Pimonidazole Binding and Tumor Vascularity Predict for Treatment Outcome in Head and Neck Cancer


ABSTRACT

Hypoxia is associated with tumor aggressiveness and is an important cause of resistance to radiation treatment. Assays of tumor hypoxia could provide selection tools for hypoxia-modifying treatments. This study correlated the exogenous 2-nitroimidazole hypoxia marker 1-[(2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride (pimonidazole) with the endogenous hypoxia-related marker carbonic anhydrase 9 (CA9) and with vascular parameters using immunohistochemical techniques and a computerized image analysis system. Tumor biopsies were obtained from patients with head and neck carcinomas that were potential candidates for a Phase II trial with accelerated radiotherapy combined with carbogen and nicotinamide (ARCON). If, after completion of the diagnostic workup, the eligibility criteria were met and informed consent was obtained, patients were treated with ARCON. Those patients that were not eligible or refused ARCON were treated with radiotherapy, surgery, or a combined modality. Forty-three biopsies were analyzed, and the results were related with treatment outcome. The distribution patterns of pimonidazole and CA9 were similar, although the CA9 signal was generally observed already at shorter distances from blood vessels. There was a weak but significant correlation between the relative tumor areas positive for pimonidazole binding and areas with CA9 expression. Locoregional tumor control was significantly lower for patients who had hypoxic tumors or tumors with low vascular density. The 2-year control rates were 48% for tumors with high and low pimonidazole binding levels (stratified by median, \( P = 0.01 \)) and 48 and 88% for tumors with low and high vascular density (stratified by median, \( P = 0.01 \)). These associations disappeared in the subgroup of patients treated with ARCON. There was no relationship between the level of CA9 expression and treatment outcome. It is concluded that pimonidazole binding to vascular density can predict tumor outcome in head and neck cancer and may be useful as selection tools for hypoxia-modifying treatments. Pimonidazole and CA9 demonstrate concordant staining patterns, but the latter is a less specific marker for hypoxia.

INTRODUCTION

In almost all solid tumors there is to some extent an imbalance between oxygen delivery and oxygen consumption resulting in hypoxia (1). Hypoxia is a powerful trigger for changes in gene expression and associated changes in the micromilieu. The altered genetic expression profile and the changed microenvironment stimulate clonal selection within the tumor cell population for cells with increased adaptation to hypoxia and drive the tumor toward a more malignant phenotype with increased resistance to anticancer treatments (2, 3).

Methods that identify hypoxic tumors could therefore provide a powerful selection tool for oxygenation modifying treatments.

Clinical studies using oxygen microelectrodes in carcinomas of the head and neck and uterine cervix have demonstrated a correlation between measurements of low tumor \( \text{pO}_2 \) and poor outcome after radiation treatment (2, 4–6). In soft tissue sarcomas and in carcinomas of the uterine cervix, hypoxia was also related with outcome after surgical treatment (2, 7, 8). A limitation of the oxygen microelectrodes is the invasive nature of the procedure and their use is restricted to accessible tumors.

Exogenous and endogenous hypoxia markers detectable by immunohistochemistry require no additional intervention beyond the initial pretreatment biopsy and may be very suitable for widespread clinical use. They also provide a more sensitive and high resolution assay of the distribution of hypoxia at the microregional level. Clinically relevant exogenous markers are the 2-nitroimidazoles, pimonidazole \(^3\) hydrochloride and [2-(2-nitro-1H-imidazole-1-yl)-\( \beta \)-(2,2,3,3,3-pentafluoropropyl)acacetamide] (EF-5) (9, 10). Hypoxic fraction estimated by pimonidazole binding agreed well with the hypoxic fraction measured using the comet assay in a number of animal and human tumors indicating that pimonidazole labeling can give a reliable estimate of radiobiologically relevant hypoxia (11). Pimonidazole has proven to be an effective and nontoxic hypoxia marker for human squamous cell carcinomas of the cervix and head and neck (9, 12–14). A disadvantage of the nitroimidazole markers is that they require i.v. injection before biopsy taking.

In addition to the well-known angiogenic factor, vascular endothelial growth factor, more recently, new and possibly more specific endogenous hypoxia-associated molecular markers have been described. Most promising at present is CA9, which is regulated via the von Hippel-Lindau and HIF-1 pathway. Carbonic anhydrases are transmembranic enzymes catalyzing the reversible hydration of carbon dioxide to carbonic acid and are involved in respiration and acid-base balance (15). Immunohistochemistry revealed high-to-

High-resolution imaging of hypoxia requires an effective hypoxia marker.
CA9 expression predicted poor survival in carcinoma of the head and neck, lung, uterine cervix, and breast (19–22).

In this study, we relate pimonidazole binding and CA9 expression in biopsy material of human squamous cell carcinomas of the head and neck. We demonstrate that the distribution pattern of the two markers relative to the vasculature is similar with increasing signal at greater distance from the blood vessels. CA9 expression was often observed at shorter distances from the vessels, suggesting that CA9 up-regulation occurs at pO2 levels higher than those required for pimonidazole binding. Although the patterns were concordant, there was only a weak correlation between the overall pimonidazole and CA9-positive tumor areas. In patients with head and neck cancer, pimonidazole binding and vascularity were predictors of outcome but CA9 expression was not. The poor outcome of hypoxic tumors could be corrected with hypoxia-modifying treatment with disappearance of the discriminative power of pimonidazole binding and vascularity. Our findings strongly support the notion that nitroimidazole markers together with vascular parameters can provide powerful selection tools for hypoxia-modifying treatment on an individual patient basis. Because CA9 is up-regulated at intermediate levels of oxygenation, its specificity and sensitivity as a hypoxia marker needs additional investigation.

MATERIALS AND METHODS

Patients. At the Department of Radiation Oncology of the University Medical Center Nijmegen, a Phase II clinical trial of ARCON in advanced squamous cell carcinomas of the head and neck has recently been completed (23). ARCON combines accelerated radiotherapy with carbogen breathing and nicotinamide to reduce diffusion-limited and perfusion-limited hypoxia. Eligibility criteria for this study were: (a) stage III or IV squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx and stage II hypopharynx carcinomas with a greatest dimension of the primary tumor >2 cm; (b) age >18 years; (c) WHO performance status of 0–2; (d) no distant metastases; (e) no severe heart or lung disease; (f) no severe liver or kidney function impairments; (g) no severe stridor; and (h) no concurrent treatment for other malignant disease outside the upper aerodigestive tract.

Patients that were potential candidates for this trial were asked to participate in the current hypoxia marker study. After giving consent, the patients received a 20-min i.v. infusion of 500 mg/m² Hypoxprobe-1 (pimonidazole hydro-

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Table 1  Clinical characteristics of 43 squamous cell carcinomas analyzed
(Union International Centre Cancer 1997)*

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>ARCON Non-ARCON*</th>
<th>Palliative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larynx</td>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td></td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Oropharynx</td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Oral cavity</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td>12</td>
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<td>T3</td>
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<td>5</td>
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</tr>
<tr>
<td>T4</td>
<td></td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>N0</td>
<td></td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>N1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>23</td>
<td>17</td>
</tr>
</tbody>
</table>

*Reasons for non-ARCON. Oral cavity/oropharynx tumors smaller than anticipated and still resectable: surgery with postoperative radiotherapy (n = 5); one larynx and one hypopharynx tumor larger than anticipated with extensive cartilage destruction: surgery with postoperative radiotherapy (n = 2); larynx carcinomas smaller than anticipated and not eligible for ARCON: radiotherapy only (n = 2); included in trial with neoadjuvant chemotherapy and radiotherapy (n = 2); and refused participation in ARCON trial, radiotherapy only (n = 6, includes 1 patient with two primary tumors).
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chlord: NPI, Inc., Belmont, MA). Approximately 2 h later, tumor biopsies were taken under general anesthesia. Biopsies were taken for routine diagnostic purposes, and additional biopsies were taken for hypoxia marker analysis. The latter were snap frozen and stored in liquid nitrogen until immunohistochemical processing. The diagnostic workup further included physical examination, chest X-ray, computed tomography scan, and/or magnetic resonance imaging scan of the head and neck area. If, after completion of the diagnostic workup, the eligibility criteria were met and informed consent was obtained, patients were treated with ARCON. Patients that were not eligible or refused inclusion in the trial were treated with radiotherapy, surgery, or a combined modality.

The ARCON trial and the hypoxia marker study were approved by the ethics committee of the University Medical Center Nijmegen.

Immunohistochemistry. From the biopsy material, frozen sections of 5 μm were cut and mounted on poly-L-lysine coated slides and stored at −80°C until staining. Before staining, sections were fixed for 10 min in acetone of 4°C and rehydrated in PBS. Between all consecutive steps of the staining procedure, sections were rinsed three times for 2 min in PBS.

The sections were incubated for 30 min at 37°C with mouse anti-CA9 antibody (Eghert Oosterwijk, Department of Urology, University Medical Center, Nijmegen, The Netherlands), diluted 1:100 in PLD (DPC Breda Diagnostic Products, Breda, The Netherlands), and pimonidazole polyclonal rabbit antibody (J. A. Raleigh (24)) diluted 1:200 in PLD. The second incubation was for 30 min at 37°C with goat-antimouse(Fab)Cy3 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) 1:300 in PLD and donkey-antirabbit-AlexaA488 antibody (Molecular Probes, Leiden, The Netherlands) 1:200 in PLD. This was then followed by 30-min incubation with donkey-antigoat(F(ab')2 tetra-methyl rhodamine isothiocyanate (Jackson ImmunoResearch Laboratories) 1:200 in PLD at 37°C to block the first mouse monoclonal. Next, to stain the vessels, the sections were incubated with the mouse antibody PAL-E (Department Pathology, University Medical Center Nijmegen) 1:6 in PLD for 30 min at 37°C, followed by 30 min incubation at 37°C with chicken-antimouse-AlexaA647 (Molecular Probes) 1:200 in PLD. The monoclonal antibody PAL-E is a marker for human endothelium, especially useful in frozen tissue sections (25). After the staining procedure, the sections were mounted in fluorostab (ICN Pharmaceuticals, Zoetermeer, The Netherlands).
**Image Acquisition and Analysis.** The sections were scanned on an 8-bit digital image analysis system with a high-resolution intensified solid-state camera on a fluorescence microscope (Axioskop, Zeiss, Göttingen, Germany) and a computer controlled motorized stepping stage. The signals captured by the camera were converted to binary images using the digital image application TCL image (TNO, Delft, The Netherlands) on a Macintosh computer as described previously (26, 27). Thresholds for the fluorescent signals were interactively set above the background staining for each individual marker. For each marker, a composite binary image was obtained from the complete biopsy. In conference with a pathologist and guided by a H&E staining of a consecutive section, the tumor area was delineated. This area was used as a mask in additional analysis excluding nontumor tissue, large necrotic areas, and artifacts. One section/biopsy was analyzed.

The VD was calculated as the number of vascular structures over the total tumor area. The RVA was defined as the PAL-E-positive area divided by the total tumor area. HFpimo and HFCA9 were defined as the tumor area positive for pimonidazole and CA9, respectively, relative to the total tumor area. To quantify the distribution patterns of pimonidazole and CA9 relative to the vasculature, zones were chosen at increasing distance from the nearest vessel (0–25 μm, 25–50 μm, 50–100 μm, and >100 μm). The area positive for pimonidazole or CA9 in a particular zone divided by the total pimonidazole or CA9-positive tumor area gave the proportion of marker distributed over that particular zone. HFpimo-distribution and HFCA9-distribution, respectively.

**Statistics.** Statistical analyses were done on a Macintosh computer using the Statistica software package. Correlations between ordinal variables were assessed using linear regression analysis. For differences in ordinal variables in relation to categorical tumor characteristics (site, T-stage, N-stage, and histopathological grade), the Kruskal-Wallis test was used. Cumulative control and survival rates were calculated from the date of pathologic diagnosis using the Kaplan-Meier method, and the log-rank test was used to test for differences. Patients who never reached complete remission after treatment (as assessed by clinical examination) were considered as having locoregional failure from time zero. Cox regression analysis was used to analyze the associations between patient and tumor variables and locoregional tumor control and disease-free survival. \( P < 0.05 \) was considered significant.

**RESULTS**

**Patients and Treatment.** Between May 1998 and February 2001, Hypoxyprobe-1 was administered to 55 patients before biopsy taking. Seven were women and 48 were men and age ranged from 37 to 85 years with a median of 59 years. Two patients had two synchronous

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**Fig. 2.** Fluorescent microscopic images of four head and neck carcinomas stained by triple immunofluorescence as described in the text. Blue, vessels; green, pimonidazole; red, CA9. Overlap of pimonidazole and CA9 signal results in bright orange. SCCNij19 is a negative control, i.e., biopsy material from a patient that was not infused with pimonidazole; apart from a few very small artifacts, this tumor shows no green fluorescence. \( N \) indicates necrosis with nonspecific staining.
primary head and neck tumors that were both biopsied. None of the patients had adverse reactions to Hypoxyprobe-1. Fourteen biopsies were excluded from additional analysis, 6 because they contained no or only very little invasive carcinoma, 6 because of poor quality attributable to mechanical damage during the biopsy procedure, and 2 because the histological diagnosis was not squamous cell carcinoma. Thus, 43 squamous cell carcinomas were analyzed. The clinical characteristics of these tumors are listed in Table 1. After completion of the diagnostic workup, 23 patients were eligible and gave consent for inclusion in the ARCON-trial. Sixteen patients were not included for various reasons and received other treatment as indicated in Table 1. One patient in this group had two primary tumors and was excluded from the analysis for locoregional control and disease-free survival. Also the 3 patients that were treated palliatively were excluded from outcome analysis. Overall, the ARCON patients had more advanced disease. Fig. 1 summarizes the patient selection for the outcome analysis.

Patients in the ARCON trial received 64–68 Gy to the primary tumor and involved neck nodes and 44 Gy to electively treated areas. The dose/fraction was 2 Gy, and two fractions/day were given during the last 1.5 weeks of the treatment with an overall treatment time of 36–38 days. During the irradiations, patients breathed carbogen, and they received nicotinamide, 60 mg/kg p.o., 1 h before the start of irradiations. This protocol has been described previously, and we refer to this publication for details of the treatment and compliance (23). Patients who underwent surgery with postoperative radiotherapy received 50 Gy in fractions of 2 Gy to the surgical bed and 64 Gy to areas at high risk for recurrence using a conventional schedule of once daily fractionation. The other patients were treated with primary radiotherapy, 3 with a conventional schedule (66–70 Gy) and 6 with an accelerated schedule to 68 Gy as described above. Of the latter, 2 patients received neoadjuvant chemotherapy with 5-fluorouracil and cisplatin. The median duration of follow-up was 18 months.

Pimonidazole Binding and CA9 Expression. The triple staining for vessels, pimonidazole and CA9 gave bright fluorescent signals with very little background, except in areas of necrosis and occasionally in the stromal components of the tumor (Fig. 2). CA9 staining was typically confined to the cell membrane, although a weaker cytoplasmic stain was occasionally observed as well. Both pimonidazole and CA9 positivity generally increased with distance from the vessels with considerable overlap of the two signals. CA9 immunoreactivity was usually observed already at shorter distances from blood vessels compared with pimonidazole (Fig. 3). There were also areas of mismatch where CA9 was found but no pimonidazole and vice versa (Fig. 4). All but 1 of 43 biopsies showed good quality of the triple staining. One biopsy demonstrated significant artifacts in the CA9 staining and was excluded from the comparison between CA9 and pimonidazole. Mean and median values and range for HFpimo, HFCA9, VD, and RVA in the biopsy material are given in Table 2. HFpimo, HFCA9, VD, and RVA were independent of site, T stage, N stage, and histopathological grading. No correlations were found between HFpimo or HFCA9 and the vascular parameters. Linear regression analysis showed a significant but weak correlation between HFpimo and HFCA9 (Fig. 5). The distribution pattern of the two markers relative to the vasculature was similar, however, with increasing signal intensity at greater distance from vessels (Fig. 6). CA9 positivity was greater in the zones 25–50 μm and 51–100 μm from vessels relative to pimonidazole positivity, which was more pronounced at distances >100 μm. The first zone (0–25 μm) directly adjacent to vessels included mainly stromal cells and extracellular matrix in the majority of the tumor samples. Only very small tumor areas were generally found in this zone. This zone was therefore excluded from the analysis because it represents mainly nontumor tissue.

Correlations of Patient and Tumor Characteristics with Treatment Outcome. Univariate Cox regression analysis demonstrated no associations between tumor site, T stage, N stage, or histopathological grade and locoregional control or disease-free survival (Table 3). HFpimo, HFCA9, VD, and RVA were tested both as continuous variables and as dichotomous variables (stratification by median values; Table 3). When entered in the model as continuous variables, only VD showed a significant association with locoregional control.
As dichotomous variables both HFpimo and VD demonstrated significant associations with locoregional control. Patients with high HFpimo or low VD did worse. Fig. 7 shows Kaplan-Meier estimates for locoregional control and disease-free survival stratified by the median of HFpimo. Locoregional tumor control at 2 years was 48% for patients with hypoxic tumors versus 87% for patients with less hypoxic tumors ($P = 0.01$). Disease-free survival was 38 and 70%, respectively ($P = 0.04$). When analyzed by treatment, ARCON versus non-ARCON, the difference in outcome between low and high HFpimo was mainly found in the non-ARCON group. The same phenomenon was observed for VD with 48% locoregional control for

![Composite binary images of four head and neck carcinomas](image)

**Table 2** Values for HFpimo, HFCA9, VD, and RVA in biopsies of 43 squamous cell carcinomas of the head and neck

<table>
<thead>
<tr>
<th></th>
<th>HFpimo (%)</th>
<th>HFCA9 (%)</th>
<th>VD (mm$^2$)</th>
<th>RVA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>6.0</td>
<td>6.4</td>
<td>20.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Median</td>
<td>5.6</td>
<td>3.5</td>
<td>16.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Range</td>
<td>0.3–17.2</td>
<td>0.6–29.4</td>
<td>6.2–58.2</td>
<td>0.5–4.2</td>
</tr>
<tr>
<td>SD</td>
<td>4.6</td>
<td>6.8</td>
<td>11.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

![Comparison between the fraction of the tumor area that binds CA9 antibody (HFCA9) with the fraction of the tumor area that binds pimonidazole antibody (HFpimo)](image) in 42 squamous cell carcinomas of the head and neck. The linear best fit is shown.

$p = 0.02$ $R = 0.36$
tumors with low vascularity and 88% for tumors with high vascularity \((P = 0.01, \text{Fig. 8})\).

**DISCUSSION**

**CA9 Expression and Hypoxia.** Induction of the CA9 protein by hypoxia has been observed in a number of cell lines, including cell lines derived from squamous cell carcinomas of the head and neck \((16, 28, 29)\). The time course of gene expression in response to hypoxia in a panel of human tumor lines was measured by real-time PCR \((17)\). CA9 had the greatest magnitude of induction among 12 hypoxia-overexpressed genes and was induced in the greatest number of tumor cell lines. Western blot analysis, using an antibody to CA9, showed that under hypoxia, protein levels steeply increased from 4 to 24 h, which was similar to the transcript level time course. Experiments with graded hypoxia in one cell line demonstrated CA9 induction from \(pO_2\) levels of \(\sim 20\) mmHg and downward \((28)\). In this study, there was concordance between CA9 expression and pimonidazole binding with increasing positivity at greater distance from the blood vessels. CA9 expression was often observed at shorter distances from the vessels, suggesting that CA9 up-regulation occurs at \(pO_2\) levels higher than required for pimonidazole binding. In earlier work we demonstrated that at a certain distance from the blood vessels, there is a rapid increase from background to maximum level of the fluorescent pimonidazole signal \((27)\). This is consistent with data from other studies, showing a steep rise in binding of the 2-nitroimidazole, misonidazole, at \(pO_2\) values \(< 10\) mmHg in multicellular spheroids \((30)\) and comparable \(K_m\) and binding patterns of pimonidazole \((31)\).

Olive et al. \((18)\) demonstrated that in spheroids from human cervical cancer cells (SiHa) only 0.5% of the cells were sufficiently hypoxic to bind pimonidazole, yet 12% of the cells bound CA9 antibodies. In tumor biopsies of patients with cervical cancer, the percentage of the tumor area with CA9 immunostaining was 2-fold the area that was stained for pimonidazole \((18)\). This indicates that CA9 expression identifies low tissue oxygenation levels as well as intermediate levels.

With double hypoxia marker experiments in xenografted head and neck tumors, although with higher pimonidazole doses, we demonstrated that the presence of pimonidazole for a period of 30 min is sufficient to stain all hypoxic areas \((32)\). Tumor regions that were only temporarily hypoxic may stain for pimonidazole but not for CA9 because up-regulation of CA9 requires at least several hours. This can explain, at least partly, the pimonidazole-positive but CA9-negative areas. Other reasons for mismatch can be additional positive or negative nonhypoxic stimuli influencing CA9 expression or defective expression of CA9 in some tumors. CA9 is involved in intra- and extracellular pH homeostasis of tumor cells. CA9 is localized normally on differentiated cells specialized in acid/base regulation (collecting ducts of the kidney, gastrointestinal gland cells), suggesting a role for CA9 in maintaining extracellular acidity in tumors \((16)\). Thus, changes in the acid/base balance may provide additional stimuli for CA9 expression. In bladder and skin carcinomas, Wykoff et al. \((28)\) observed pimonidazole staining extending beyond CA9 immunoreactivity, the latter being more tightly limited to perinecrotic regions. This is in contrast to the results of this study and those of Olive et al.

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**Table 3 Correlations of tumor characteristics with treatment outcome (Univariate Cox regression analysis)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Locoregional control</th>
<th>Disease-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>n.s.(^a)</td>
<td>n.s.</td>
</tr>
<tr>
<td>(T) stage</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>(N) stage</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Grade</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>HFpimo</td>
<td>Continuous, (P = 0.09)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Dichotomous, (P = 0.02)</td>
<td>(P = 0.09)</td>
</tr>
<tr>
<td>HFCA9</td>
<td>Continuous, n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Dichotomous, n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>VD</td>
<td>Continuous, (P = 0.02)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Dichotomous, (P = 0.02)</td>
<td>n.s.</td>
</tr>
<tr>
<td>RVA</td>
<td>Continuous, n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Dichotomous, n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

\(^a\) n.s., not significant.
Whether this truly represents differences between tumors of different origin and histology or if this should be explained by differences in immunohistochemical techniques is yet unclear. In the skin and bladder carcinomas, no overall correlation between the percentage of tumor stained for pimonidazole and CA9 was found. It can be concluded that, although CA9 is indicative for hypoxia, it is less specific compared with pimonidazole, which can explain the weak correlation between relative pimonidazole and CA9-positive areas in this study (Fig. 5).

The transmembrane carbonic anhydrases are regulated by HIF-1, which is considered to be a master regulator integrating physiological responses to hypoxia. HIF-1 itself is also gaining interest as an intrinsic hypoxia marker. Immunohistochemical detection of HIF-1 demonstrated patterns similar to those of its target gene products with typical perinecrotic staining (33, 34). However, more diffuse expression patterns with staining in proximity of blood vessels are also reported (33, 34). In cervical cancer xenografts, maximal HIF-1 expression generally occurred at shorter distances from blood vessels.
than the maxima of \([2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyloxy)acetamide] (EF-5) binding (33). This suggests that HIF-1 up-regulation occurs at higher \(pO_2\) levels than required for nitromidazole binding, similar to what we observed for CA9. HIF-1α expression was inversely correlated with local failure-free survival, disease-free survival, and overall survival in squamous cell carcinomas of the oropharynx treated with radiotherapy (34).

**Prognostic Value of Tumor Vascularity and Hypoxia-related Markers.** In this study, there was a significant association between VD and locoregional tumor control. With our automated image processing system, we analyzed complete tissue sections, and the average number of vascular structures was calculated per mm² tumor area. This is different from hot-spot counting, where vessel counting is restricted to selected areas of high vascularity, yielding what is generally referred to as MVD. Three studies in head and neck carcinomas found an association between MVD and local tumor control after radiation treatment (35–37). One study demonstrated an increased risk of local recurrence with low MVD in larynx carcinomas but another showed the opposite in oropharynx tumors. The largest study on head and neck tumors thus far published found a U-shaped relationship between MVD and survival after combined radiotherapy and chemotherapy (37). An intermediate MVD defined a better survival, whereas both low MVD and high MVD were linked to poor outcome. This observation was explained by a poor treatment response attributable to insufficient oxygen and drug availability in the poorly vascularized cases and an increased metastatic potential in the highly vascularized tumors. The association between low VD and poor oxygenation and, consequently unfavorable outcome after radiotherapy, is compatible with the results of this study. The suggestion is strengthened by the fact that the outcome of poorly vascularized tumors improved with ARCON. A weakness in this hypothesis is, however, the absence of a correlation between VD and HFPimo in the current material.

Retrospective clinical studies support the notion that CA9 represents tumor aggressiveness. High CA9 expression predicted poor survival in carcinoma of the head and neck, lung, uterine cervix, and breast (19–22). One older study, however, showed the reverse in early cervical cancers (38). The association of CA9 expression with local tumor response is still equivocal. One retrospective study in patients with head and neck carcinomas treated with concurrent chemoradiotherapy did show a correlation with local control rate, whereas a study in patients with cervical carcinomas treated with radiotherapy did not (19, 22). In this study, we found no correlation with locoregional control nor with disease-free survival. Although not consistently so, the available data are supportive of a role for CA9 as a predictor of tumor aggressiveness reflected in patient survival. Whether CA9 can serve as a reliable marker of tumor hypoxia and predictor of local outcome remains unclear to date.

To our knowledge, this is the first report presenting data on the predictive value of pimonidazole. Direct measurements of hypoxia with oxygen microelectrodes have been associated with treatment outcome in squamous cell carcinomas of the head and neck and uterine cervix (2, 4–6). Likewise, the pimonidazole binding assay, also a direct indicator of tumor hypoxia, demonstrated a significant association with locoregional control and disease-free survival in this study. Patients with hypoxic tumors showed a worse initial response to the treatment and more locoregional recurrences within the first 15 months of follow-up (Fig. 7A). After this initial period, there was no additional divergence of the course of the locoregional control or disease-free survival curves (Fig. 7, A and C). This suggests that the worse outcome of hypoxic tumors is mainly determined by early locoregional failures and not so much by later events such as distant metastases. Follow-up is still short, however, and distant metastases may occur later than 2 years. The observation that the association with locoregional control exists mainly in the non-ARCON treatment group but hardly in the ARCON treatment group (Fig. 7B) is a strong indication that pimonidazole binding indeed reflects hypoxic radiation resistance. This also indicates that the nitromidazole binding assay, together with vascular parameters, may provide a selection tool for hypoxia-modifying treatments on an individual patient basis.

An important limitation of this study is the relatively small sample size and the heterogeneity of the patient selection with regard to tumor site and stage. This could partly explain why no correlations were found between these classical clinical parameters and outcome. However, also in a larger series of 215 ARCON-treated patients, T stage was no longer a prognostic indicator, which may well be the result of the very high local control rates with this treatment (23). Furthermore, treatment assignment was not randomized, and various treatment modalities were used in the non-ARCON group and follow-up is still short. This precludes firm conclusions to be based on this material, and verification of the results is needed in a larger patient cohort. A recently started randomized trial comparing ARCON with accelerated radiotherapy in laryngeal cancer with a projected accrual of 344 patients will provide sufficient material to validate the conclusions of this study.

**REFERENCES**

Pimonidazole Binding and Tumor Vascularity Predict for Treatment Outcome in Head and Neck Cancer


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