

Carbonic Anhydrase (CA IX) Expression, a Potential New Intrinsic Marker of Hypoxia: Correlations with Tumor Oxygen Measurements and Prognosis in Locally Advanced Carcinoma of the Cervix¹

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ABSTRACT

There is increasing evidence that hypoxia-regulated gene expression influences tumor aggressiveness, contributing to the poorer outcome of patients with hypoxic tumors. The role of the transcriptional complex hypoxia-inducible factor-1 as an important mediator of hypoxia-regulated gene expression is one of the best documented pathways. Recently, it has emerged that certain tumor-associated carbonic anhydrases (CAs) can be added to the list of known hypoxia-inducible factor-responsive genes. Here we show that the immunohistochemical expression of the tumor-associated CA IX is correlated with the level of hypoxia in human cervical tumors. We performed a prospective study in 68 patients where needle electrodes were used to make direct measurements of tumor oxygenation levels. CA IX expression was evaluated immunohistochemically in pre-treatment tumor biopsies. There was a significant positive correlation between the level of tumor hypoxia (HP5) and the extent of CA IX expression. A retrospective study of 130 squamous cell cervical carcinomas demonstrated that a semiquantitative immunohistochemical analysis of CA IX expression in tumor biopsies is a significant and independent prognostic indicator of overall survival and metastasis-free survival after radiation therapy. These studies provide clinical evidence that CA IX expression is up-regulated in hypoxic human cervical tumors and is associated with a poor prognosis. CA IX may act as an intrinsic marker of tumor hypoxia and poor outcome after radiation therapy. The level of CA IX expression may be used to aid in the selection of patients who would benefit most from hypoxia-modification therapies or bio-reductive drugs.

INTRODUCTION

Hypoxia is a key factor in the pathogenesis of many diseases, including cancer, where its importance in relation to treatment outcome is well established. The intrinsic biological aggressiveness of hypoxic tumors is explained, in part, by the up-regulation of a number of hypoxia-inducible genes mediated by the activation of the transcription factor, HIF-1.³ The HIF-1 protein is stabilized under hypoxia and binds to hypoxia-responsive elements in many genes, including erythropoietin, VEGF, and glucose transporter (GLUT-1) leading to the induction respectively of erythropoiesis, angiogenesis, and glycolysis. Recently, the tumor-associated CAs were reported to be a new class of HIF-1 responsive gene, the role in which the hypoxic tumor microenvironment is yet to be elucidated (1).

CAs catalyze the reversible hydration of carbon dioxide to carbonic acid (2) and, therefore, play a role in pH regulation. To

date, 14 human isoforms (11 active and 3 CA-related proteins; Ref. 3) have been reported, but it is the CA IX protein that is strongly induced by hypoxia in a broad range of cell types (1). CA IX, initially described as a tumor-associated MN, was originally detected in HeLa cells where its expression was found to be cell density dependent (4). It is a transmembrane glycoprotein, the extracellular domain of which contains the essential features and activity of CAs. Evidence supporting the role of the CA IX protein in neoplastic progression includes its association with tumorigenic phenotypes in human cell hybrids and neoplastic transformation of mouse 3T3 cells after transfection with MN cDNA (4). The gene product is expressed in many types of human cancer and is usually absent from their normal tissue counterparts. Its role as a biomarker of malignancy has been demonstrated in renal cell, cervical, lung, and colorectal tumors (5–7). CA IX protein was found to be expressed in 90% of cervical squamous cell carcinomas (6). In colonic lesions, a progressive increase in CA IX expression has been noted from hyperplastic polyps through adenomas to carcinomas (8). Little is known about the role of CA IX in malignancy. Its site on the plasma membrane suggests a role in cell-cell and cell-matrix interactions (9). It has also been implicated in cellular proliferation (4, 8) and more recently hypoxia (1).

In view of the tight regulation of the CA 9 gene by an HIF-1-dependent hypoxia-responsive element, it was suggested that CA IX may be a useful endogenous marker of tumor hypoxia (1). Interest in the latter stems from the need to find an alternative to the current Eppendorf pO₂ histogram method for assessing tumor hypoxia. Although the Eppendorf method provides prognostic information in a variety of tumor types, it is limited to tumors accessible for microneedle insertion. A method based on the immunohistochemical expression of a hypoxia marker would be attractive. In this study, therefore, we have explored the role of CA IX as a marker of hypoxia and prognosis in cancer. Two hypotheses were generated: (a) there is a relationship between tumor hypoxia and CA IX expression in human tumors; and (b) the extent of CA IX expression is prognostic for patient outcome. The hypotheses were tested in carcinoma of the cervix by comparing CA IX expression with oxygen electrode measurements of tumor hypoxia and by examining the relationship between expression and outcome after radiation therapy.

MATERIALS AND METHODS

Patients. All patients had locally advanced squamous cell carcinoma (International Federation of Gynecologists and Obstetricians stage Ib–IVa) of the uterine cervix. Patients were treated with radiation therapy with curative intent according to the standard techniques of the Manchester school (10). Tumor biopsies were taken at the time of their staging examination under anesthesia. Signed informed consent was obtained in all cases. Two separate studies were undertaken: (a) a prospective study, including 68 patients, was initiated to examine the relationship between CA IX expression and oxygen electrode measurements of tumor oxygenation (local ethical approval was obtained for

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³The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; CA, carbonic anhydrase; MN, membrane antigen.

carrying out the oxygen electrode measurements); and (b) a retrospective study of 130 patients was performed to evaluate the prognostic value of CA IX expression in archival tumor biopsy material. Information about treatment outcome was obtained from the patients' hospital records. All patients in the study had been reviewed regularly for treatment in specialist oncology clinics, ≤ 5 years after treatment, using a standard follow-up protocol. Additional follow-up information was obtained from questionnaires to general practitioners. For surviving patients, the median follow-up was 60 months (range 27–115 months). The sites of any disease relapse were identified clinically and radiologically and, where appropriate, were confirmed on biopsy. The recurrences were then classified as being either local (*i.e.*, within the radiation field) or metastatic (*i.e.*, outside the radiation field). Data were also available on the inherent radiosensitivity measured *in vitro* as the surviving fraction at 2Gy (SF2; Ref. 11) in 78 of the tumors in the retrospective series. Information about tumor differentiation was obtained from the hospital pathology department.

CA IX Immunohistochemistry. Formalin-fixed, paraffin-embedded, 4- μ m sections were prepared from the pretreatment biopsy specimens. After dewaxing and rehydration, an endogenous peroxidase block (DAKO Envision) was applied for 5 min. The samples were then washed and incubated with 10% casein (Vector) in Tris-buffered saline (blocking buffer) for 10 min. A mouse monoclonal antihuman antibody (M75) raised to the external domain of CA9 (12) was applied at a 1/50 dilution for 30 min at room temperature. Secondary polymer from the Envision kit (DAKO) was applied for 30 min at room temperature. Visualization of CA IX was by diaminobenzidine substrate. After rinsing in water, slides were lightly counterstained with hematoxylin, dehydrated, and mounted. Substitution of the primary antibody with Tris-buffered saline was used as a negative control.

Analysis of CA IX Staining. The extent and distribution of the CA IX staining was evaluated under low magnification, without the observer's knowledge of outcome data or oxygen measurements. The sections were scored in a semiquantitative fashion by estimating the percentage area of tumor cells that had been stained: 0 for $<1\%$, 1 for 1–10%, 2 for 10–30%, and 3 for $>30\%$ immunostaining. These values were chosen to achieve similar patient numbers in each group. Regions of necrosis were excluded from the analysis. Batch-to-batch variation was taken into account by including a high and low scoring section from each batch in the following batch. All of the retrospective series were scored independently by two observers (J. A. L. and C. M. L. W.). Where more than one biopsy was available for assessment, the average of the scores was used to represent the tumor.

Eppendorf Measurements. In the prospective series of patients, measurements of tumor oxygenation were made using a sterile polarographic needle electrode. The methods are described in detail elsewhere (13). Briefly, measurements were made with patients in the lithotomy position and under general anesthetic maintained using propofol infusion and nitric oxide. Measurements were made at the 12 and 6 o'clock positions, starting at a depth of ~ 4 mm and taken every 0.7 mm. A distance of ≥ 5 mm separated measurement tracks. Data were stored in the Eppendorf computer system and subsequently processed using the pO₂ pool program (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The individual patients' tumor oxygenation results were expressed as the median pO₂ and as the percentage of values <5 -mm Hg (HP5).

Statistical Analysis. Survival was analyzed using the Kaplan-Meier method, and prognostic factors were assessed by Log-rank analysis. Univariate and bivariate analyses were made of disease-specific survival (based on the number of patients who did not die from cervical cancer), metastasis-free survival, and local recurrence-free survival. CA IX score, and other putative prognostic factors (age, stage, tumor differentiation, and SF2), were used to stratify patients. A stepwise multivariate Cox regression analysis was also performed to further test the independence of CA IX expression from other parameters. The distribution of the CA IX score in relation to tumor and patient characteristics was investigated using the χ^2 test. Correlations between variables were obtained using Spearman's rank correlation. All tests were two-sided, and a significance level of 0.05 was used throughout.

RESULTS

Scoring Reproducibility. To assess intraobserver variability, 30 sections were selected at random and scored twice by the same observer (J. A. L.). There was a significant correlation between the

two independent scores ($r = 0.9$, $P < 0.001$). To assess interobserver variability, all sections in the retrospective series were scored independently by two observers (J. A. L. and C. M. L. W.). There was a significant correlation between the initial scores from the two observers ($r = 0.9$, $P < 0.001$), and any differences in the scores were by a single scale point. In the 34 of 130 cases where the initial scores differed, the slides were reanalyzed in conference, and a final consensus score agreed. Batch-to-batch variation was assessed by choosing two sections from each batch, one with low and one with high intensity of expression, and running additional sections from the same biopsy in the subsequent batch. All repeat sections scored identically.

CA IX Expression. Staining was predominantly on the plasma membrane. The intensity of the staining, where present, was usually strong, with minimal intra- and inter-tumor variation. There was clear demarcation between stained and unstained areas. For this reason, the extent, rather than the intensity, of the expression was scored. Very weak cytoplasmic staining was considered negative because of the lack of specificity (14). The distribution of staining was focal and intense around, but not in, areas of necrosis. There was an absence of staining close to blood vessels, consistent with diffusion-limited hypoxia. Stromal staining was rare. Examples of typical CA IX immunostaining are shown in Fig. 1. Definite CA IX expression was seen in 64 of 68 (94%) and 92 of 130 (71%) of tumors in the prospective and retrospective series, respectively.

Correlation with Tumor Oxygenation Measurements. A median of four oxygen electrode tracks (range, 1–7) was made per tumor, resulting in a median of 128 oxygen measurements (range, 32–300). The median pO₂ level was 4-mm Hg (range, 0–45-mm Hg), and the median HP5 (percentage of values <5 -mm Hg) was 54% (range, 0–97%). There was a significant positive correlation ($r = 0.50$, $P < 0.001$) between HP5 and CA IX expression (Fig. 2). There was also a significant correlation between CA IX expression and the fraction of values <10 -mm Hg ($r = 0.45$, $P < 0.001$), <2.5 -mm Hg ($r = 0.43$, $P < 0.001$), and the median pO₂ ($r = -0.44$, $P < 0.001$).

Distribution of Patients According to CA IX Expression. Table 1 summarizes the distribution of tumor CA IX expression in relation to patient characteristics in the retrospective series of 130 patients. Information on tumor differentiation was available for only 114 of the patients. For 78 patients, data were also available for inherent tumor radiosensitivity measured *in vitro* as surviving fraction at 2 Gy (SF2). Using Fisher's exact test, CA IX expression was independent of tumor cell differentiation and radiosensitivity. There were borderline significant trends for a higher level of tumor expression in older women and more advanced disease stage.

Correlation with Outcome. Fig. 3 illustrates the relationship between CA IX expression and treatment outcome. The level of expression was a significant prognostic factor for disease-specific ($P = 0.018$) and metastasis-free ($P = 0.022$) survival but not local control ($P = 0.73$). The significance of CA IX expression as a predictor of outcome increased slightly when stage IV tumors were excluded from the analysis [$P = 0.013$, $P = 0.015$, and $P = 0.80$, respectively (results not illustrated)]. The analyses were repeated stratifying patients by the absence ($<1\%$) or presence ($>1\%$) of immunostaining. The prognostic significance of CA IX expression increased for both disease-specific ($P = 0.0041$) and metastasis-free ($P = 0.0049$) survival (Fig. 4). The sensitivity of CA IX staining to predict poor outcome was 85 and 86% for disease-specific and metastasis-free survival, respectively. Table 2 summarizes the results of univariate Log-rank analyses of outcome after radiation therapy. Tumor stage and CA IX expression were the strongest prognostic factors for disease-specific and metastasis-free survival. Radiosensitivity (SF2) was the strongest prognostic factor for local control.

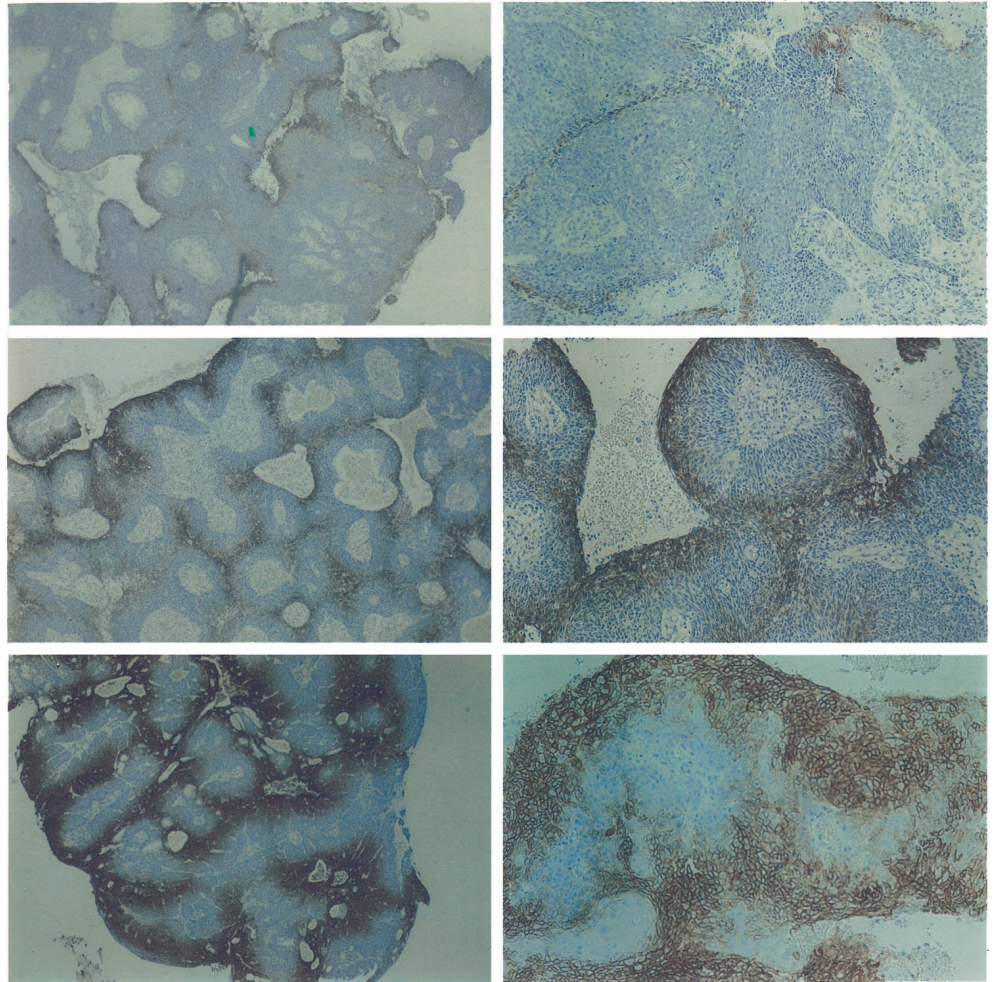


Fig. 1. Immunohistochemical analysis of CA IX expression in tumor biopsies. Sections are stained with anti-CA IX antibody M75. Left-hand panels, magnification $\times 25$. Right-hand panels, magnification $\times 50$. Top panels, CA IX score = 1. Middle panels, CA IX score = 2. Bottom panels, CA IX score = 3. The spatial relationship with tumor vasculature demonstrates a pattern consistent with chronic, diffusion-limited hypoxia.

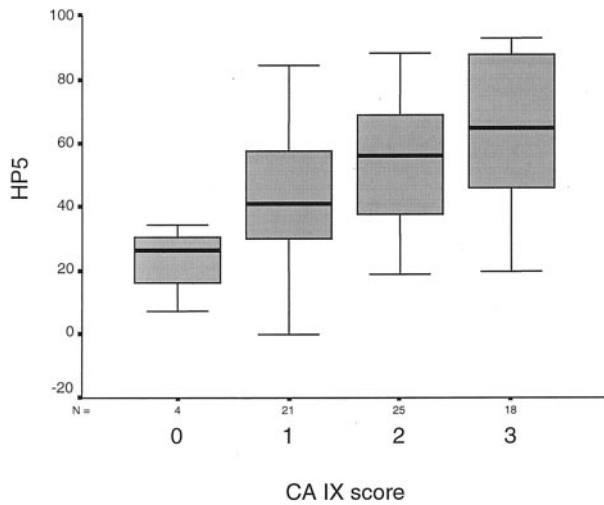


Fig. 2. Relationship between CA IX expression and tumor hypoxia in the 68 patients of the prospective study. Box and whisker plots indicate the median, interquartile range, and minimum and maximum values.

Multivariate Analyses. Bivariate analyses were carried out examining the prognostic significance of CA IX expression after allowing for disease stage, tumor grade, patient age, and SF2 (Table 3). Expression remained a significant predictor of disease-specific and metastasis-free survival after allowing for stage, age, and tumor grade. Finally, a Cox multiple regression analysis was performed. This

included CA IX (absence/presence) and the conventional prognostic indicators: stage, age, and grade. There were 114 patients available for this analysis. For survival, stage ($P < 0.0010$) and CA IX ($P = 0.050$) were statistically significant independent prognostic indicators. Stage ($P = 0.002$), CA IX ($P = 0.021$), and age ($P = 0.047$) were significant independent prognostic factors for metastasis-free survival. Only stage ($P = 0.007$) was a significant independent prognostic factor for local control.

Table 1 Summary of distribution of patients in the retrospective series, according to CA IX expression

Parameter	No.	CA IX				P^a
		0	1	2	3	
Stage						
I	36	15	7	6	8	0.041
II	38	12	14	3	9	
III	47	9	12	14	12	
IV	9	2	1	5	1	
Age						
<52 yrs	66	19	23	11	13	0.062
>52 yrs	64	19	10	17	18	
Differentiation						
Well	24	6	5	4	9	0.26
Moderate	73	17	21	20	15	
Poor	17	7	5	2	3	
Unknown	16	8	2	2	4	
SF2 ^b						
Low	40	10	12	9	9	0.92
High	38	12	11	7	8	

^a Statistical significance using Fisher's exact test.

^b Surviving fraction at 2 Gy.

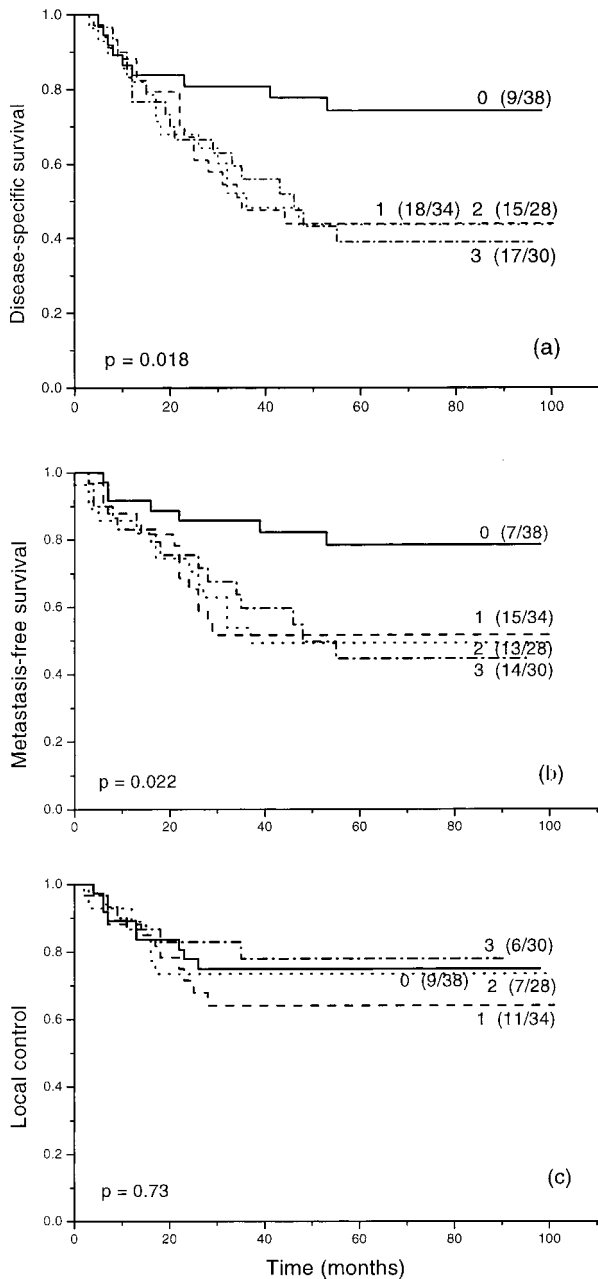


Fig. 3. Disease-specific survival (a), metastasis-free survival (b), and local control (c) in relation to CA IX expression in the 130 cervical cancer patients of the retrospective series treated with radical radiotherapy. Patients were stratified according to the extent of CA IX expression (0 = <1%, 1 = 1–10%, 2 = >10–30%, and 3 = >30% of tumor cells immunostained).

DISCUSSION

CA IX Expression and Tumor Hypoxia. The majority of human cervical tumors studied were immunostained for CA IX. The semi-quantitative scoring system used was rapid, reproducible, and simple to perform, without the requirement of image analysis software. A significant correlation was seen between the level of staining and hypoxia in the tumors. Recently, we reported the glucose transporter GLUT-1 to be a potential intrinsic marker of hypoxia in cervical cancer (15), although the present study has shown that CA IX has a stronger correlation with oxygen electrode measurements and a greater predictive value. Other groups have explored the potential of VEGF expression in this role but found no correlation with tumor

hypoxia measurements in human tumors (16). The lack of relationship was thought to be attributable to the regulation of VEGF expression by nonhypoxic stimuli and diffusion of the protein from its cell of origin. In contrast, the CA9 gene product is expressed at the site of production, and as the protein distribution is linked to tissue hypoxia

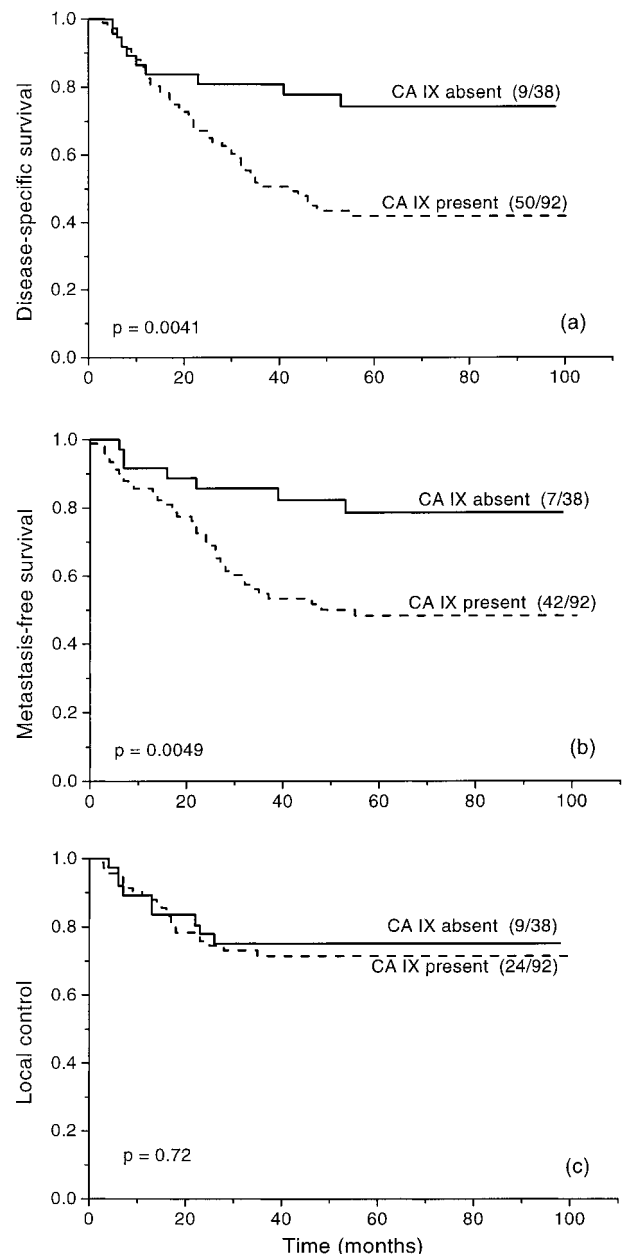


Fig. 4. Disease-specific survival (a), metastasis-free survival (b), and local control (c) in relation to the presence or absence of significant CA IX expression in 130 cervical cancer patients treated with radical radiotherapy.

Table 2 Univariate Log-rank analysis of putative prognostic factors for outcome after radiation therapy for carcinoma of the cervix. The P values for each factor are given.

Parameter	No.	Disease-specific survival ^a	Metastasis-free survival	Local control
Stage	130	<0.0001	<0.0001	0.041
Differentiation	114	0.97	0.89	0.75
Age	130	0.52	0.060	0.19
SF2 ^b	78	0.0054	0.010	0.0038
CA IX expression	130	0.0041	0.0049	0.72

^a On the basis of the number of patients who did not die from cervical cancer.

^b Surviving fraction at 2 Gy.

Table 3 Bivariate stratified Log-rank analyses showing the significance of the presence of CA IX expression as a prognostic factor after allowing for the listed parameters

Parameter	No.	Overall survival	Metastasis-free survival	Local control
Stage	130	0.049	0.015	0.81
Differentiation	114	0.0015	0.0088	0.44
Age	130	0.045	0.038	0.89
SF2 ^a	78	0.032	0.098	0.14

^a Surviving fraction at 2 Gy.

at the micro-regional level, the extent of expression is more representative of the level of hypoxia.

However, although there was a high *P* for the correlation between CA IX expression and hypoxia, the correlation coefficient was only 0.5, and there are a number of possible reasons for this. First, like VEGF, CA IX may also be up-regulated by other nonhypoxic stimuli. Second, p53 may exert a repressive effect on CA IX expression via its inhibition of HIF-1 transcription (17) and degradation of HIF-1 α (18). Although the cervix tumors studied are likely to have diminished levels of wild-type p53 because of degradation by the human papillomavirus E6 protein, hypoxia may induce accumulation of p53 by uncoupling its interaction with E6 (19). Third, oxygen electrode measurements are likely to be dominated by the level of acute (perfusion limited) rather than chronic (diffusion limited) hypoxia. In contrast, CA IX expression probably reflects chronic hypoxia (1). Although the exact level and duration of exposure to hypoxia required to up-regulate CA IX expression is unknown, tissue culture experiments showed the protein to be stable and accumulate after a period of 4-h hypoxia with maximum up-regulation at 16 h (1). Finally, sampling is heterogeneous, and the two methods are not comparing the same part of a tumor. Despite these factors possibly influencing the correlation between the two methods of estimating the extent of tumor hypoxia, there was nevertheless a substantial relationship between the two end points.

Prognostic Role of CA IX Expression. The prognostic value of needle electrode measurements has been demonstrated in many studies but is not predictive of outcome in ~30% of cases (20). This may, in part, reflect the technical limitations of needle electrodes, sampling errors, and other clinical/biological confounding factors. In addition, the relative prognostic importance of acute versus chronic hypoxia has still to be determined. A method of detecting chronic hypoxia alone may not necessarily provide the same prognostic information as an Eppendorf pO₂ histogram. We found CA IX expression to be a significant and independent prognostic factor for disease-specific and metastasis-free survival after radiation therapy in locally advanced cervical carcinoma. Its independence from disease stage implies that information about CA IX expression together with stage could highlight large differences in patient outcome. However, if CA IX expression is reflecting tumor hypoxia, which is known to limit radioresponsiveness, it might also be expected to predict local recurrence. The lack of relationship between CA IX expression and local control in this study may again reflect the absence of information on acute hypoxia that is likely to result in resistance to therapeutic agents. Our results suggest that CA IX expression reflects the enhanced metastatic potential of hypoxic tumors but not the relative radioresistance of hypoxic cells. In support of this, no relationship was seen between CA IX expression and intrinsic tumor cell radio-sensitivity measured as SF2.

The molecular basis for the involvement of CA9 in carcinogenesis remains unclear. There is some evidence for a role in aberrant cell-cell and cell-matrix interactions that facilitate loss of contact inhibition and anchorage independence of cancer cells (21). It has also been suggested that the role of the CA domain in regulating acid-base

balance may optimize conditions in the tumor microenvironment in favor of tumor invasion (22). Alternatively, our findings may simply reflect global HIF-regulated up-regulated gene expression, rather than a direct influence on tumor behavior.

Implications for Patient Management. Our results have a number of implications for cancer patient management. First, the rapid identification of hypoxic tumors before treatment remains a goal for many of those involved in the management of solid tumors. Of the various methods explored to date, most have limited value in the routine clinical setting. Any potential intrinsic marker of hypoxia has the advantage of being assessable on routine clinical biopsies without the need for specialist equipment or administration of exogenous hypoxia markers. Once hypoxic tumors can be quickly and reliably identified, it will be possible to select patients who not only have a poorer prognosis but also are most likely to benefit from hypoxia-modifying therapy or bio-reductive drugs. Second, the findings provide a potential link between tumor hypoxia, pH regulation, and treatment outcome, which could be exploited therapeutically. CA inhibitors have already been shown in tumor models to inhibit the invasion of renal cell carcinoma lines (22) and to have synergistic effects with other chemotherapeutic agents in animal models (23). Third, the MN/CA IX antigen has been shown to be a strong biomarker of malignancy and may be a good target for immunotherapy (24, 25). Finally, the induction of CA IX in hypoxic conditions may be used in the refinement of gene therapy vectors seeking to target therapeutic gene expression to hypoxic regions of tumors.

In conclusion, we have identified a potential intrinsic marker of hypoxia validated by oxygen electrode measurements. Scoring CA IX expression was rapid, reproducible, and simple to perform, yielding significant independent prognostic information on treatment outcome. The level of CA IX expression may have potential to aid selection of patients who might benefit most from hypoxia-modification therapies or bio-reductive drugs.

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