Colocalization of Carbonic Anhydrase 9 Expression and Cell Proliferation in Human Head and Neck Squamous Cell Carcinoma

Ilse J. Hoogsteen,1 Henri A.M. Marres,2 Karien I.E.M. Wijffels,2 Paul F.J.W. Rijken,1 Johannes P.W. Peters,1 Francisiscus J.A. van den Hoogen,2 Egbert Oosterwijk,3 Albert J. van der Kogel,1 and Johannes H.A.M. Kaanders1

Departments of 1Radiation Oncology, 2Otorhinolaryngology/Head-Neck Surgery, and 3Urology, University Medical Center, St. Radboud, Nijmegen, the Netherlands

ABSTRACT

Purpose: Tumor cells undergo a variety of biological changes under sustained hypoxic conditions, allowing cells to survive and retain their clonogenic potential. The purpose of this study is to relate the expression of the hypoxia marker carbonic anhydrase 9 (CA9) to the uptake of iododeoxyuridine (IdUrd), a marker of proliferation, in head and neck squamous cell carcinomas. Colocalization of IdUrd and CA9 may identify an important subpopulation of tumor cells that might be responsible for repopulation and disease progression.

Experimental Design: Expression of CA9, IdUrd labeling, and colocalization between IdUrd and CA9 was examined by immunohistochemistry in biopsies of head and neck squamous cell carcinomas. Biopsies were taken from 51 patients recruited between 1998 and 2001 after administration of the proliferation marker IdUrd.

Results: A large variation was observed between the tumors in CA9 expression (range 0-39%), IdUrd labeling (range 0-81%), and colocalization between IdUrd and CA9 [FID(CA9); range 0-53%], FID(CA9) the fraction of IdUrd-labeled cells positive for CA9, was highest at an intermediate distance from the blood vessels (100-150 μm). IdUrd labeling was higher in T4 carcinomas relative to lower stage tumors (P = 0.04). High FID(CA9) correlated with the worst disease-free survival rates (P = 0.04).

Conclusions: Colocalization between IdUrd labeling and CA9 expression was observed in head and neck squamous cell carcinomas, suggesting the presence of a population of tumor cells under intermediate hypoxic conditions which still has proliferative capacity. The size of this subpopulation may be indicative of tumor aggressiveness and is associated with the worst disease-free survival rates.

INTRODUCTION

There is a growing awareness that malignant neoplasms could escape from anticancer treatments through several mechanisms. Tumor cell repopulation and hypoxia are important factors determining the response of squamous cell carcinomas to radiation treatment. Tumor hypoxia is associated with a poor prognosis in several types of cancer such as carcinomas of the uterine cervix, and head and neck squamous cell carcinomas (1-3). Under sustained hypoxic conditions, tumor cells undergo a variety of biological responses and changes in their genetic expression profiles including activation of several signaling pathways for the regulation of proliferation, angiogenesis, and apoptosis (4, 5). These adaptation mechanisms allow clonogenic tumor cells to survive and even proliferate under hypoxic conditions. Also, these processes contribute to a more aggressive and malignant tumor phenotype (1, 4, 6). Eventually, clonogenic tumor cells may survive treatment and are triggered to repopulate even more effectively forming local recurrences (7).

Several methods have been described to measure tumor oxygenation. These include the Eppendorf polarographic oxygen electrode (8), the use of bioreductive drug markers such as the 2-nitroimidazoles pimonidazole hydrochloride and EF5 (9, 10), or detection of endogenous hypoxia markers. In a previous study, we showed a significant association between the pimonidazole binding assay and loco-regional control and disease-free survival in patients with head and neck carcinomas (3). A disadvantage of this technique however, is that it requires i.v. administration of the hypoxia marker before biopsy.

Endogenous hypoxia markers are currently receiving great interest as a new and convenient way for the detection of hypoxia. One interesting group of gene products is the tumor-associated carbonic anhydrases (CA), of which CA9 is the most promising one. Several studies have reported the overexpression of CA9 in several solid tumors, including uterine cervix (11-13), breast (14-16), bladder (17, 18), renal cell (19), colorectal (20, 21), non–small cell lung cancer (22, 23), and head and neck carcinomas (3, 24-26), albeit being absent in normal tissues.

This family of carbonic anhydrases catalyzes the reversible hydration of carbon dioxide to carbonic acid (27), and participates in a variety of biological processes including the regulation of pH, respiration, and calcification (28). The tumor-associated CA9 plays a role in maintenance of pH homeostasis in several solid tumors, including uterine cervix (11-13), breast (14-16), bladder (17, 18), renal cell (19), colorectal (20, 21), non–small cell lung cancer (22, 23), and head and neck carcinomas (3, 24-26), and may be a marker of tumor aggressiveness (7).
CA9 was found to be induced by hypoxia in a wide range of malignant cells in vitro including bladder, breast, uterine cervix, and lung cancer cell lines. It has been shown that up-regulation of CA9 was directly regulated through constitutive activation of the hypoxia-inducible factor-1 (HIF-1) pathway after inactivation of the von Hippel-Lindau tumor suppressor gene (30). Immunohistochemical expression of CA9 is usually described as a typical “perinecrotic” pattern consistent with the distribution of diffusion-limited hypoxia (25). Previous studies have shown substantial, but incomplete, overlap with pimonidazole in cancer of the uterine cervix (12), and head and neck carcinomas (3).

Proliferative potential has also been recognized as an important determinant of the efficacy of radiotherapy. Various studies in several tumor types have examined the prognostic significance of proliferation on local control and survival after radiotherapy (31–35). Results have been variable, some studies showing a correlation, whereas others did not. Different markers were used for assessment of proliferation. These include endogenous markers such as Ki-67, proliferating cell nuclear antigen (PCNA), or the i.v. administration of 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.

Despite numerous studies investigating hypoxia or proliferation in relationship to treatment outcome, little is known about the combination of these parameters. Colocalization between proliferating cells and hypoxia may define important subpopulations in the tumor that might be responsible for repopulation and disease progression. The purpose of the present work is to relate expression of CA9 to the presence of IdUrd in S-phase cells in head and neck carcinomas. The amount of colocalization between the two markers relative to the total tumor area and in relationship to the vasculature was analyzed and related to loco-regional control and disease-free survival.

MATERIALS AND METHODS

Patients. Between May 1998 and November 2001, 68 patients with head and neck squamous cell carcinomas were included in our study at the University Medical Center, Nijmegen. Patients with primary stage II to IV squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx were included. Other inclusion criteria were: (a) age over 18 years, (b) WHO performance status of 0–2, (c) no severe heart or lung disease, (d) no severe liver or kidney dysfunction, (e) no severe stridor, (f) no distant metastases, and (g) written informed consent. Approval from the local ethics committee was obtained.

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Approximately 20 minutes before biopsies were taken, patients received 200 mg of the proliferation marker 2′- deoxy-5-iodouridine (IdUrd; Centre Hospitalier Univeristaire, Lausanne, Switzerland) dissolved in 100 mL NaCl 0.9% and given i.v. over 5 minutes. Biopsies were taken for routine diagnostic purposes and additional biopsies were taken for CA9 and proliferation marker analysis. The latter were snap-frozen and stored in liquid nitrogen until immunohistochemical processing.

Immunohistochemical Staining of IdUrd, CA9 and Vessels. From the frozen biopsy material, 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 minutes in acetone at 4°C and rehydrated in PBS 0.1 mol/L (pH 7.4). Between all consecutive steps of the staining procedure sections were rinsed thrice for 2 or 5 minutes in PBS. The sections were incubated for 30 minutes at 37°C with mouse anti-CA9 antibody (E. Oosterwijk, Department of Urology, University Medical Center, Nijmegen, the Netherlands) diluted 1:100 in polyclonal liquid diluent (PLD; DPC Breda Diagnostic Products, Breda, the Netherlands). The second incubation was for 30 minutes at 37°C with goat anti-mouse (Fab′)-Cy3 antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) 1:600 in PLD. This was followed by an incubation period of 30 minutes at 37°C with donkey anti-goat F(ab′)2 tetramethyl rhodamine isothiocyanate (Jackson ImmunoResearch Laboratories) diluted 1:600 in PLD to block the first mouse monoclonal. Next, the sections were denatured with HCl 2N and Borax 0.1 mol/L to neutralize the pH, both for 10 minutes. For IdUrd staining, the tissue sections were incubated with mouse anti-IdUrd (Caltag Laboratories, Burlingame, CA, USA) 1:1,000 in monoclonal liquid diluent (DPC Breda Diagnostic Products) for 30 minutes at 37°C. This was then followed by incubation for 30 minutes with donkey anti-mouse Biotin (Jackson Immunoresearch Laboratories) 1:200 in PLD and for 30 minutes with STREP-Alexa 350 (Molecular Probes, Eugene, OR, USA) 1:200 in PLD both at 37°C. To completely block the mouse anti-IdUrd antibody, the sections were incubated overnight at a temperature of 4°C with rabbit anti-mouseFab (Jackson Immunoresearch Laboratories) 1:50 in PLD. After this overnight procedure, the sections were thoroughly rinsed thrice with PBS for 5 minutes and stained for vessels by incubation with the mouse antibody PAL-E (Department of Pathology, University Medical Center, Nijmegen, the Netherlands) 1:6 in monoclonal liquid diluent for 30 minutes at 37°C. This was followed by incubation for 30 minutes at 37°C with chicken anti-mouse Alexa647 (Molecular Probes) 1:100 in PLD. The monoclonal antibody PAL-E is a marker for human endothelium, especially useful in frozen tissue sections (36). After the staining procedure, the sections were mounted in fluorostab (ICN Pharmaceuticals, Zoetermeer, the Netherlands).

After image acquisition of the CA9, IdUrd, and vessel signals (see below), all nuclei were stained with fast blue (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). The second incubation was for 30 minutes with STREP-Alexa 350 (Molecular Probes, Eugene, OR, USA) 1:200 in PLD both at 37°C. To completely block the mouse anti-IdUrd antibody, the sections were incubated overnight at a temperature of 4°C with rabbit anti-mouseFab (Jackson Immunoresearch Laboratories) 1:50 in PLD. After this overnight procedure, the sections were thoroughly rinsed thrice with PBS for 5 minutes and stained for vessels by incubation with the mouse antibody PAL-E (Department of Pathology, University Medical Center, Nijmegen, the Netherlands) 1:6 in monoclonal liquid diluent for 30 minutes at 37°C. This was followed by incubation for 30 minutes at 37°C with chicken anti-mouse Alexa647 (Molecular Probes) 1:100 in PLD. The monoclonal antibody PAL-E is a marker for human endothelium, especially useful in frozen tissue sections (36). After the staining procedure, the sections were mounted in fluorostab (ICN Pharmaceuticals, Zoetermeer, the Netherlands).

Image Acquisition. The tissue sections were scanned with a digital image analysis system consisting of a fluorescence microscope (Axiophot, Zeiss, Göttingen, Germany) and a computer-controlled motorized stepping stage, using IPLab software (Scanalytics, Inc., Fairfax, VA, USA) on a Macintosh computer, as described previously (37). Each tissue section was sequentially scanned for the CA9, IdUrd, and vessel signals. The resulting composite gray scale images, captured by a 12-bit CCD camera (MicroMax, Roper Scientific, Inc., Trenton, NJ, USA) were converted to binary images using the digital imaging application IPLab. Thresholds for the fluorescent signals
were interactively set above the background staining for each individual marker. After the scanning procedure, the corresponding composite binary images were superimposed into one pseudocolored image. With H&E staining of a consecutive section and under the supervision of a pathologist, the tumor area was delineated. This area was subsequently used as a mask in additional analysis from which nontumor tissue, large necrotic areas, and artifacts were excluded.

**Analysis.** To facilitate the analysis and calculations of the amount of colocalization between CA9 (membrane staining) and IdUrd (nuclear staining), a binary closing operation on the CA9 signal was done. The fraction of CA9 (FCA9) was defined as the tumor area positive for CA9 relative to the total tumor area. The labeling index (IdUrd-LI) was determined as the area of IdUrd positively stained nuclei relative to the total area of nuclei. Based on IdUrd staining, the biopsies were categorized into the four proliferation patterns as previously described by Bennett et al. (38) and Wijffels et al. (39). The marginal pattern shows proliferating cells in the basal and suprabasal layers. The second, intermediate pattern shows proliferating cells not only in the basal and suprabasal layers but also in more distinct layers. The random pattern shows a diffuse distribution of proliferation without obvious organization. The mixed proliferation pattern shows a combination of these patterns.

The fraction of IdUrd-labeled cells positive for CA9 [FId(Urd,CA9)] was defined as the area that stained positive for both IdUrd and CA9 divided by the total IdUrd-positive area. The vascular density was calculated as the number of vascular structures per square millimeter and the relative vascular area was defined as the PAL-E positive area divided by the total tumor area.

**Statistics.** Statistical analyses were done on a Macintosh computer using the Prism 4.0 software package. To test for differences between overall IdUrd-LI analyses, the nonparametric t test was used. Linear regression analysis was used to assess correlations between the different parameters. Survival rates were calculated from the date of histologic diagnosis using the Kaplan-Meier method, and the log-rank test was used to test for differences between survival rates.

**RESULTS**

**Patients and Treatment.** This study included 68 patients, and in total, 74 biopsies were collected. IdUrd was given to all patients before biopsy and none of them had adverse reactions. Of these patients, 8 were women and 60 were men and their ages ranged between 36 and 84 years with a median of 58 years. Seventeen patients were excluded from the analysis, five patients because their biopsies contained no or too little tumor tissue, one because of histologically confirmed adenocarcinoma, nine because the biopsy was of poor quality attributable to mechanical damage during biopsy or because of large areas of necrosis, and two patients whose biopsies were not available for analysis. One patient had two synchronous primary head and neck tumors, an oropharyngeal and a hypopharyngeal carcinoma that were both biopsied. Multiple biopsies were available from five patients. Thus, 52 histologically confirmed squamous cell carcinomas of the head and neck were analyzed. The primary tumors were localized in the oral cavity (3), oropharynx (15), hypopharynx (14), and in the larynx (20). Table 1 shows the T and N classification of the tumors.

After discussion in the head and neck working group, patients received different treatments. Primary radiotherapy was given to 33 patients, of which 24 were included in a phase II trial of ARCON (accelerated radiotherapy combined with carbogen and nicotinamide). This treatment regimen combines accelerated radiotherapy with carbogen breathing and nicotinamide (40). Of the patients that received radiotherapy alone, five were treated with conventional radiotherapy and four received an accelerated schedule. The other patients were treated with surgery followed by postoperative radiotherapy (9), or radiotherapy and neoadjuvant chemotherapy with 5-fluorouracil, cisplatin, and methotrexate (6). Three patients died before any treatment could be started. The median duration of follow-up for all 51 patients was 22.7 months and for surviving patients 44.3 months.

**CA9 Expression and IdUrd Binding.** All tissue sections were stained for CA9, IdUrd, and vessels. The triple staining gave strong and bright fluorescent signals with very little background except in areas of necrosis where some nonspecific staining was sometimes observed. If staining of the markers was present, all 57 biopsies showed good quality of staining. Figure 1 shows examples of different expression patterns of CA9 in head and neck squamous cell carcinomas. Staining of CA9 was predominantly confined to the cell membrane. CA9 expression was generally found at an increasing distance from blood vessels but it was also sometimes observed in close proximity to vessels.

IdUrd binding was observed in the nuclei of tumor cells as it is incorporated in the DNA of S-phase cells. Based on IdUrd staining, tumors were classified into four proliferation patterns as described earlier. The proliferation pattern was defined as marginal in 16 tumors, intermediate in 12, random in 16, and mixed in 5 tumors. In 3 tumors, hardly any IdUrd-positive nuclei could be detected and they were not classified. Of the 5 tumors of which multiple biopsies were available, 2 showed intratumoral differences in proliferation pattern. IdUrd labeling index generally decreased with distance from blood vessels, whereas CA9 expression increased with distance from the blood vessels. The variation in distribution of CA9 expression and IdUrd labeling between biopsies is shown in Fig. 2.

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<th>Table 1</th>
<th>T and N classification of 52 head and neck squamous cell carcinomas</th>
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*Fifty-one patients were analyzed. One patient had two synchronous primary tumors.

All patients were M0.
The highest fraction of IdUrd-labeled cells positive for CA9 was usually observed at intermediate distances (100-150 μm) from the blood vessels. Variable degrees of colocalization between IdUrd and CA9 were seen between the biopsies (Fig. 2C). Figure 3 shows different examples of colocalization. No correlations were found between Fld(CA9) and tumor site, stage, or proliferation pattern.

The IdUrd labeling increased with T stage. The overall IdUrd labeling of the T4 tumors (median 7%) was significantly different \((P = 0.04)\) from the overall IdUrd labeling of T1-3 tumors (median 5%). This relationship did not exist for Fld(CA9).

Using linear regression analysis, no significant correlations were found between FCA9, LI, Fld(CA9), and the vascular density, and relative vascular area.

**Correlations With Loco-Regional Control and Disease-Free Survival.** For survival analysis, the patient group was divided by the median values of FCA9, IdUrd-LI, and Fld(CA9) and, because of the skewed distribution and many low percentages of CA9-positive areas (Fig. 2A), by the 75th percentile of FCA9 as well. Figure 4 shows the Kaplan-Meier estimates for loco-regional control and disease-free survival by the 75th percentile of FCA9 and the median of IdUrd-LI. No significant differences in survival were noted between either the subgroups of patients or with stratification by the median of FCA9. Figure 5 shows Kaplan-Meier estimates for loco-regional control, metastasis-free survival, and disease-free survival stratified by the median of Fld(CA9). No significant difference was observed between the two subgroups for loco-regional control \((P = 0.39)\) or metastasis-free survival \((P = 0.20)\). Five-year disease-free survival, however, was 27% for patients with high Fld(CA9) versus 64% for patients with low Fld(CA9) \((P = 0.04)\).
**DISCUSSION**

Many normal tissues, particularly continually renewing systems, consist of functionally and kinetically distinct populations of cells. This heterogeneity is also a common feature of many tumors, which can be attributed to microenvironmental factors and changes in gene expression. Differences in oxygenation and proliferation within a tumor are often seen and show the dynamic nature of evolving and regressing subpopulations of cells, each of them with different implications for tumor growth and treatment response (41). Tumor hypoxia is associated with adverse outcome for several solid tumors independent of treatment modality (1–3). Also, rapid proliferation is regarded as an important cause of treatment resistance (33). Furthermore, in normal tissues as well as in tumors, gradients of oxygen and nutrients exist. The population of hypoxic cells is therefore heterogeneous both in terms of duration of hypoxia, pO2 level, and proliferative activity. This heterogeneity of the hypoxic tumor cell population may have important implications for cancer treatment.

**CA9 Staining.** Externally administered 2-nitroimidazoles such as pimonidazole and EF5 are probably the most robust markers of cellular hypoxia in clinical studies. They are reductively activated in tissues at pO2 values < 10 mm Hg (9). Wykoff et al. (30) showed that CA9 was strongly induced by hypoxia in a broad range of cell types. They showed that the CA9 promoter was tightly regulated by a hypoxia-inducible factor-1 response element and that CA9 expression was overlapping with pimonidazole. In previous work from our group, concordance was also found between CA9 expression and pimonidazole binding, although areas of mismatch were observed as well. CA9 expression was often seen at shorter distances from blood vessels suggesting that up-regulation of CA9 occurs at higher pO2 levels than pimonidazole binding (3). Experiments with cells exposed to increasing degrees of hypoxia, showed induction of CA9 expression after 16 hours at pO2 levels < 20 mm Hg (30). In this study, CA9 expression was often observed at intermediate distance from blood vessels, which confirms earlier observations (3, 30). However, Hedley et al. (13) did not find a significant correlation between CA9 expression and pO2 levels (< 5 mm Hg) measured by the Eppendorf probe method in cervix carcinomas. They explained the lack of association by intratumoral heterogeneity at the microregional level and the action of biological factors other than hypoxia, such as pH influencing the expression of CA9. It can be concluded that CA9 expression can be indicative of low and intermediate oxygenation levels. However, the exact biological relationship between CA9 expression, hypoxia, and pH is complicated.

**Proliferation.** Proliferation, measured by IdUrd labeling and other static measurements will be affected by several factors such as the duration of S phase, the differentiation status, microenvironmental factors governing oxygen and nutrient availability, local growth factor levels, and cell cycle gene regulation. Our observation of decreasing proliferative activity with distance from vessels confirms the findings of other investigators (42, 43). Differences between tumors can be categorized into four proliferation patterns: marginal, intermediate, random, and mixed (38, 39). Between the patterns, however, no differences were found in the Flid(CA9) nor could a correlation with outcome be found. An interesting observation is the finding that the IdUrd labeling increased with T stage. This may suggest
that the more advanced T4 carcinomas harbor an actively proliferating cell population resulting in a more extended and perhaps a more aggressive tumor. This, however, was not reflected by an increase in colocalization between IdUrd and CA9. Colocalization of IdUrd and CA9 is mainly found at intermediate distances (100-150 μm) from the blood vessels. Green, CA9; red, IdUrd; white, vessels.

**Hypoxia and Proliferation.** With the triple staining method employed in this study, we showed substantial colocalization between IdUrd binding and CA9 expression both in proximity and more distant from vessels. A number of studies have been done in recent years comparing tumor hypoxia, using...
administration of bioreductive drugs, and tumor cell proliferation in canine tumors, xenografts and human malignancies (42–49). Zeman et al. (44) described a “geographic” relationship between the distribution of proliferating and hypoxic tumor cells in spontaneous canine tumors. They found apparent overlap between hypoxic areas labeled with hexafluoromisonidazole (CCI-103F) and proliferating cell nuclear antigens. Using a similar technique, Raleigh et al. (42) described a small degree of overlap between the two markers that seemed to occur along the edges of hypoxia marker labeled areas. In the same study, they showed a larger overlap between the hypoxic and proliferating compartments after radiation therapy with 15 Gy of cobalt-60 gamma rays in canine tumors. Ljungkvist et al. (43) made a similar observation in xenografts from human head and neck squamous cell carcinomas by using pimonidazole and bromodeoxyuridine. They showed the coexistence of hypoxia and proliferation even at 100 to 150 μm from vessels. Experiments by Durand and Raleigh (50) 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 tumor. It can be concluded from these studies that proliferation sparsely occurs at pO2 levels below 10 mm Hg required for nitroimidazole binding.

Only limited data exist on the relationship between CA9 and proliferation. Pastorek et al. (51) were the first to show that CA9 promoted cell proliferation in fibroblasts. In an immunohistochemical study of colorectal tumors, Saarnio et al. (20) showed abnormal expression of CA9 in colorectal neoplasms and coexpression of CA9 and Ki-67 in colorectal adenocarcinomas indicating a relationship between CA9 and proliferation. They also found colocalization in normal colorectal crypt mucosa. It was suggested that CA9 might play a role in intercellular communication and cellular proliferation. Colocalization between IdUrd and CA9 was seen in the present study, clearly showing tumor cells both in the S phase of the cell cycle and expressing CA9. FId(CA9) was highest at an intermediate distance from blood vessels and decreased at large distances, most likely caused by deficiency of nutrients, lower pO2, and low pH. This suggests that a population of cells can be identified which exists under intermediate hypoxia and is still able to proceed through the cell cycle and proliferate slowly. Several definitions explaining colocalization between proliferating cells and CA9 have been considered in this study, making use of absolute numbers, total tumor area, CA9-positive and IdUrd-positive areas. The denominator was an important factor in determining colocalization, particularly in CA9-positive areas; therefore, it was decided to express colocalization as a ratio with the IdUrd-positive area being the denominator as defined by FId(CA9).

The Cell Cycle and Hypoxia. An important population of tumors seems to be the cells with slower proliferation rates. It is in these cells that entry and exit to the cell cycle will occur (41). It is a heterogeneous population consisting of cells whose fate is apoptosis and cells that persist in a viable state for a long time and could be recruited back in the cell cycle under better conditions (41). Hypoxia has been shown to induce arrest predominantly in the G0/G1 phase or induce apoptosis (48). Cell cycle analysis by flow cytometry revealed an increase of arrested cells in the G0/G1 phase with a concomitant decrease in S-phase

**Fig. 4** Kaplan-Meier estimates of loco-regional control (A and C) and disease-free survival (B and D). Stratification is by the 75th percentile of the FCA9 and the median of IdUrd-LI. Comparison by log-rank test showed that differences between the curves were not significant.
cells (48). Webster et al. (49) showed that hypoxia delays progression of the tumor cell through the cell cycle leading to an accumulation of cells in the G2 phase. These cells may have completed a hypoxic S phase and progress slowly or are unable to continue into mitosis (49). The positive staining of cells with IdUrd seen in this study may well indicate cells that are progressing slowly through a hypoxic S phase before entering the G2 phase. Cells that are arrested in the G2 phase are not labeled by IdUrd and it is therefore possible that we might have missed such an “invisible” and perhaps important clonogenic subpopulation of tumor cells.

**Regulation of pH.** Low pH contributes to a more malignant tumor phenotype. It has been shown that a low pH and accumulation of lactic acid are able to inhibit cell proliferation and survival (48). CA9 is known to be involved in the regulation of pH (28), and may therefore be a crucial enzyme in cell survival decisions and tumor progression. In their study, Schmaltz et al. (48) showed that the trigger for apoptosis in hypoxic cultures is acid accumulation, providing a selective tool for apoptosis-resistant malignant clones. Furthermore, they showed in *vitro* that under hypoxia, but in non-acidotic conditions, cells have lost the hypoxic G0/G1 checkpoint and continue to proliferate slowly. Another study by Helmlinger et al. (52) described a frequent discordance between hypoxic and acidic regions in solid tumors *in vivo*. Under hypoxia, changes in gene expression occur and favor clonal selection of tumor cells expressing a more malignant phenotype. CA9-positive areas may contribute to this phenotype by regulating the pH in tumor tissue, thereby giving the opportunity for tumor cells to proliferate under intermediate hypoxic conditions without apparent acidosis. Eventually, under severe hypoxia, accumulation of acid may have become too severe for tumor cells to survive (48).

**Prognostic Value.** Several studies have reported a relationship between poor survival and high expression of CA9 (11, 15, 22, 23, 25, 26). Our results are consistent with these findings although we could not show a statistically significant correlation. No correlations were found between IdUrd labeling, loco-regional control, and disease-free survival, nor could a significant association be found between Fld[CA9] and loco-regional control. There was however, a significant correlation between Fld[CA9] and disease-free survival. Figure 5 shows that the worst outcome for tumors with a high Fld[CA9] is mainly determined by distant metastases. Interpretation of these data should be done carefully because of the small sample size and relatively short follow-up period.

To our knowledge, this is the first study investigating colocalization between expression of CA9 with IdUrd labeling and the correlation with disease control and survival. A relationship between hypoxia and the development of metastases, and not with local failure, has been shown earlier in cancer of the uterine cervix and soft tissue sarcomas (1, 53). Graeber et al. (6) showed that hypoxia promotes the selection of tumor cell phenotypes with diminished apoptotic potential. This is in agreement with the observation that hypoxic tumors of the uterine cervix with a low apoptotic potential exhibited very aggressive clinical behavior with a high metastatic ability to the lymph nodes (54). Tumor cells with diminished apoptotic potential might be able to actively proliferate. This supports our measurements demonstrating a higher metastatic potential for tumors with a high fraction of IdUrd-labeled cells positive for CA9, which is not reflected in clinical stages. The proliferating cells found in CA9-positive areas under intermediate hypoxic conditions could possibly represent a critical subpopulation contributing to treatment failure. An interesting contribution is a recent observation that overexpression of CA9 reduces the E-cadherin–mediated cell-cell adhesion capacity, which could be of significance in tumor progression (55).
In conclusion, using immunohistochemical triple staining and computerized simultaneous analysis of multiple parameters, we were able to quantify colocalization between IdUrd and CA9 positivity in head and neck squamous cell carcinomas. This may represent a subpopulation of proliferating cells in intermediate hypoxic conditions that might be relevant for clinical outcome. A correlation was found between the relative amount of dual-labeled cells and disease-free survival but not with loco-regional tumor control. This suggests that this subpopulation of tumor cells might play a role in the metastatic potential of these tumors. The exact nature of this relationship remains largely unclear and requires additional study. Further determination of the role of CA9 in the regulation of tumor pH and its contribution to treatment resistance and malignant tumor progression is needed. Currently, we are investigating the dynamics of hypoxic and proliferating cell subpopulations in human tumors and xenographs using multiple markers of hypoxia and proliferation.

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