Carbonic Anhydrase IX Expression, Hypoxia, and Prognosis in Patients with Uterine Cervical Carcinomas

David Hedley,1,4,5 Melania Pintilie,3 Jennifer Woo,1 Andrew Morrison,1 Diana Birle,1,6 Anthony Fyles,2 Michael Milosevic,2 and Richard Hill1,8

1Division of Experimental Therapeutics and Departments of 2Radiation Oncology, 3Biostatistics, and 4Medical Oncology and Hematology, Ontario Cancer Institute/Princess Margaret Hospital, Toronto, Ontario, and 6Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

Purpose: Hypoxia is associated with adverse outcome for a number of solid tumors, including cervical carcinomas. Direct pO2 measurement requires specialized equipment and expertise that is not generally available. Immunohistochemical measurement of intrinsic tissue markers of hypoxia is an alternative approach. Recent studies suggest that carbonic anhydrase IX (CA IX), which is regulated via hypoxia-inducible factor 1, is a useful intrinsic marker of tumor hypoxia.

Experimental Design: Biopsies were obtained from 110 patients with locally advanced cervical carcinoma treated with radiotherapy or chemoradiotherapy. Tissue sections were labeled using an immunofluorescence technique and CA IX expression in the viable tumor area measured using a semiautomated fluorescence image analysis technique. Results were compared with direct pO2 values obtained using an Eppendorf probe and to patient outcome. Intratumoral heterogeneity of CA IX expression was examined in a subgroup of patient who underwent multiple biopsies.

Results: The median percentage of tumor area staining for CA IX was 3.56 (range, 0.01–58.85). CA IX staining did not correlate with the Eppendorf pO2 measurements. Whereas the latter values were predictive of patient outcome, the CA IX levels were not. Measurement of CA IX in multiple biopsies indicated that intratumoral heterogeneity accounted for 41% of the total variance in the data set.

Conclusions: In contrast to some recent studies, we did not find significant associations between CA IX expression and tumor pO2 levels or patient outcome in locally advanced carcinomas of the cervix. Probable explanations relate to the problems of sampling error using single biopsies and the existence of biological factors other than hypoxia that influence CA IX levels.

INTRODUCTION

Carbonic anhydrases are expressed on the outer cell membrane and play a role in pH regulation by catalyzing the reaction between carbon dioxide and water to form carbonic acid (1). CA IX (2, 3) is activated by HIFs (2, 3). Several groups have reported that elevated CA IX levels are predictive of hypoxia in various types of cancer and that the tissue distribution is correlated with other markers of tumor hypoxia such as the distance from blood capillaries or the binding of nitroimidazole probes for hypoxia (4–10).

To date, most studies of hypoxia in cancer patients have been based on direct pO2 measurements using instruments such as the Eppendorf probe, and this is still regarded as the gold standard (11). Overall, this work confirms an adverse effect of hypoxia on prognosis for a number of tumor types (12–15). Interestingly, although hypoxia has long been regarded as a cause of primary radioresistance, several recent studies, including work from our own group, have suggested additional adverse effects of hypoxia on patient outcome. These effects include increased metastatic potential and enhanced tumor cell survival (15–18). Consequently, attention has refocused on the interaction between hypoxia, gene expression, and the regulation of molecular pathways (19).

Although direct measurement of tumor hypoxia using needle electrodes is robust and reproducible, this is an invasive technique that requires special equipment and expertise that is expensive to acquire and maintain. An alternative is to use tissue markers of hypoxia that can be identified on histological sections. This has the additional advantage of allowing assessment of the microregional distribution of oxygen within tissue and its association with other morphological or molecular features. The two approaches currently available involve either the use of nitroimidazole probes that are administered to the patient before biopsy and become activated to reactive intermediates under hypoxic conditions (20, 21) or the use of intrinsic markers of hypoxia. Of the latter, the HIF-1α subunit of the HIF-1 transcription factor is particularly attractive because the mechanisms of protein accumulation under hypoxic conditions are well characterized at the molecular level (19, 22). We have found that immunohistochemical measurement of HIF-1α in...
biopsies obtained from cervix cancer patients is correlated with direct pO₂ measurements made using the Eppendorf probe (23). However, in our hands, this marker is technically difficult to standardize using patient material. CA IX, which is up-regulated by HIF-1, is an attractive alternative because the protein is highly expressed on the cell surface and has a long half life in hypoxic tissue (6, 7). Furthermore, specific, high-affinity monoclonal antibodies suitable for immunohistochemistry have been

Fig. 1 Representative CA IX staining in a patient sample. A shows the H&E staining of a serial section, with the viable tumor areas outlined. B is the grayscale CA IX image and C the corresponding binary image. This is then overlaid on the H&E section in D. Scale bars = 1 mm.
developed. We therefore used a wide field digital fluorescence imaging method to examine the expression of CA IX in a series of biopsies obtained from patients with uterine cervical carcinomas who had undergone Eppendorf probe measurements at the time of biopsy and were treated using a standardized protocols involving radical radiotherapy.

MATERIALS AND METHODS

Patients. Biopsies were obtained from patients with locally advanced cervical carcinoma (International Federation of Gynecologists and Obstetricians stages IB–IIIB) who were enrolled in a translational research program examining the biology of tumor hypoxia. After written informed consent according to a protocol approved by the University Health Network/University of Toronto Research Ethics Board, patients underwent examination under anesthesia as part of their pretreatment evaluation. During this procedure, measurements were made of tumor oxygenation using an Eppendorf probe and IFP using a wick-in-needle technique, as described previously (15, 24). The results from the pO2 measurements were expressed as the HP5 value, which is the percentage of pO2 readings < 5 mm Hg. Punch biopsies were obtained, placed in cryovials containing ornithine carbamyl transferase embedding medium (Sakura Finetek, Torrance, CA), and stored in a liquid nitrogen freezer. Multiple biopsies were available from a subset of 9 patients who consented to a parallel project examining intratumoral heterogeneity, and core biopsies were obtained from an additional subset of 7 patients using a Trucut needle. Before February 1999, patients were treated uniformly with external beam radiotherapy followed by a single intracavitary brachytherapy application (15). After the publication of randomized clinical trials showing benefit from concurrent chemotherapy in this patient population (25, 26), the treatment protocol was modified in February 1999 with the addition of cisplatin given weekly during external beam radiotherapy at a dose of 40 mg/m2.

CA IX Labeling. Serial sections were cut at 5-μm thickness using a cryostat, air-dried, and fixed in 4% formaldehyde at 4°C for 20 min. One section was stained using H&E and scanned at low optical resolution. Using the original slide for fine detail, a pathologist (D. B.) outlined areas of viable tumor tissue on this digital image. Biopsies that did not contain viable tumor tissue (n = 13) were excluded from analysis. The next section was labeled for CA IX using a mouse monoclonal antibody (M75) at a dilution of 1/50 for 1 h. An Alexa-647-conjugated secondary antibody obtained from Molecular Probes (Eugene, OR) was then used at a dilution of 1/300 for 30 min. Slides were counterstained using the nuclear DNA dye 4′,6-diamidino-2-phenylindole. Fluorescence imaging of the entire tumor surface was done using a 20× n.a. objective lens on an Olympus BX50 fluorescence microscope fitted with a cooled CCD camera and computer controlled autostage, as described previously (27). Grayscale images were converted to 16-bit TIFF files and image analysis done using software modules written in Interactive Data Language (IDL 5.6; Research Systems, Inc., Boulder CO). The levels of CA IX staining were expressed both as the percentage of pixels staining above a

![Fig. 2 CA IX staining in the viable tumor areas as a bivariate plot of the percent-labeled area versus mean integrated optical density values.](clincancerres.aacrjournals.org)
threshold value and as the mean integrated optical density (IOD), a parameter describing the total amount of antibody labeling in the section.

To test the validity of the immunofluorescence CA IX staining protocol used for the patient samples, we first examined a series of SiHa human cervical carcinoma xenografts grown in SCID mice that were treated with the nitroimidazole probe EF5 (gift of Dr. Cameron Koch, University of Pennsylvania) 4 h before sacrifice. The excised tumors were processed similar to the protocol used for the clinical samples and stained using a triple immunofluorescence technique for EF5 and HIF-1α, as recently reported from our laboratory (27), combined with the CA IX technique used in this article.

To estimate the effects of intratumoral heterogeneity on
CA IX measurements, multiple biopsies and core biopsies obtained from the deeper tumor tissue were labeled with the same antibody and developed with an immunohistochemical technique. Slides were scanned at 10×10 objective using an autostage and RGB camera. The areas of viable tumor tissue were outlined, and the extent of CA IX-labeled tumor area was obtained by setting a threshold value to identify CA IX-positive pixels, similar to the immunofluorescence measurements. Heterogeneity within the core biopsies was examined by measuring the CA IX labeling at 1-mm intervals along the core length.

Statistical Analysis. The scatter plots of the percent CA IX-positive and other continuous variables (CA IX mean intensity, HP5, IFP) were visually inspected and the Pearson and Spearman correlation coefficients were calculated.

The outcome was DFS, defined as the time between diagnosis and the first failure, relapse, or death. The patients who did not respond completely to treatment were considered failures at time 0. At the time of the analysis, 44 of the 102 patients who were eligible for the outcome analysis had events. The median follow-up time was 2.4 years, range 0.8–6.4 years. The effect of the percent CA IX positive (as a continuous variable) on DFS was tested using Wald statistics within the Cox proportional hazards model. The percent CA IX was tested alone in the model, as well as when the model was adjusted for the known important clinical factors (tumor size and nodal status). To illustrate the effect of CA IX on DFS, the variable was dichotomized at the median, and Kaplan-Meier curves were drawn.

The intratumor heterogeneity was estimated using variance component analysis.

RESULTS
Measurement of CA IX in Tumor Tissue
In preliminary experiments to validate the labeling technique used, we confirmed that the distribution of CA IX staining in human cervical carcinoma xenografts correlated closely, but not exactly, with that of the nitroimidazole probe EF5 and HIF-1α, similar to studies by others (8, 28). Biopsies were available from 123 patients, and viable tumor tissue was identified in 110 of these. As previously described by others, tumor tissue showed discrete areas of bright CA IX staining that was predominantly at the cell surface. These areas of positive CA IX staining were often apparent surrounding regions of necrosis. Although positive staining was seen in almost all cases, there was considerable heterogeneity in the extent of staining between individual patients. Fig. 1 shows the representative immunofluorescence staining pattern from a patient sample, with the corresponding H&E sections outlining tumor tissue. This figure also illustrates the manipulations used to obtain the values used for analysis of the correlates of CA IX in this patient population. As seen in Fig. 2, there was a strong correlation between the percentage of pixels positively labeled with CA IX, and the CA IX values expressed as mean IOD (correlation coefficients: Pearson = 0.93, Spearman = 0.82). Because the former is more intuitive and consistent with most of the published literature, it was used for most of the subsequent statistical analyses. Positive staining CA IX involving >1% of the tumor area was found in 70% of cases, similar to that previously reported for locally advanced disease.

Table 2 Patients’ characteristics (for the subset that was eligible for outcome analysis)

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advanced cervical carcinomas by Loncaster et al. (6). The median percent CA IX positive was 3.4%, range 0.01–62.8%, and the median for HP5 was 52%, range 0–93% (Table 1).

Assessment of Intratumoral Heterogeneity of CA IX Labeling

Multiple Tumor Surface Biopsies. A subset of 9 patients had three separate biopsies taken. Tumor tissue was present in all biopsies in 7 cases and in 2 samples in the remaining 2 patients. The biopsies were stained for CA IX using an immunohistochemical technique and imaged at ×10 using a scanning stage and RGB camera. The percentages of CA IX-positive tumor area for each biopsy are shown in Fig. 3A. Analysis of these data shows that intratumoral heterogeneity accounts for 41% of the total variance in the data set.

Core Biopsy Samples. Frozen sections from core biopsies were stained by immunohistochemistry, and the entire length of the core scanned using a ×10 objective similar to the multiple biopsies. A representative image is shown in Fig. 3B. These images were analyzed by measuring the percentage of viable tumor area positively labeled for CA IX at 1-mm intervals along the length of the core, and the values are shown in Fig. 3C. The intratumor heterogeneity accounts for 43% of the total variance.

Relations between CA IX Staining and Other Variables

CA IX staining, expressed either as percent labeled area or as mean IOD, was not correlated with direct tumor pO2 measurements made using the Eppendorf probe. As seen in Fig. 4A, no association was found between the percent CA IX-positive tumor area and HP5 (correlation coefficients: Pearson = 0.04, Spearman = −0.03). We also examined the CA IX values obtained from the entire tissue section area but again found no significant correlation with the HP5 values (Fig. 4B). Analysis of the relationships between CA IX staining and IFP measurements, which is related to tumor blood flow and prognostically significant in this group of patients (24), and to standard clinicopathological features, including tumor size, presence of lymph node metastases, and histological subtype also failed to identify any statistically significant correlations.

Relations of CA IX to Patient Outcome

Of the 110 patient samples available for correlative analysis, 8 were excluded from analysis of outcome because of: overt metastatic disease identified (4 cases); primary tumor treated by surgical excision (2 cases); small cell histology on pathology review (1 case); and patient not treated at our institution (1 case). The characteristics of the patients eligible for the outcome analysis (n = 102) are in Table 2. DFS at 3 years was 51%, similar to results reported by our group previously (15). Tumor size, nodal status, HP5, and IFP were all significant prognostic features for these patients (Fig. 5).

In this data set, CA IX, expressed as percent-labeled area, failed to show a significant prognostic effect for DFS, whether it was tested alone in the model (P = 0.67) or the model was adjusted for tumor size, nodal status, and treatment (P = 0.5). When CA IX expression was dichotomized at the median (median = 3.66) the P based on log-rank test was still not significant (P = 0.76; Fig. 6). The extent of CA IX-positive area was also divided into categories of <1, 1–10, 10–30, and >30%, as used by Loncaster et al. (6), but this did not identify significant effects on patient outcome.

Assessment of Impact of Intratumoral Heterogeneity on Data Analysis

Fig. 5 DFS curves for patients, dichotomized at the median values for tumor size (A), HP5 (B), and IFP (C).
between CA IX and outcome, assuming that one truly exists. To investigate this possibility, statistical simulations were done based on the observed distribution of CA IX in this study and the heterogeneity of CA IX expression from the patients who had multiple biopsies. The independent variable representing CA IX was assumed to be linearly associated with outcome, and noise was added to simulate the effect of intratumoral heterogeneity and sampling error. The simulation was executed 5000 times for each of the four following scenarios: no heterogeneity; heterogeneity with one sample; heterogeneity with two samples; and heterogeneity with three samples.

In the ideal situation with no heterogeneity, the probability of failing to detect the true underlying association between CA IX and outcome was 13%. This increased to 28% when only one biopsy was obtained from a heterogeneous tumor. The error was 21 and 18% for two and three biopsies, respectively. Detailed description of these statistical simulations is given in the “Appendix.”

DISCUSSION

The goal of this work was to assess the usage of CA IX labeling as an indicator of tumor hypoxia and clinical outcome in a series of well-characterized patients with locally advanced uterine cervical carcinoma. We used a digital fluorescence imaging technique to scan the entire tumor surface, followed by manual editing based on morphological identification of viable tumor areas done by a pathologist who was blinded to the extent of antibody labeling. Compared with conventional scoring of staining intensities by immunohistochemistry, this technique is relatively free of observer bias and partly addresses the problem of intratumoral heterogeneity to the extent that the entire tissue section is analyzed. Using simple image analysis tools, the percentage of pixel area staining above a threshold value was found to be closely correlated with the mean IOD value for CA IX staining.

In contrast to some recently published series, no significant association was found between CA IX labeling and the extent of tumor hypoxia determined at the time of biopsy using the Eppendorf probe. The Eppendorf probe measurements were made under anesthesia, including 40% inspired oxygen, which might potentially influence the pO2 measurements. However, no significant differences in pO2 values were seen in a multicenter study where measurements were made in cervix cancer patients with or without general anesthesia (29), and we do not think this is an important factor for interpretation of the current data. Furthermore, although we have recently shown that the Eppendorf values provide prognostic information in these patients (15), there was no significant correlation between CA IX and patient outcome or other clinicopathological features.

Although most published series examining CA IX levels in human cancers report positive associations with pO2 measurements or patient outcome (9, 30, 31), this is not a universal finding (8). A possible explanation for these discrepancies is the introduction of sampling errors caused by intratumoral heterogeneity. This was partially addressed in subgroups of patients who had undergone either multiple superficial biopsies or core biopsy in addition to superficial biopsy according to protocol modifications. The number of samples available was relatively small, but the data illustrated in Fig. 3 show that based on a single biopsy, some tumors could be assigned to either hypoxic or nonhypoxic categories. Although the analysis of the variance performed on both the core biopsy and multiple biopsy data showed that the intratumoral heterogeneity was smaller than the intertumoral heterogeneity, statistical simulations indicated that the chance to miss a significant association between CA IX and outcome doubles if CA IX is measured imprecisely because of intratumoral heterogeneity (13–28%).

The existence of intratumoral heterogeneity is being increasingly recognized as a problem in the assessment of tumor hypoxia using tissue markers. Although the distribution of hypoxic regions within solid tumors appears to be fairly uniform (32), individual regions are relatively large-scale structures, as...
seen in Fig. 1. Potential approaches to this problem include the use of multiple biopsies, as described by others (10, 29, 33), and used in a limited number of patients in the present study or the use of multiple sections cut at different levels from the same tissue block (34).

An additional factor that could explain lack of significant correlation between CA IX and Eppendorf probe measurements is the action of biological factors additional to hypoxia that influence the levels of CA IX. Its association with hypoxia is attributable to the existence of an hypoxia response element in the promoter region that is sensitive to the HIF-1 transcription factor (3). Although stabilization of the HIF-1α subunit is sensitive to hypoxia, this effect occurs at pO₂ values that are greater than those associated with radiobiologically important hypoxia (28). The expression levels of HIF-1α are also influenced by signaling pathways acting via phosphatidylinositol 3’-kinase/Akt pathway, including mTOR and the forkhead transcription factor FOXO4 (35–37). Currently, it is not clear to what extent these effects might have on the relation between hypoxia and HIF-1α levels in solid tumor tissue.

On the basis of the data presented in the present study and the published literature, CA IX appears to be a useful marker of tumor hypoxia. However, measurements in clinical samples are influenced by the effects of intratumoral heterogeneity, and the tissue expression of CA IX and probably other intrinsic markers of hypoxia, can be influenced by other factors, including alterations in some signal transduction pathways. Given the emerging importance of hypoxia in the biology of human cancers, additional studies are indicated to establish guidelines for adequate tumor sampling and to characterize other biological markers for tumor hypoxia.

APPENDIX

To measure the power lost when the data are subjected to intratumor variation, we used Monte Carlo techniques. In the first step, we generated a variable distributed similarly to the observed CA IX. In the second step, we added noise to it to create the value of CA IX subject to within variability (\(\tilde{\text{CAIX}}\)). Each of these was tested using Cox regression for its relationship with outcome. The outcome variable was generated such that CA IX was linearly associated with outcome. The loss in power because of within variability of CA IX was quantified as the difference between the proportion of significant tests for CA IX and the proportion of significance tests for \(\tilde{\text{CAIX}}\).

Details of the Simulation. Because the observed CA IX was not normally distributed, we first generated the arcsine transformed CA IX values: \(\tilde{\text{trCA IX}}\) was not normally distributed, we first generated the arcsine transformation of CA IX and the proportion of significance tests for CA power because of within variability of CA IX was quantified as the p-value with outcome. The outcome variable was generated such that it correlated with CA IX and \(\tilde{\text{CAIX}}\). In the second step, we added noise to it to create the value of CA IX subject to within variability (\(\tilde{\text{CAIX}}\)). In the second step, we added noise to it to create the value of CA IX subject to within variability (\(\tilde{\text{CAIX}}\)).

\[
\begin{align*}
\text{trCA IX} & = \ln(1 + \text{CA IX}) \\
\tilde{\text{CAIX}} & = \text{CA IX} + N(0, \sigma = 7.66)
\end{align*}
\]

The outcome of interest was the DFS. The observed DFS, \(\text{DFS}_0\), was 0.51, which yields an observed hazard rate \(\lambda = 0.22\). We chose the effect size for the association between DFS and CA IX to be \(\beta = \ln(0.90) = 0.03\).

The baseline hazard \(\lambda_0\) was calculated based on:

\[
\lambda_0 = \lambda_0 e^{0.22}, \quad \text{where } \bar{x} \text{ is the mean of CA IX (} \bar{x} \equiv 11), \text{ and} \quad \lambda_m = 0.22
\]

Hence, the hazard rate for each record is \(\lambda_i = \lambda_0 e^{\mu_i}\), with \(\mu_i = \text{CA IX for record } i\).

We have generated \(T_i \sim \text{EXP} (\lambda_i)\).

The simulation was repeated 5000 times.

Results and Conclusions. We found that although the CA IX was significant 87% of times, the \(\tilde{\text{CAIX}}\) was significant only 72% of times. This means that due to intratumor heterogeneity, the chance to miss an effect doubled (from 13 to 28%). When we considered a reduced SE caused by multiple biopsies/patient, the chance to miss an effect was 21% for two biopsies and 18% for three biopsies.

REFERENCES


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