguished: perfusion-limited and diffusion-limited hypoxia. Perfusion-limited or acute hypoxia is often transient and may be due to severe structural and functional abnormalities of the tumor microvessels. These abnormalities cause disturbances in the blood supply, leading to temporal shutdown of vessels, gradients of oxygen and nutrients, and even reversal of blood flow. Hypoxia can also be caused by an increase in diffusion distances, resulting in diffusion-limited hypoxia, leaving cells chronically deprived of oxygen and other nutrients. In most tumors, both types coexist and contribute to greater or lesser extent. Oxygen measurements in locally advanced primary tumors revealed that tumors are often heterogeneous in their oxygen levels. In approximately 60% of investigated lesions, hypoxia existed with pO2 values below 2.5 mm Hg and median oxygen partial pressures below 10 mm Hg. As a consequence of adaptation of tumor cells to hypoxia, a more aggressive tumor phenotype may develop. Several mechanisms have been identified as key factors to hypoxia tolerance, such as the inhibition of apoptosis, regulation of angiogenesis, and the increase in anaerobic glycolysis.

Many classical radiobiological studies showed that cells with oxygen partial pressures below 0.5 mm Hg are maximally resistant to the lethal effects of irradiation. However, it was suggested by Wouters et al. that cells at intermediate oxygen levels between 0.5 and 20 mm Hg might be more important in determining the response to fractionated radiotherapy. Cells under severe chronic hypoxia (pO2 < 0.5 mm Hg) are not likely to survive, whereas there is a greater chance of viability under intermediate hypoxia with adapta-
Tumor Hypoxia and Anemia. A significant association exists between low hemoglobin (Hb) levels and poor prognosis in head and neck cancer treated with either surgery or radiotherapy.9,22–24 To understand the link between low Hb-levels and outcome, it was postulated that a decrease in oxygen-carrying capacity of the blood might further exacerbate tumor hypoxia and increase treatment resistance. This alleged relationship between anemia, tumor hypoxia, and outcome has been investigated in several animal and human studies.9,24,26,27 In a rat model, Kelleher et al25 demonstrated that tumor-related anemia resulted in a significant decrease of tumor pO2 measured with polarographic needle electrodes. Correction of anemia with either blood transfusion or the administration of recombinant human erythropoietin (rhEPO) only partially led to reduction of tumor hypoxia in small tumors. Clinical studies in head and neck carcinomas9,24,26,27 demonstrated significant albeit very weak correlations between Hb concentration and oxygen level in the tumor measured with polarographic needle electrodes. Because of the absence of a strong association with oxygen level, Hb concentration may not be a good indicator of tumor hypoxia. The ability to detect proteins that are upregulated under hypoxic conditions, such as the hypoxia inducible factors (HIF) 1α and 2α and carbonic anhydrase IX (CA-IX), made it possible to evaluate the association of Hb-levels with the presence of hypoxia-activated pathways. However, Koukourakis et al26 could not show an association between Hb-levels and upregulation of hypoxia-regulated proteins, microvessel density, or the activation of angiogenic pathways such as vascular endothelial growth factor (VEGF) in endometrial adenocarcinomas and head and neck tumors.

Treatments. The first approach to counteract hypoxic treatment resistance was the development of compounds that mimic oxygen and sensitize hypoxic cells to radiation. The most successful compound thus far is nimorazole, a nitroimidazole. The Danish Head and Neck Cancer Study Group (DAHANCA) demonstrated significant beneficial tumor response with this drug in carcinomas of the supraglottic larynx and pharynx demonstrating high tumor control rates for tumors of the larynx (80%) and the oropharynx (87%).33 These results support the concept of increased susceptibility of tumors to the biologically based approach of ARCON offering excellent opportunities for organ preservation. Currently, a phase III trial with ARCON for laryngeal cancer is ongoing.

Another attempt to counteract hypoxic treatment resistance was the development of compounds that mimic oxygen and sensitize hypoxic cells to radiation. The most successful compound thus far is nimorazole, a nitroimidazole. The Danish Head and Neck Cancer Study Group (DAHANCA) demonstrated significant beneficial tumor response with this drug in carcinomas of the supraglottic larynx and pharynx when combined with a conventional radiotherapy schedule.34 Although the drug-related toxicity of nimorazole was limited, this study is overshadowed by negative studies with older generations of sensitizers which were more toxic. This approach is therefore not generally accepted and is only standard practice in Denmark.

The third strategy is to exploit hypoxia by using cytotoxins that specifically target hypoxic cells or by using gene therapy.35 Tirapazamine is the first hypoxic cytotoxin introduced in the clinic.31 In a recent phase II clinical trial, tirapazamine showed promising results when used in
combination with cisplatin and radiotherapy in patients with head and neck cancer. It was demonstrated that the regimens used were feasible, had acceptable toxicity profiles, and showed promising efficacy. Based on this trial, tirapazamine is currently being evaluated in phase III trials.

Although several approaches have been successful in counteracting tumor hypoxia, this has not yet resulted in a treatment that has been generally accepted in the clinic. It has become clear that not only hypoxia is an important factor determining the response to treatment but that a combination of microenvironmental factors should be targeted. Currently, a number of new approaches combining different treatment modalities are under investigation in phase III trials.

**PROGNOSTIC INDICATORS AND PREDICTIVE ASSAYS**

Despite advances in treatment, head and neck squamous cell carcinomas still carry a significant mortality rate, partly due to uncontrolled locoregional disease. At present, the site of origin and the tumor stage are the main prognostic factors for survival. Although tumors may have clinically equivalent stages, head and neck carcinoma is a heterogeneous disease with distinct patterns of presentation and biological behavior and different responses to treatment. This suggests that additional genetic and molecular markers could be used to supplement TNM staging and to improve selection of patients for new treatment strategies. Identification of these key genetic and molecular markers may lead to the development of predictive assays. The term “predictive assay” refers to a test designed to predict the response of tumors to therapy on the basis of biological tumor characteristics. Predictive assays need to be distinguished conceptually from “prognostic factors.” The latter have been determined empirically and, although they can be powerful predictors of treatment outcome, they simply indicate favorable or unfavorable response but offer no basis for selecting possibly superior alternative treatments. A predictive assay, on the other hand, is mechanistically based, thereby offering the possibility of rational early interventions to improve therapeutic outcome.

Many studies have focused on the identification of molecular markers as prognostic factors, but few clinical studies have explored the potential of these markers as a predictive assay. Tumor response is almost certainly multifactorial, and therefore, molecular markers may not be predictive on their own; however, in conjunction with other markers a specific profile may arise that could characterize tumors and predict treatment outcome. A next step would be to link certain marker profiles to specific treatment resistance mechanisms, such as hypoxia, as a guide to therapy customization.

**ASSESSMENT OF HYPOXIA IN SOLID TUMORS**

Several techniques have become available for measurement of tumor oxygenation and include invasive techniques such as polarographic needle electrodes and minimal invasive methods such as exogenous hypoxia markers, endogenous molecular markers, and functional imaging with positron emission tomography (PET) and dynamic contrast-enhanced MRI. An overview of endogenous hypoxia-related markers will be presented in the next section.

**Polarographic Needle Electrodes.** Polarographic needle electrodes provide the most widely used method for direct measurement of pO2 in human cancers. They have been used to measure pO2 distributions in a large number of experimental and human tumors, demonstrating large intra- and inter-tumor heterogeneity. Several studies in head and neck squamous cell carcinomas showed that low pO2 measurements correlate with poor outcome after treatment. The needle-electrode system, however, has important drawbacks. Most primary tumors are not accessible for electrode measurements, and in the head and neck area the available data are mainly from nodal metastases. Also, the method cannot distinguish necrosis with very low pO2 from severe hypoxia in viable tumor areas and provides no information about how hypoxia is related to the histological architecture and the microenvironment of the tumor.
tionship between hypoxia, vasculature, and other microenvironmental factors. It has been demonstrated that the pimonidazole binding assay can be used to determine tumor control and disease-free survival in patients with head and neck cancer and can provide a selection tool for hypoxia-modifying treatments on an individual patient basis.\(^5\)

**Radiologic and Nuclear Medicine Imaging Techniques.** Noninvasive methods with repetitive assessment capacity allowing visualization of the whole tumor include radiologic and nuclear medicine imaging techniques such as PET imaging using specific tracers or dynamic contrast-enhanced MRI (DCE-MRI).

\([18F]\)-fluorodeoxyglucose (18F-FDG) PET is now routinely used for cancer detection, staging, and monitoring of response in several tumor types. Although glucose utilization is indirectly related to the proliferative activity and the oxygenation status of the tumor, 18F-FDG uptake at best correlates weakly with these aspects of the tumor biology and more specific radiopharmaceuticals are currently available. A number of small clinical studies assessed the value of these tracers in head and neck tumors.\(^{46-48}\) The most widely used tracer for hypoxia imaging by means of PET is \([18F]\)-labeled fluromisonidazole (18F-FMISO). Preliminary data suggest that outcome after radiotherapy is associated with the kinetic behavior of 18F-FMISO in tumor tissue.\(^{46}\) There is also an indication that early resolution of abnormal 18F-FMISO uptake during treatment may be predictive for local tumor control.\(^{47}\) Also, tumor cell proliferation can be imaged with PET. Currently, the most promising tracer for tumor cell proliferation appears to be \(3'-\)deoxy-\(3'-[18F]\)-fluorothymidine (18F-FLT). 18F-FLT is phosphorylated by thymidine kinase 1 (TK1) and trapped intracellularly. TK1 activity is increased during DNA synthesis, and thus 18F-FLT can be used as an S-phase marker. The clinical experience with 18F-FLT-PET is very limited. One study investigated the feasibility of 18F-FLT-PET for the detection of laryngeal cancer and concluded that the performance of 18F-FLT and 18F-FDG was equal. The potential of 18F-FLT-PET as a prognostic or predictive tool remains to be investigated.\(^{48}\) A limitation of PET is the low resolution relative to other imaging modalities.

DCE-MRI is used both experimentally and clinically to monitor the functionality of the tumor vasculature after administration of the contrast agent gadolinium-DTPA. Because of the high spatial resolution of MRI heterogeneities in blood flow, vascular volume and permeability of blood vessels within a tumor can be detected.\(^{49}\) In a small study of patients with head and neck cancer, this method was used to assess tumor perfusion before and after radiotherapy.\(^{50}\) Durable local controls were seen mainly in those tumors with a diminished perfusion at the post-radiotherapy assessment. Another study in head and neck carcinomas employing the blood oxygen level dependent MRI (BOLD MRI) effect demonstrated that this MRI technique enables the assessment of improved tumor blood oxygenation by carbogen breathing.\(^{51}\)

These preliminary clinical results indicate that PET and DCE-MRI can become important clinical tools for determining vascular function and hypoxia in vivo and for monitoring the effect of therapeutic agents.

**ENDOGENOUS HYPOXIA-RELATED MARKERS**

Several promising methods for detection of tumor hypoxia have been developed, each having strengths and weaknesses. Major limitations are the invasive nature of some of these procedures, the use restricted to accessible tumors, and the inability to analyze archived tumor material. This stimulated the search for endogenous hypoxia-related markers.
Hypoxia Inducible Factor-1α. A key regulator of oxygen homeostasis is the transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimer composed of 2 subunits: HIF-1α and HIF-1β. Regulation by oxygen is through the HIF-1α subunit, which is constitutively synthesized and subsequently rapidly destroyed under normal oxygen conditions by proteasomal degradation. This process is rapid and complete with the HIF-1α subunits having a very short half-life in normoxic cells. The von Hippel-Lindau tumor (VHL) suppressor protein plays an essential role in targeting HIF-1α for degradation. Inactivation of the VHL gene leads to stabilization of the HIF-1α subunits and activation. Under hypoxia, the proteolytic process is suppressed leading to the high amplitude upregulation of HIF-1α.

In response to low oxygen conditions, HIF-1α is known to transactivate more than 70 genes whose protein products function either to increase O2 availability or to mediate metabolic adaptation to O2 deprivation. These genes encode proteins that are required for angiogenesis (VEGF), regulation of blood vessel tone and vascular remodelling, pH regulation (CA-IX), cell proliferation (cyclin G2), cell survival (IGF) and apoptosis, erythropoiesis (EPO), drug resistance (MDR), and energy and glucose metabolism (GLUT1, GLUT3).

Immunohistochemical analysis of human tumor biopsies demonstrated that overexpression of HIF-1α is common in solid tumors and their metastases. Overexpression of HIF-1α has been associated with increased failure to achieve locoregional control and impaired disease-free and overall survival in head and neck cancers. Several studies evaluated the relationship between hypoxia and HIF-1α in head and neck squamous cell carcinomas in vitro and in vivo with inconsistent and inconclusive results. Some studies demonstrated strong induction of HIF-1α by hypoxia, but HIF-1α induction is also shown to be dependent on other microenvironmental conditions such as glucose availability. Two reports compared HIF-1α expression with pimonidazole binding using immunohistochemistry in biopsies of head and neck squamous cell carcinomas or flow cytometry in FaDu (human pharyngeal carcinoma) xenografts. Both studies demonstrated no correlation between HIF-1α expression and pimonidazole positive cells. Given the association between HIF-1α expression and outcome, it is likely that HIF-1α indicates tumor aggressiveness. However, it is debatable whether it is also a reliable marker of tumor hypoxia.

Carbonic Anhydrase IX. Carbonic anhydrases (CAs) form a large family of genes encoding zinc metalloenzymes. They catalyze the reversible hydration of carbon dioxide to carbonic acid, thereby maintaining a stable intracellular pH at the cost of acidification of the extracellular tumor microenvironment. The CAs participate in a variety of biological processes including the regulation of pH, respiration, and calcification. There are 14 known members of this family, which may be subdivided on the basis of cellular localization: membrane associated, cytosolic, mitochondrial, and secreted.

Two tumor-associated transmembrane carbonic anhydrases, CA-IX and CA-XII, have been identified. CA-IX was first characterized as a tumor-associated gene product in HeLa cells and was initially called MN protein. Immunohistochemistry showed high-to-moderate CA-IX expression in normal tissues such as the gastrointestinal tract and mainly in highly specialized cells. High expression levels of CA-IX were also found in many different cancer cell lines and tumor tissues (Figure 2). It has become clear that CA-IX expression is directly regulated through consecutive activation of the HIF-1 pathway after inactivation of the VHL tumor suppressor gene. Furthermore, in vitro studies demonstrated that CA-IX was strongly induced by hypoxia in a broad range of tumor cells. After 16 hours of exposure to hypoxic conditions, the level of CA-IX increased with decreasing oxygen tensions from 5% to 0.1%. It was also shown that CA-IX positive cells were more resistant to killing by ionizing radiation than CA-IX negative cells. In vivo studies are consistent with these findings and revealed that poorly perfused tumor areas expressed the most CA-IX and that CA-IX staining was preferentially located in perinecrotic
regions. In head and neck squamous cell carcinomas, a gradient of CA-IX expression could be observed with the highest levels adjacent to necrosis. A significant positive correlation between tumor hypoxia measured with polarographic needle electrodes and the extent of CA-IX expression has been shown in cervix carcinomas. Furthermore, considerable colocalization with pimonidazole staining was observed, although areas of mismatch were also found. The latter suggests that both markers may operate at different oxygenation levels. Another explanation for this mismatch may be the time course of CA-IX upregulation, which requires several hours such that temporal fluctuations of hypoxia are not likely to be reflected in CA-IX expression.

The tumor-associated CA-IX plays a role in the maintenance of pH homeostasis in tumor cells. It protects cells from intracellular acidification, thereby producing an acidic extracellular environment, which stimulates development toward a more malignant phenotype. Two retrospective studies in patients with head and neck squamous cell carcinomas support the notion that CA-IX upregulation indicates tumor aggressiveness and demonstrate a correlation with local tumor control and overall survival. Another study, however, did not find a correlation with treatment outcome. Currently, CA-IX is the most promising endogenous marker of hypoxia, demonstrating concordant staining patterns with pimonidazole, although less specific. However, its clinical value as a predictive factor has not yet been established.

**Glucose Transporters.** Also controlled via the HIF-1 pathway and of interest as potential hypoxia markers are the glucose transporters (GLUT) in particular GLUT-1 and GLUT-3. They mediate cellular glucose uptake and thus facilitate anaerobic glycolysis. GLUT have been identified in many different human tissues and tumors, including head and neck squamous cell carcinomas. Immunohistochemical staining demonstrated GLUT-1 expression mainly at a distance from perfused vessels adjacent to necrosis.
indicating diffusion-limited hypoxia. The relationship with tumor hypoxia has not been investigated as well as for HIF-1 or CA-IX. Only in advanced carcinomas of the uterine cervix the oxygenation status has been compared with the microregional expression of GLUT. A weak but significant correlation between GLUT-1 expression and low oxygen microelectrode measurements has been reported in 1 study, whereas another more recent study did not find any correlation. Also in carcinomas of the uterine cervix, a significant correlation between GLUT-1 expression and pimonidazole staining and between GLUT-1 and CA-IX was found, suggesting a relationship between GLUT and tumor hypoxia. For head and neck squamous cell carcinomas, this relationship remains unclear and is still under investigation (Figure 2). There are some indications that GLUT-1 and GLUT-3 are related to tumor aggressiveness. A few studies found a correlation between GLUT-1 or GLUT-3 expression and disease-free and overall survival.

**Erythropoietin Receptor.** EPO is a 34-kDa glycoprotein hormone that regulates the production of red blood cells by supporting proliferation, survival, and terminal differentiation of erythroid progenitor cells that reside in the bone marrow. It is produced in the kidney in response to hypoxia. The biologic effects of EPO are mediated through the interaction with its specific transmembrane erythropoietin receptor (EPOR). Hypoxia-dependent expression of EPO and EPOR is primarily regulated by HIF-1α. EPO was long considered to be exclusively produced in the kidney and the fetal liver as a specific regulator of erythropoiesis. However, more recently it has been shown that EPO and EPOR are also produced and expressed by various nonerythroid tissues such as the nervous system, endothelial cells, and breast and endometrial tissues. In these tissues, EPO exhibits different functions, including cytoprotection against ischemic injury, stimulation of angiogenesis, and acceleration of the wound-healing process. In recent years, it has become clear that EPO and EPOR are also expressed in many human malignancies. Studies in vitro demonstrated an increase in EPO and EPOR expression in a variety of tumor cell lines under severe hypoxia. Uregulation of functional EPOR along with EPO in malignant tumors may contribute to the hypoxia-mediated selection of cells with diminished apoptotic potential and tumor progression. Unexpected results from 2 randomized trials investigating the effect of EPO in breast cancer and head and neck cancer have strengthened these considerations. Both studies reported worse survival, and the head and neck study also showed worse locoregional tumor control in patients receiving recombinant human erythropoietin (rHuEPO, epoetin) compared with those on placebo. It has been suggested that EPO signaling might have promoted cancer progression and contributed to worse outcome in the experimental arms. A few studies of head and neck carcinomas, cancer of the uterine cervix, and breast carcinomas showed coexpression of EPO with HIF-1α and pimonidazole binding. In biopsies of head and neck squamous cell carcinomas, Arcasoy et al demonstrated a positive correlation between EPO levels and tumor hypoxia as defined by pimonidazole, although EPO and pimonidazole did not always colocalize. A second study in head and neck cancer also could not demonstrate colocalization between EPOR and pimonidazole. In fact, EPOR expression seemed to be strongest in better oxygenated areas. A possible explanation could be that EPO is produced in relatively hypoxic tumor areas and subsequently stimulates EPOR expressing cells in more viable tumor areas as a mechanism for tumor survival. From the available observations, it must be concluded that EPO and EPOR most likely are not useful as markers for hypoxia in comparison with the exogenous hypoxia marker pimonidazole. However, one should be aware of the potential of EPO to promote tumor growth in head and neck cancer and possibly other tumor types as well. Future studies addressing this issue should be supported because this is relevant for the use of EPO in patients with cancer.

**Other Potential Endogenous Hypoxia Markers.** The current interest in tumor hypoxia has provoked many studies searching for potential future hypoxia marker candidates. Identification of hypoxia-regulated genes and proteins occurs not only with immunohistochemistry but also by DNA, proteomic, or tissue array profiling. Several genes, transcription factors, and proteins have been investigated in head and neck squamous cell carcinomas. Examples of other hypoxia-inducible proteins under investigation include IkB kinase β (IKKβ), involucrin, osteopontin, the hypoxia-regulated transcription factor DEC1, the epidermal growth factor receptor (EGFR), lactate dehydrogenase-5 (LDH-5), and the activating transcription factor 4 (ATF4). These proteins
have mainly been tested in vitro in cell cultures derived from human head and neck squamous cell carcinomas, and only a few of them have also been tested in vivo in clinical samples from patients with head and neck cancer. After exposure to hypoxia it was demonstrated that protein levels were increased, leading to the assumption that they are triggered by hypoxic stress. Whether these hypoxia-related factors are actually upregulated in hypoxic tumor areas has not yet been sufficiently investigated.

Osteopontin emerged as a potential marker with clinical relevance in recent studies in head and neck cancer. It was demonstrated by Le et al that plasma osteopontin levels correlated with tumor hypoxia in head and neck carcinomas as measured by Eppendorff microelectrodes. The DAHANCA 5 randomized trial further showed that high plasma concentrations of osteopontin are associated with poor outcome after radiotherapy. Most interesting was that this poor outlook could be improved by use of the hypoxic sensitizer nimorazole. The importance of this observation is not so much the identification of hypoxia itself, but rather the proof of principle that variations of the level of hypoxia between tumors can be used to identify patients in whom outcome can be improved by hypoxic modification.

On the microregional level, only the immunostaining patterns of involucrin and pimonidazole have been compared, showing an incomplete overlap between involucrin expression and pimonidazole binding. Furthermore, it appeared that involucrin was induced by hypoxia in moderately differentiated tumor cells and not in poorly differentiated cells, indicating that differentiation grade coregulates its transcription status.

It is becoming clear that other tumor and microenvironmental factors such as differentiation, availability of glucose, and pH also control the expression of hypoxia-related gene and gene products. A proper understanding of these other factors regulating gene induction and protein expression is needed to assess the potential of these gene products as markers of tumor hypoxia.

**TURNOVER RATE OF HYPOXIC CELLS**

The tumor microenvironment is considered to be a constantly changing environment as a result of tumor cell proliferation and temporal and spatial variations in blood supply. Studies in human biopsies and xenografts of head and neck carcinomas revealed heterogeneities of the vascular architecture, and distribution of hypoxic and proliferating cell populations and tumors have been categorized according to these distribution patterns. Also, in vitro studies demonstrated that the lifetime of hypoxic cells varies between tumors from a few hours to several days under severe hypoxic conditions. These observations support the notion that the distribution, lifetime, and viability of hypoxic cells is dependent on not only the blood supply but also other microenvironmental factors such as cell proliferation, metabolism, cell migration, and cell loss.

In 1955, Thomlinson and Gray postulated their theory that new cells were formed in the proliferating cell compartment that consequently pushed tumor cells away from the blood vessels resulting in a gradual depletion of oxygen and nutrients. Tumor cells in the hypoxic cell compartment would be pushed further down the oxygen gradient and eventually die of oxygen deficiency and starvation. Since then, however, only a limited number of assays have become available to study the dynamics of tumor oxygenation and hypoxic cells in vivo. Recently, a method has been developed that allows analysis of changes in tissue hypoxia at the microregional level. This involves the consecutive injection of 2 different bioreductive hypoxia markers at variable times before harvest of the tumor. In a recent study, this double marker assay was used to analyze the dynamics of hypoxic tumor cells in xenografted human head and neck carcinomas. It was demonstrated that over time pimonidazole-positive cells were being pushed away from the vasculature and cell debris with pimonidazole adducts appearing in the necrotic regions. Meanwhile, new hypoxic cells appeared at the “hypoxic front,” which could be identified by the second marker, CCI-103F, administered at a later point in time. The hypoxic cell turnover rate ranged from 17 to 49 hours, and the speed of this phenomenon differed significantly between different xenograft models. The tumors with a fast hypoxic cell turnover rate had a considerable amount of necrosis as well as a high proliferation index. It was concluded that the dynamics and lifetime of hypoxic cells depend on the proliferative activity of the tumor, the availability of nutrients, and other microenvironmental factors. These dynamics and the interplay with the proliferating cell compartment may eventually affect the response of tumors to different treatment modalities and should be taken into account in the design and sequencing of combined treatment strategies.
PROLIFERATION AND HYPOXIA

As indicated in the previous section, the proliferative activity of the tumor is another important element of the tumor microenvironment. Proliferative potential of a tumor has been recognized as an important determinant in the efficacy of the treatment of head and neck squamous cell carcinomas. Radiotherapy and chemotherapy are given in multiple doses over time to allow recovery of normal tissues. Although the number of tumor cells is greatly reduced during cytotoxic treatment, cells that survive are triggered to repopulate more effectively during the intervals between treatments, and this process of repopulation is an important cause of treatment failure. In the last decade, randomized trials have convincingly shown that accelerated radiotherapy, ie, delivery of the radiation dose in shorter time, can counteract this repopulation and improve tumor control probability. Tumor cell repopulation might also limit the effectiveness of chemotherapy and combined modality approaches, especially when the intervals between the treatments are long.

Different markers for assessment of proliferation have been used. These include the endogenous markers Ki-67, proliferating cell nuclear antigen (PCNA), and members of the cyclin group or the IV. administration of exogenous markers such as the thymidine analogues bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd). These thymidine analogues have a short half-life and are rapidly incorporated into the DNA of S-phase cells. In a multicenter study, it was demonstrated that a high BrdUrd or IdUrd labeling index (LI) was weakly associated with worse local control after radiotherapy of head and neck carcinomas. This indicates that repopulation rate may be 1 factor, but not the only factor, determining the effect of radiotherapy.

Wijffels et al used the endogenous marker Ki-67 to quantify proliferation patterns in relationship to vasculature. They found proliferating tumor cells not only near the blood vessels but also at distances greater than 70 μm from the vasculature, indicating that these cells might exist under conditions of relative hypoxia. It is likely that these cells behave differently and show different responsiveness than well-oxygenated tumor cells. Hypoxia is generally thought to induce arrest in the G0/G1 phase of the cell cycle or to induce apoptosis. Experiments by Durand and Raleigh using flow cytometry clearly showed that proliferating cells were usually distinct from hypoxic cells. Although some dual-labeled cells were seen, they constituted <2% of the total tumor cell population. This is supported by several studies showing only a small degree of overlap between proliferating cells and the hypoxia marker pimonidazole, indicating that proliferation sparsely occurs at PO2 levels below 10 mm Hg. It was, however, recently demonstrated in biopsies of head and neck squamous cell carcinomas that a considerable amount of proliferating cells were present in CA-IX–positive areas and that this population was associated with worse disease-free survival rates (Figure 4). CA-IX upregulation occurs al-
CONCLUSION

Tumor hypoxia and repopulation have for many years been the subject of investigation as they play a critical role in treatment resistance. Successful approaches have been developed to counteract tumor hypoxia and tumor cell repopulation, although some of these treatments are accompanied by an increase in side effects. Thus far, no treatment modality targeting tumor hypoxia is widely accepted in clinical practice, but several phase III trials are currently investigating new strategies. For these treatment modalities to be successful, it is important to provide better selection of patients and better prediction of tumor response by pretreatment testing of microregional parameters. New methods for qualitative and quantitative assessment of functional microenvironmental parameters such as hypoxia, proliferation, and vasculature have identified several candidate markers for future use. The increasing interest in endogenous proteins as markers of tumor hypoxia has also demonstrated the complex biology of head and neck squamous cell carcinomas as these proteins are not only controlled by hypoxia but also by other factors. It is therefore important to develop microregional marker profiles reflecting the complex biology of a tumor. Whether these profiles can be used in the clinic for the customized design of treatment for individual patients with head and neck squamous cell carcinomas remains to be investigated in prospective trials comparing standard treatment against experimental treatments targeting the relevant microregional factors.

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