Carbonic Anhydrase IX as a Marker for Poor Prognosis in Soft Tissue Sarcoma

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ABSTRACT

Purpose: **Hypoxia is associated with malignant progression and poor outcome in several human tumors, including soft tissue sarcoma. Recent studies have suggested that carbonic anhydrase (CA) IX is an intrinsic marker of hypoxia, and that CA IX correlates with poor prognosis in several types of carcinoma. The aim of this study was to quantify the extent of CA IX expression and to investigate whether CA IX is a marker for poor prognosis in soft tissue sarcoma patients at high risk of developing metastasis.**

Experimental Design: **Archival paraffin-embedded blocks were retrieved from 47 patients with deep, large, high-grade soft tissue sarcoma. Sections from two separate and representative tumor areas were immunostained for CA IX, and the CA IX-positive area fraction was quantified by image analysis, excluding areas of normal stroma and necrosis that were identified from serial H&E-stained sections. Patients were then subject to survival analysis.**

Results: **CA IX-positive area fractions of viable tumor tissue varied significantly between tumors (range, 0–0.23; median, 0.004), with positive membranous CA IX staining in 66% (31 of 47) of the tumors. Patients with CA IX-positive tumors had a significantly lower disease-specific and overall survival than patients with CA IX-negative tumors ($P = 0.033$ and $P = 0.044$, respectively).**

Conclusions: **These data suggest that CA IX, a potential intrinsic marker of hypoxia, predicts for poor prognosis in patients with deep, large, high-grade soft tissue sarcoma. Larger studies are required to determine whether CA IX has independent prognostic value in this group of tumors.**

INTRODUCTION

Many human tumors develop regions of hypoxia during growth, and the fraction of hypoxic cells can differ substantially among individual tumors of the same histological type (1, 2). Several clinical studies have demonstrated that hypoxia is related to poor response to radiation and chemotherapy (3). Experimentally, it has been shown that hypoxia may promote the development of metastatic disease (4–6), and a relationship between hypoxia and metastasis has been demonstrated clinically in cervical carcinoma (7–9) and soft tissue sarcoma (10, 11). In these clinical studies tumor oxygenation was measured with the Eppendorf pO_2 histograph. This method, although robust and reproducible, is invasive and requires special equipment and expertise. An alternative approach is to use tissue markers of hypoxia that can be identified on histological sections, allowing for assessment of the microregional distribution of oxygen within the 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 or the use of intrinsic markers of hypoxia (12).

Carbonic anhydrase (CA) IX, a membrane protein first detected by Pastorekova and co-workers (13, 14) and recognized as a tumor marker in cervical carcinoma (15, 16) and renal cell carcinoma (17), has recently been suggested to be a potential intrinsic marker of hypoxia. Wykoff *et al.* (18) showed that the *CA9* gene contains a hypoxia-responsive element in its promoter region and is activated by hypoxia-inducible factor 1. Specific, high-affinity monoclonal antibodies suitable for immunohistochemistry have been developed, making it useful as a marker in retrospective studies of paraffin-embedded material. CA IX levels have been associated with hypoxia in cervical carcinoma (19), and the tissue distribution of CA IX has been found to correlate with other markers of tumor hypoxia such as the distance from blood vessels or the binding of nitroimidazole probes (20–25). CA IX expression has been shown to be a prognostic marker for poor outcome in cervical (19), head and neck (22), non-small cell lung (24, 26), nasopharyngeal (27), and invasive breast carcinoma (28), although there are other studies showing no correlation in cervical (29), head and neck (25), and bladder carcinoma (30) or a correlation with good outcome in renal clear cell carcinoma (31).

Among the independent prognostic factors of extremity

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soft tissue sarcoma are tumor size, depth, histological grade, presentation status (primary tumor *versus* local recurrence), and adverse histotype (32–34). Patients with large, high-grade primary soft tissue sarcoma of the extremities have a 10-year disease-specific survival of approximately 55% (33), and metastasis to the lung is the common cause of death. Recently, it was shown that hypoxia predisposes for metastatic disease in patients with high-grade soft tissue sarcoma (10). A similar result showing that hypoxia is associated with poor disease-specific survival in soft tissue sarcoma was found by Nordsmark *et al.* (11), although it was not clear from that study whether the role of hypoxia was independent of tumor grade. Here we hypothesized that CA IX is a potentially useful marker for hypoxia in soft tissue sarcoma and investigated whether CA IX expression can be used to identify patients at high risk of developing metastatic disease in a retrospective study of patients with deep, large, high-grade soft tissue sarcoma. This first report of CA IX expression in soft tissue sarcoma shows CA IX staining patterns consistent with the existence of hypoxia. CA IX expression levels were quantified by image analysis, and a negative correlation was found between CA IX expression and disease-specific and overall survival in these patients.

MATERIALS AND METHODS

Patient and Tumor Characteristics. This retrospective study involves 47 patients with primary soft tissue sarcoma of the extremities presented to Princess Margaret Hospital/Mount Sinai Hospital between 1990 and 2001. All had surgery as their first line of management, and some had also been treated with postoperative radiotherapy to a total dose of 66 Gy (33 × 2-Gy fractions in 6–7 weeks). The patients were selected as being at high risk for developing lung metastasis, having deep, large (>5 cm), and high-grade tumors (35). Median follow-up time among the survivors was 73 months (range, 1–117 months). Chemotherapy was given to some of the patients after relapse, but this decision was not uniform and depended on comorbidity, age, resectability of lung tumors, and patient preferences. All patients had prospectively signed a consent allowing for molecular evaluation of their resected tumors and comparison of molecular testing with clinical outcome. The patient and tumor characteristics are summarized in Table 1.

Immunostaining. Blocks from surgical resections had been fixed in 10% buffered formalin and embedded in paraffin. Representative blocks from two separate tumor areas were selected, and 5-μm-thick tissue sections were cut, and the histopathology was reviewed. Sections for immunostaining were deparaffinized in xylene for 10 min and rehydrated through graded ethanol, peroxidase activity was blocked in 3% H_2O_2 for 10 min, and sections were rinsed in distilled water. They were incubated with M75 antibody reactive with CA IX (Ref. 13; a gift from Dr. Adrian L. Harris and Dr. Nigel J. Beasley) at room temperature for 16 h. The sections were then incubated with antimouse IgG biotin-conjugated linking antibody (Signet multi-link kit; Signet, Dedham, MA) for 30 min followed by streptavidin-horseradish peroxidase (Signet kit; Signet) for 30 min. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DakoCytomation, Glostrup, Denmark). Slides were counterstained with hematoxylin.

Additional serial sections were double stained for CA IX and CD31 to visualize the relationship of CA IX with vessels within the tumor. CA IX immunostaining was performed first, followed by staining for CD31. This entailed microwave antigen retrieval in a pressure cooker. Slides were immunostained with antibody reactive with CD31 at room temperature for 1 h. They were then incubated with antimouse IgG biotin-conjugated linking antibody (Signet multi-link kit; Signet) for 30 min, followed by two rinses in PBS. Slides were incubated with streptavidin-alkaline phosphatase (DakoCytomation), washed in PBS, and incubated with Vector Red substrate (Vector, Burlingame, CA) for up to 30 min; alternatively, they were incubated with horseradish peroxidase for 30 min followed by incubation with Nova Red substrate (Vector) for 5 min. Slides were counterstained with hematoxylin.

CA IX Quantification. Single-stained sections were reviewed by light microscopy, and if no immunopositivity for CA IX was observed, they were assigned a CA IX-positive area fraction of 0 with no further analysis. If immunopositivity for CA IX was present, tiled images of the whole sections were acquired on a Zeiss Axioscope (×10 magnification) using a Sony color charge-coupled device camera, or the section was scanned with a Polaroid SprintScan 4000 slide scanner that gave smaller and more manageable image sizes due to lower resolution. Images were analyzed using Adobe Photoshop version 5.5 software. Normal stroma and necrosis were identified from serial H&E-stained sections, and image masks of the total tumor area and the necrotic tumor area were created using editing tools. A mask of the viable tumor area resulted from overlaying these individual masks. CA IX-positive areas were segmented using intensity thresholding, followed by editing to remove artifacts and noise if necessary, and overlaid with the mask of viable tumor area. The area (number of pixels) of each mask was found using the histogram function, and the CA IX-positive area fraction of total or viable tumor tissue was found by dividing the

Table 1 Patient and tumor characteristics

Age at surgery (yrs)	Median	70
	Range	20–90
Gender	Male	28
	Female	19
Tumor site	Upper extremities	10
	Lower extremities	37
Grade	3	47
Depth	Deep	47
Tumor size	>5 cm	47
Tumor volume (cm^3)	Median	293
	Range	14–8681
Treatment	Surgery	13
	Surgery + irradiation	34
Follow-up time survivors (mo)	Median	73
	Range	1–117
Histopathology	Malignant fibrous histiocytoma	17
	Liposarcoma	8
	Neurosarcoma	8
	Leiomyosarcoma	6
	Synovial sarcoma	2
	Others	6
Resection margin	Negative	37
	Positive	10

CA IX-positive area by the total area or the viable tumor area, respectively. All CA IX quantification was done in a blinded manner before survival analysis.

Statistical Analysis. The Spearman rank correlation coefficient was used to calculate the correlation between two parameters. Overall survival was defined as time between surgery and death by any cause; disease-specific survival was defined as the time between surgery and death caused by soft tissue sarcoma, whereas freedom from distant relapse was defined as the time between surgery and detection of lung metastases. Kaplan-Meier survival curves were calculated, and they were compared using the log-rank test (two groups) or the log-rank test for trend (three groups; GraphPad Prism statistical software). P values of <0.05 were considered statistically significant. All P values were determined from two-sided tests. Calculations to assess the probability of misclassifying a tumor are given in the Appendix.

RESULTS

Table 1 shows the patient and tumor characteristics for this study. There was a predominance of male patients, and most of the tumors were located in the lower extremities. Thirteen patients underwent surgery alone, and 34 patients received postoperative irradiation. Malignant fibrous histiocytoma was the dominant histological subtype. Thirty-seven patients had a negative resection margin, and 10 had a positive resection margin.

Thirty-one cases showed positive CA IX staining, and the staining was distinct and found in the cell membrane, similar to what has been reported by others (18). The specificity of the staining was verified by using a human renal cell carcinoma sample as a positive control and by observing no staining in serial sections from nine of the patients when the primary antibody had been omitted from the staining protocol. CA IX-positive cells were usually found distant from blood vessels (Fig. 1*A*) and, in cases with necrosis, adjacent to the necrotic regions (Fig. 1, *C* and *D*), *i.e.*, in regions expected to be hypoxic. CA IX-positive cells were also found close to blood vessels (Fig. 1*B*), indicating temporarily or permanently impaired oxygen supply from these vessels.

No correlation was found between the CA IX-positive area fraction of total tumor tissue and the extent of necrosis in the CA IX-positive sections that were subject to image analysis (Fig. 2).

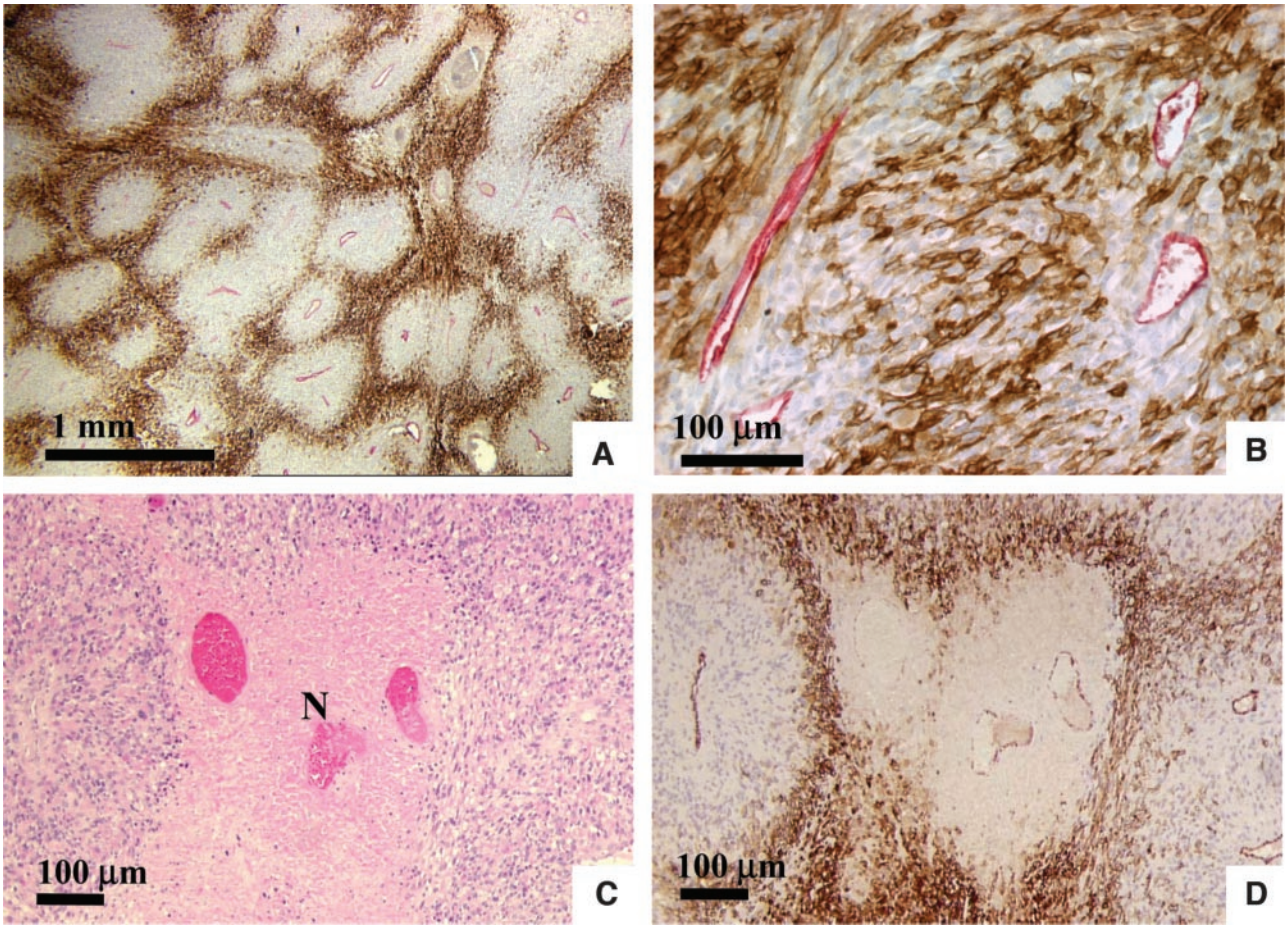

Fig. 1 Carbonic anhydrase (CA) IX (*brown stain*) and blood vessels (*pink* or *red stain*) in human soft tissue sarcoma. *A*, chord-like structures with CA IX-positive staining distant from blood vessels. *B*, CA IX-positive membrane staining in cells close to blood vessels. *C*, H&E staining showing necrotic region (*N*). *D*, perinecrotic CA IX staining in the same region as shown in *C*.

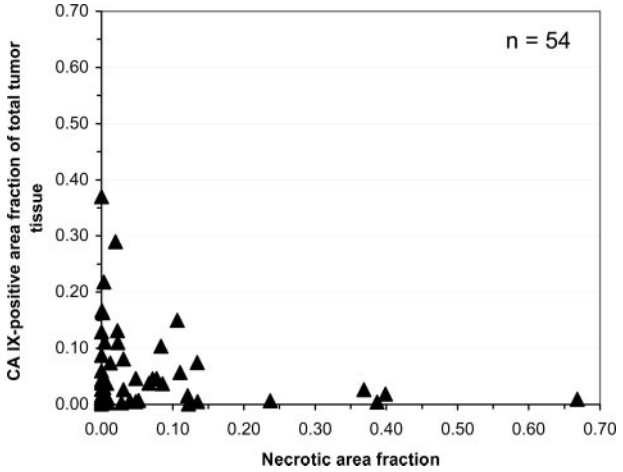

Fig. 2 Carbonic anhydrase (CA) IX-positive area fraction of total tumor tissue *versus* fraction of necrosis. Each *symbol* represents one CA IX-positive tumor section.

A wide range of CA IX-positive area fractions of viable tumor tissue were found among the tumors (Fig. 3), with 66% (31 of 47) of the tumors showing detectable CA IX levels. The highest average CA IX-positive area fraction of viable tumor tissue for a tumor was 0.23, and the median CA IX-positive area fraction of viable tumor tissue for all tumors was 0.004. CA IX-positive area fractions of viable tumor tissue were relatively consistent between two separate areas from the same tumor, although the intratumor heterogeneity increased with increasing CA IX levels. Nineteen percent (9 of 47) of the tumors showed positive CA IX staining in one section but not in the other. CA IX-positive area fractions estimated semiquantitatively from the same sections in a subset of 20 tumors correlated with but showed generally higher values than those obtained by image analysis (data not shown).

Patients were divided into three groups based on their CA IX-positive area fractions of viable tumor tissue (one CA IX-negative group and two CA IX-positive groups of low and high expression, divided at a discontinuity point near the median value of 0.025 for the CA IX-positive sections; Fig. 3), and Kaplan-Meier survival analysis was performed. There was significantly lower disease-specific survival ($P = 0.024$) and overall survival ($P = 0.047$) with higher CA IX expression levels, and the survival curves for tumors with low and high CA IX expression levels were similar (Fig. 4, *A* and *B*). When the two CA IX-positive groups were pooled and patients were stratified according to absence or presence of CA IX expression, there was a significantly lower disease-specific survival ($P = 0.033$) and overall survival ($P = 0.044$) in the CA IX-positive group (Fig. 5, *A* and *B*). There was a trend for lower freedom from distant relapse with higher CA IX levels (Figs. 4*C* and 5*C*), but this was not statistically significant ($P = 0.12$ and $P = 0.20$, respectively).

DISCUSSION

There is some evidence that hypoxia predicts for metastatic disease in patients with soft tissue sarcoma. Brizel *et al.* (10) showed, in a group of 22 patients with high-grade soft tissue sarcoma, that patients with the more hypoxic tumors (*i.e.*, with a median pO_2 less than the sample median of 10 mm Hg) had a higher risk of developing metastases to the lung than those with well-oxygenated tumors, suggesting that hypoxia has additional prognostic value to the standard assessment of advanced disease. However, the number of patients was small, and the median follow-up time was only 9 months (range, 6–28 months) in that study. Somewhat similar results were obtained by Nordsmark *et al.* (11), showing that those with hypoxic tumors (*i.e.*, tumors with a median pO_2 less than the sample median of 19 mm Hg) had poorer disease-specific survival and overall survival than those with well-oxygenated tumors ($n = 28$). The higher sample median pO_2 in that study probably reflects the fact that low-grade tumors were included, and most of these were well oxygenated. Hence, it is not clear from that study whether hypoxia has predictive value independently of tumor grade.

The aim of the study undertaken here was to further explore the relationship between hypoxia and metastasis in a larger number of soft tissue sarcoma patients with a longer follow-up time. We performed a retrospective study in which we investigated the potential prognostic value of CA IX in archival paraffin-embedded tissue obtained at surgery in patients with deep,

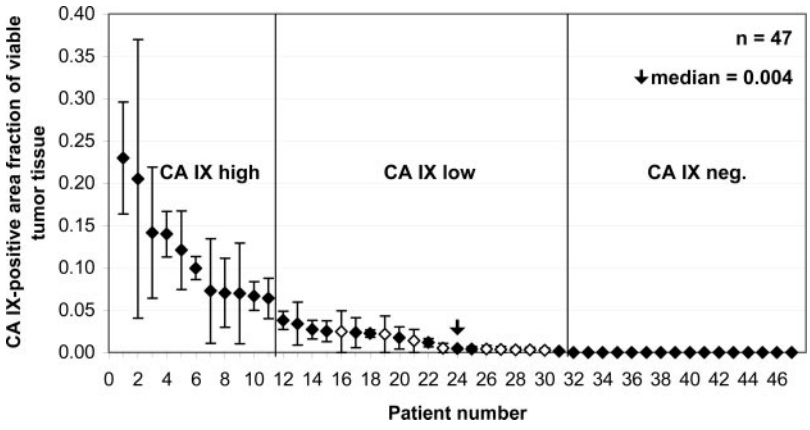

Fig. 3 Distribution of carbonic anhydrase (CA) IX-positive area fractions of viable tumor tissue. Each *symbol* represents the mean value of two sections from one tumor, and the *error bars* represent the range. *Closed symbols* indicate tumors in which both sections were either CA IX positive or CA IX negative, whereas *open symbols* indicate tumors with one CA IX-positive section and one CA IX-negative section. For the survival analysis, tumors were divided into three groups based on their CA IX levels as indicated by the *vertical lines*. The median CA IX-positive area fraction of viable tumor tissue is indicated by an *arrow*.

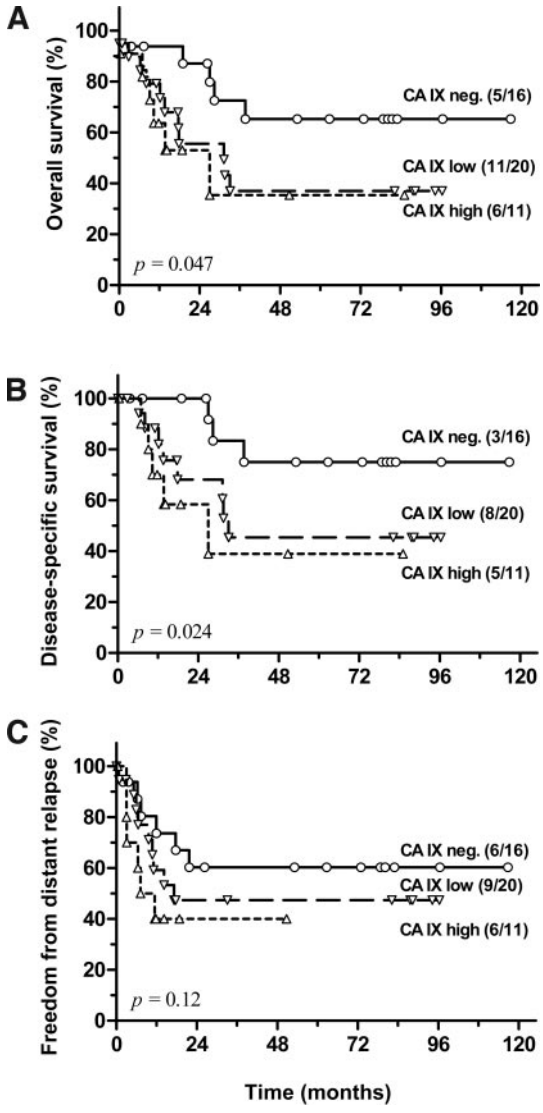

Fig. 4 Overall survival (*A*), disease-specific survival (*B*), and freedom from distant relapse (*C*) in patients with high or low positive carbonic anhydrase (CA) IX staining or negative CA IX staining as indicated in Fig. 3. The number of events/number of patients in each group is indicated in *parentheses*.

large, high-grade soft tissue sarcoma. No correlation was found between CA IX levels and tumor size (Spearman's correlation = 0.17; $P = 0.26$). Patients were selected who had not received preoperative irradiation because this might affect the levels of hypoxia and hence the CA IX expression levels being measured in the tumors.

The major finding of this study was that there is a negative correlation between CA IX levels and disease-specific and overall survival in patients with deep, large, high-grade soft tissue sarcoma, showing that CA IX has potential additional prognostic value in this group of patients. The largest difference in survival was found when patients were stratified according to the presence or absence of CA IX, which was also the case in a recent study of cervical carcinoma (19). All cases of disease-specific deaths in our study had distant relapse to the lung. However, there was only a trend between CA IX levels and freedom from distant relapse. This apparent discrepancy, similar to what has recently been observed in bladder cancer (36), may be related to inadequate power to detect significant differences in distant relapses due to the low number of cases, especially in the CA IX-negative group. It could also reflect that the effect of CA IX on prognosis is smaller than that for pO_2 because they represent different biological measures (10). Alternatively, CA IX possibly predicts for patients at higher risk of early death from soft tissue sarcoma because of a higher metastatic burden. The latter could be explained by higher tumor cell proliferation rates, which have previously been found to correlate weakly with lowered oxygenation in soft tissue sarcoma (37). Also, a correlation between CA IX-positive cells and the Ki67 proliferation index was found in colorectal cancer (38). *Ad hoc* analysis, after the planned survival analysis, showed that 7 of 14 patients with relapse received subsequent chemotherapy and

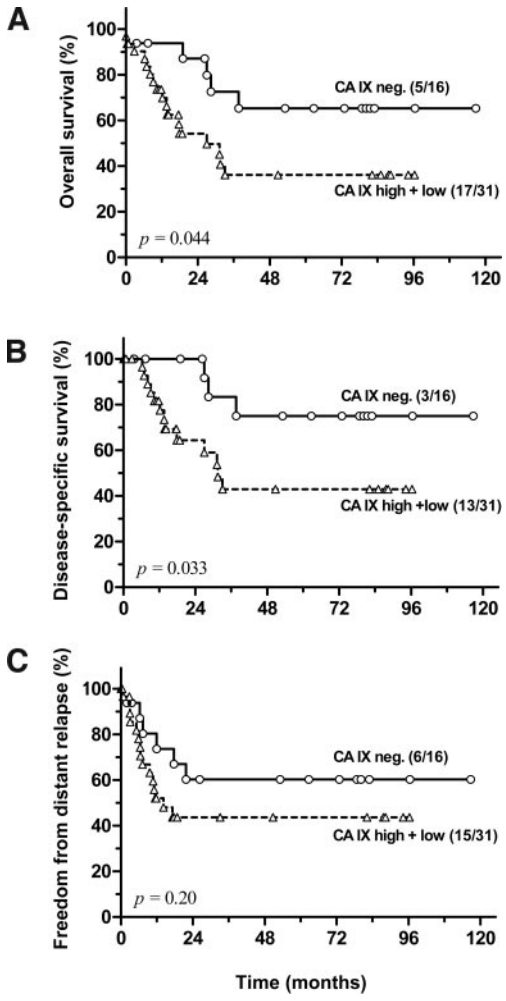

Fig. 5 Overall survival (*A*), disease-specific survival (*B*), and freedom from distant relapse (*C*) in patients with positive (*high + low*) *versus* negative carbonic anhydrase IX staining as indicated in Fig. 3. The number of events/number of patients in each group is indicated in *parentheses*.

that only 1 of these cases was CA IX negative, ruling out the possibility that the prolonged survival after onset of metastases in the CA IX-negative group was due to the effect of chemotherapy.

Several studies have found a correlation between CA IX expression levels and hypoxia and/or outcome in a variety of tumor types (19, 22, 24, 26–28). Most of these studies have looked at only one section per tumor and scored CA IX levels semiquantitatively. We attempted to account for intratumor heterogeneity by looking at sections from two different tumor regions and to perform a more stringent quantification of the CA IX levels using image analysis. Interestingly, we found a good correlation between the CA IX levels in two tumor areas (Fig. 3). Nineteen percent (9 of 47) of the patients had one CA IX-negative section and one CA IX-positive section, and the probability of misclassification using two sections per tumor was estimated to be in the order of 3–9% when stratifying the patients according to CA IX being present or absent (see Table A4 in "Appendix" for details). Furthermore, assuming that the observed effect size is large enough to yield a power of 0.80, simulations showed that probabilities of misclassification of 3% and 9% would reduce the power to 0.76 and 0.62, respectively. Previously, it was shown in solid canine tumors that the greatest source of variation in areas labeled with the nitroimidazole hypoxia marker CCI-103F was at the microscopic level and that four sampling areas of $\leq$25 mm^2 were adequate for estimating the overall labeled area fraction of a tumor (39). The larger areas of our sections (usually in the order of 100–200 mm^2) and the good correlation observed between two sections suggest that sections from two to three separate tumor areas are probably sufficient to correctly classify patients as positive or negative for CA IX staining. Also, in a subgroup of 20 patients, we found a correlation but generally higher CA IX levels when they were scored semiquantitatively as compared with image analysis (data not shown). Overall, these data suggest that a semiquantitative scoring of the absence or presence of CA IX in two or three tumor sections can have additional prognostic value in patients with deep, large, high-grade soft tissue sarcoma.

The evidence for CA IX being a marker of hypoxia has been reviewed elsewhere (12) but is indirect in this study, showing CA IX staining distant from blood vessels and in perinecrotic areas. Also, serial sections from three of the tumors with strong and distinct CA IX staining and from two of the tumors with negative CA IX staining were stained for glucose transporter 1, another hypoxia-inducible factor 1-regulated protein. They showed similar staining patterns with CA IX, although background levels were higher for glucose transporter 1 (data not shown). CA IX has been reported to be a stable protein that accumulates over a long period of hypoxia in tissue culture, suggesting that it is a marker of chronic hypoxia (40). We have observed up-regulation of CA IX at the protein level in HT1080 human fibrosarcoma cells after hypoxic exposures *in vitro* to 0.2% O_2 for 6 h or longer, and perinecrotic CA IX staining was found in HT1080 tumors when grown i.m. in severe combined immunodeficient mice (data not shown), further supporting a role of long-term hypoxia in the regulation of CA IX in sarcoma. Additional studies are required to evaluate the role of hypoxia in regulating CA IX in human soft tissue sarcoma.

There is conflicting evidence from clinical trials concerning whether adjuvant chemotherapy is effective in soft tissue sarcoma (41, 42). However, if a marker such as CA IX was recognized as identifying patients with high-grade tumors who were at particularly high risk for development of early metastatic disease, the use of chemotherapy in higher risk patients might be appropriate. Further evaluation of CA IX and other measures of hypoxia as markers for early death in soft tissue sarcoma is certainly justified by the results of this study.

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APPENDIX

The Probability of CA IX Misclassification.

The aim is to calculate the probability of misclassifying tumors based on CA IX levels measured in two sections from a sample of 47 patients when CA IX levels are scored as positive or negative with a cutoff value of 0. The observed data are given in Table A1.

The true probability of CA IX-positive cases in the population is denoted p, and the probability of CA IX-negative cases in the population is $1 - p$. It is assumed that the probability of misclassifying a section from a CA IX-negative tumor as positive, x, is less than the probability of misclassifying a section from a CA IX-positive tumor as negative, kx, *i.e.*, $k \geq 1$. This reflects the fact that it is unlikely to observe CA IX positivity in a CA IX-negative tumor, whereas it is possible to score a CA IX-positive tumor as CA IX negative due to sampling error. It follows that two CA IX-negative sections (neg,neg) can be observed either with probability k^2x^2 if the tumor is CA IX positive or with probability $(1 - x)^2$ if the tumor is CA IX negative, and the overall probability of observing two CA IX-negative sections is as follows.

$$P(\text{neg, neg}) = pk^2x^2 + (1 - p)(1 - x)^2 \qquad \text{(Eq. A)}$$

Similarly, it can be found that the probability of observing one CA IX-positive section and one CA IX-negative section is, as follows,

$$P(\text{pos, neg}) = 2pkx(1 - kx) + 2(1 - p)x(1 - x) \qquad \text{(Eq. B)}$$

and the probability of observing two CA IX-positive sections is as follows.

$$P(\text{pos, pos}) = p(1 - kx)^2 + (1 - p)x^2 \qquad \text{(Eq. C)}$$

The observed values for P(neg,neg), P(pos,neg), and P(pos,pos) can be inserted from Table A1, resulting in a system of three equations with three unknowns. These equations are dependent and can be solved as a function of k for the two other unknowns as shown in Table A2.

It is unlikely that the probability of misclassification in the CA IX-positive group is larger than 10-fold relative to the CA IX-negative group (*i.e.*, $k = 10$), and in fact, the values tend to reach a plateau as k increases.

Table A1 Observed data

Combinations of CA[a] IX scores from two sections	Proportion of patients, P
Neg, neg	16/47
Pos, neg	9/47
Pos, pos	22/47

[a] CA, carbonic anhydrase.

In general, the overall probability of misclassifying a tumor is given as

$$P(\text{miscl}) = P(\text{ClNeg/POS}) + P(\text{ClPos/NEG}) \qquad \text{(Eq. D)}$$

where P(ClNeg/POS) is the probability of classifying a CA IX-positive tumor as negative (*i.e.*, the tumor is observed as negative but is truly positive), and P(ClPos/NEG) is the probability of classifying a CA IX-negative tumor as positive (*i.e.*, the tumor is observed as positive but is truly negative). Hence, the overall probability of misclassification when observing one section can be calculated as follows.

$$P(\text{miscl})_1 = pkx + (1 - p)x \qquad \text{(Eq. E)}$$

Table A3 shows that the overall probability of misclassification when observing one section is close to 11%.

When observations are based on two sections the observed values can be (neg,neg), (pos,neg), or (pos,pos). There are two ways of classifying observations for a tumor as being positive or negative: (*a*) Rule 2-A, if (neg,neg), then the tumor is ClNeg; otherwise, it is ClPos; and (*b*) Rule 2-B, if (neg,neg) or (pos,neg), then the tumor is ClNeg; otherwise, it is ClPos.

The corresponding probabilities of misclassification are as follows.

$$P(\text{miscl})_{2\text{-}A} = pk^2x^2 + (1 - p)[2x(1 - x) + x^2] \qquad \text{(Eq. F)}$$

$$P(\text{miscl})_{2\text{-}B} = p[k^2x^2 + 2kx(1 - kx)] + (1 - p)x^2 \qquad \text{(Eq. G)}$$

Table A4 shows that Rule 2-A gives the lowest probability of misclassification. As seen in Fig. 3, it was this rule that was applied in the current study.

Table A2 Calculated probabilities

k	P	x	kx
1	0.581	0.107	0.107
2	0.622	0.067	0.134
3	0.639	0.048	0.145
4	0.648	0.038	0.151
5	0.654	0.031	0.154
6	0.658	0.026	0.157
7	0.661	0.023	0.159
8	0.663	0.020	0.16
9	0.665	0.018	0.161
10	0.666	0.016	0.162

Table A3 Overall probability of misclassification when observing one section

k	$P(\text{miscl})_1$[a]
1	0.107
2	0.109
3	0.110
4	0.111
5	0.112
6	0.113
7	0.113
8	0.113
9	0.113
10	0.113

[a] $P(\text{miscl})_1$, overall probability of misclassification when observing one section.

Table A4 Overall probability of misclassification when observing two sections

k	$P(\text{miscl})_{2\text{-A}}$[a]	$P(\text{miscl})_{2\text{-B}}$
1	0.092	0.122
2	0.060	0.157
3	0.047	0.173
4	0.041	0.181
5	0.037	0.187
6	0.034	0.191
7	0.032	0.193
8	0.030	0.195
9	0.030	0.197
10	0.030	0.198

[a] $P(\text{miscl})_{2\text{-A}}$, overall probability of misclassification using Rule 2-A; $P(\text{miscl})_{2\text{-B}}$, overall probability of misclassification using Rule 2-B.

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