The Korean Journal of Pathology 2010; 44: 384-9
DOI: 10.4132/KoreanJPathol.2010.44.4.384

Expression of Carbonic Anhydrase IX Correlates with Histologic Grade and Metastasis in Osteosarcoma

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Received : December 29, 2009
Accepted : March 16, 2010

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*This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 20090091346).

Background : Carbonic anhydrase IX (CA9) is reportedly overexpressed in several types of carcinomas, but little is known about the expression pattern of CA9 in osteosarcoma. We aimed to assess the prevalence of CA9 expression and its prognostic implications in osteosarcoma patients. **Methods :** We compared immunohistochemical expression of CA9 between conventional, high-grade and low-grade, central osteosarcomas. Specimens were obtained before chemotherapy and stained with anti-human CA9 antibody. We also evaluated the histologic grade, presence of metastasis, and patient prognosis. **Results :** Among 38 samples of conventional high-grade osteosarcoma, 22 (57.9%) tumors displayed CA9 overexpression. Twenty-five cases of low-grade central osteosarcomas were all negative ($p < 0.0001$). CA9 expression was significantly associated with the presence of metastasis ($p = 0.0010$). The overall survival rate was significantly reduced with increased CA9 expression ($p = 0.0012$), higher histologic grade ($p < 0.0001$), and younger age ($p = 0.0140$). However, the overall survival rate was not significantly correlated with gender, tumor size, or American Joint Committee on Cancer stage. **Conclusions :** CA9 expression is a frequent and tumor-specific event in osteosarcoma. CA9 expression is associated with higher grade tumors, metastasis and poor prognosis for the osteosarcoma patients.

Key Words : Osteosarcoma; Neoplasm metastasis; CA9 protein, human

Hypoxia is a common feature of human solid tumors. Tumor hypoxia causes tumor cells to undergo adaptive changes that enable them to survive and proliferate. Hypoxia is associated with malignant progression and poor outcome in several human tumors.[1] In addition, several clinical studies have demonstrated that hypoxia is associated with poor response to radiation and chemotherapy.[2] Development of a marker for hypoxic tumors could allow assessment of the biologic aggressiveness of individual tumors, which could, in turn, facilitate individually tailored treatments.

Carbonic anhydrase IX (CA9) is a transmembrane glycoprotein that is involved in acid-base homeostasis by reversibly converting carbon dioxide and water to carbonic acid. CA9 has been shown to play an important role in pH regulation and its expression in response to hypoxia has been suggested to reduce pericellular pH thus facilitating breakdown of the extracellular matrix.[3] In addition, CA9 may play a role in cell proliferation and cellular transformation as well as in adaptation of tumor cells to hypoxic conditions.[4,5]

Recent studies have suggested that CA9 is an intrinsic marker of hypoxia, and that CA9 correlates with poor prognosis in several types of carcinoma.[3,6-8] Numerous CA9 inhibitors have been developed as antitumoral agents in the past few years, and studies suggest that CA9 may be a good therapeutic target.[9] For soft tissue sarcoma, patients with CA9-positive tumors had a significantly lower disease-specific and overall survival than

CA9-negative patients.[10] However, little is known about the prevalence of CA9 expression and its clinical correlates in patients with osteosarcomas. In this study, we analyzed the prevalence of CA9 expression and its prognostic implications in patients with osteosarcomas.

MATERIALS AND METHODS

Patients and samples

A total of 63 patients with osteosarcoma were enrolled in the present study, including 38 and 25 cases of conventional high-grade and low-grade central osteosarcomas respectively. Medical records were reviewed and information about age, gender, tumor location, presence of metastasis, and follow-up data were retrieved. Clinical data for tumor size and American Joint Committee on Cancer (AJCC) stage were only available for 33 patients with high-grade conventional osteosarcomas and information for neo- and adjuvant chemotherapy was available for only 25 patients. All specimens were primary osteosarcoma tissue obtained prior to chemotherapy. One of the conventional high-grade osteosarcomas included a metastatic pulmonary lesion. Two pathologists reviewed all sections from each case to confirm the diagnosis and determine the histologic subtype and grade. Tumors were subclassified as osteoblastic, chondroblastic, or fibroblastic according to the predominant histological element, and were graded using a two-tiered system of low-grade (1 and 2) and high-grade (3 and 4).[11] This study was approved by the Institutional Review Board at Kyung Hee University Hospital.

Immunohistochemistry

The avidin-biotin complex method was done on 4 μm-thick tissue sections for immunohistochemical analysis. Sections were deparaffinized with xylene for 15 minutes and pretreated in a microwave oven using 0.01 M citrate buffer (pH 6.0) for 30 minutes. Sections were incubated with rabbit polyclonal antibody directed against CA9 (1 : 1,000, NB100-417, Novus Biologicals, Inc., Littleton, CO, USA) for 30 minutes at room temperature. Both positive and negative controls were used in each experiment. Sections from renal cell carcinoma specimens were used as a positive control for CA9. The utility of all antibodies after decalcification was confirmed using a decalcified renal cell carcinoma specimen.

Evaluation

Consensus judgment was adopted to determine immunohistochemical positivity of the tumors based on the distribution of positive cells: negative, focal (positive cells $\leq$ 30%), and diffuse (positive cells > 30%). The intensities of CA9 expression were similar in the positive samples. Two independent pathologists judged immunoreactivity.

Statistical analysis

All statistical analyses were done with the DBSTAT program (ver. 4.1, DBSTAT Co., Seoul, Korea). Relationships between clinicopathologic variables and CA9 expression were determined using the chi-square test. The clinicopathologic variables studied were histologic grade, age at diagnosis, gender, tumor location, histologic subtype, tumor size, AJCC stage and presence of metastatic disease at any time during the progression of the disease. Overall survival was defined as the time from diagnosis until the date of the final follow-up or death. Overall survival rates were calculated using the Kaplan-Meier method, and differences in survival were compared using the log-rank test. When the p-value was less than 0.05, the statistical difference was regarded as significant.

RESULTS

Patient characteristics

The clinical and pathological features of 63 patients are shown in Table 1. In total, we analyzed 38 cases of conventional high-grade and 25 low-grade central osteosarcomas. For 63 patients with osteosarcomas, the age distribution ranged from 7 to 66 years. Of these cases, 35 were male and 28 were female. Among the histologic subtypes of conventional high-grade osteosarcomas, 21 were osteoblastic, ten were chondroblastic, and five were fibroblastic types. Each case of telangiectatic and giant cell rich type was included. The anatomical sites of the primary tumor were femur (n = 27), pelvic bone (n = 12), tibia (n = 10), facial bone (n = 6), humerus (n = 4), fibula (n = 2), skull (n = 1), and clavicle (n = 1).

CA9 expression

Immunohistochemistry showed that CA9 was present both

Table 1. Correlations between carbonic anhydrase IX (CA9) expression and clinicopathologic variables in 63 osteosarcomas

Variables	CA9			p-value
	Negative	Focal	Diffuse	
Histologic grade				< 0.0001*
Low (n = 25)	25	0	0	
High (n = 38)	16	12	10	
Age (yr)				0.4257
≤ 20 (n = 26)	14	6	6	
> 20 (n = 37)	27	6	4	
Gender				0.4101
Male (n = 35)	23	5	7	
Female (n = 28)	18	7	3	
Tumor location				0.6096
Long bone (n = 43)	28	7	8	
Flat bone (n = 20)	13	5	2	
Histological subtype				0.0032*
Osteoblastic (n = 21)	9	7	5	
Chondroblastic (n = 10)	3	4	3	
Fibroblastic (n = 5)	3	1	1	
Low-grade central (n = 25)	25	0	0	
Others (n = 2)	1	0	1	
Tumor size (cm)				0.8128
≤ 8 (n = 17)	7	6	4	
> 8 (n = 16)	5	6	5	
AJCC stage				0.4192
I (n = 16)	6	7	3	
II (n = 9)	4	3	2	
III (n = 1)	0	1	0	
IV (n = 7)	2	1	4	
Metastasis				0.0010*
Absent (n = 54)	39	10	5	
Present (n = 9)	2	2	5	

Clinical data including size and stage are only for high-grade conventional osteosarcomas (five cases are data missing).
*Statistically significant difference (chi-square test, $p < 0.05$).
AJCC, American Joint Committee on Cancer.

Table 2. Analysis of clinicopathological variables for overall survival in 63 osteosarcoma patients

Variables	Overall survival p-value
CA9 expression No/Focal/Diffuse	0.0012*
Histologic grade Low/High	< 0.0001*
Age (yr) ≤ 20/> 20	0.0140*
Gender Male/Female	0.3260
Tumor size (cm) ≤ 8/> 8	0.2315
AJCC stage I/II/III/IV	0.2996

*Statistically significant difference (Kaplan-Meier method using log-rank test).
CA9, carbonic anhydrase IX; AJCC, American Joint Committee on Cancer.

along the cytoplasmic membrane and in the cytoplasm of osteosarcoma cells (Fig. 1). The distribution of CA9 positivity was focal or diffuse. In chondroblastic osteosarcoma, CA9 expression was mainly noted in the peripheral cellular spindle cell areas. The normal bone and cartilage adjacent to the tumors did not display expression of CA9. In a positive control using renal cell carcinoma, CA9 expression was noted along the cytoplasmic membrane of tumor cells. Twenty-two cases (57.9%) of the 38 conventional high-grade osteosarcomas showed increased staining for CA9. Twelve cases (31.6%) were considered focal and 10 (26.3%) were diffuse. Twenty-five low-grade central osteosarcoma samples were also analyzed for CA9 expression and all negative. CA9 expression showed a significant correlation with higher histologic grade ($p < 0.0001$) (Table 1). However, no significant differences were found in CA9 expression with respect to gender ($p = 0.4101$), age ($p = 0.4257$), or tumor location ($p = 0.6096$).

Among the variable histological subtypes in high-grade osteosarcomas, no significant differences were found in CA9 expression ($p = 0.7351$). Among all 38 cases of high-grade conventional osteosarcoma, nine cases had a history of metastasis, and seven showed CA9 expression. CA9 expression was significantly associated with the presence of metastasis ($p = 0.0010$). For one patient who died with pulmonary metastasis, paraffin blocks were available. They exhibited diffuse CA9 expression in both primary bone and pulmonary metastatic lesions (Fig. 1D). However, no significant differences were found in CA9 expression with respect to tumor size ($p = 0.8128$) or AJCC stage ($p = 0.4192$).

Overall survival data

Follow-up data were available for 24 patients with high-grade osteosarcomas and all 25 patients with low-grade central osteosarcomas. The follow-up period ranged from 1 to 275 months. In the high-grade group, 13 patients died while the other 11 patients survived. On the other hand, all 25 patients with low-grade central osteosarcomas survived. CA9 expression ($p = 0.0012$) (Fig. 2), histologic grade ($p < 0.0001$), and age ($p = 0.0140$) were found to be prognostically relevant in terms of overall survival by univariate analysis (Table 2). However, overall survival rate was not significantly correlated with gender ($p = 0.3260$), tumor size ($p = 0.2315$), or AJCC stage ($p = 0.2996$).

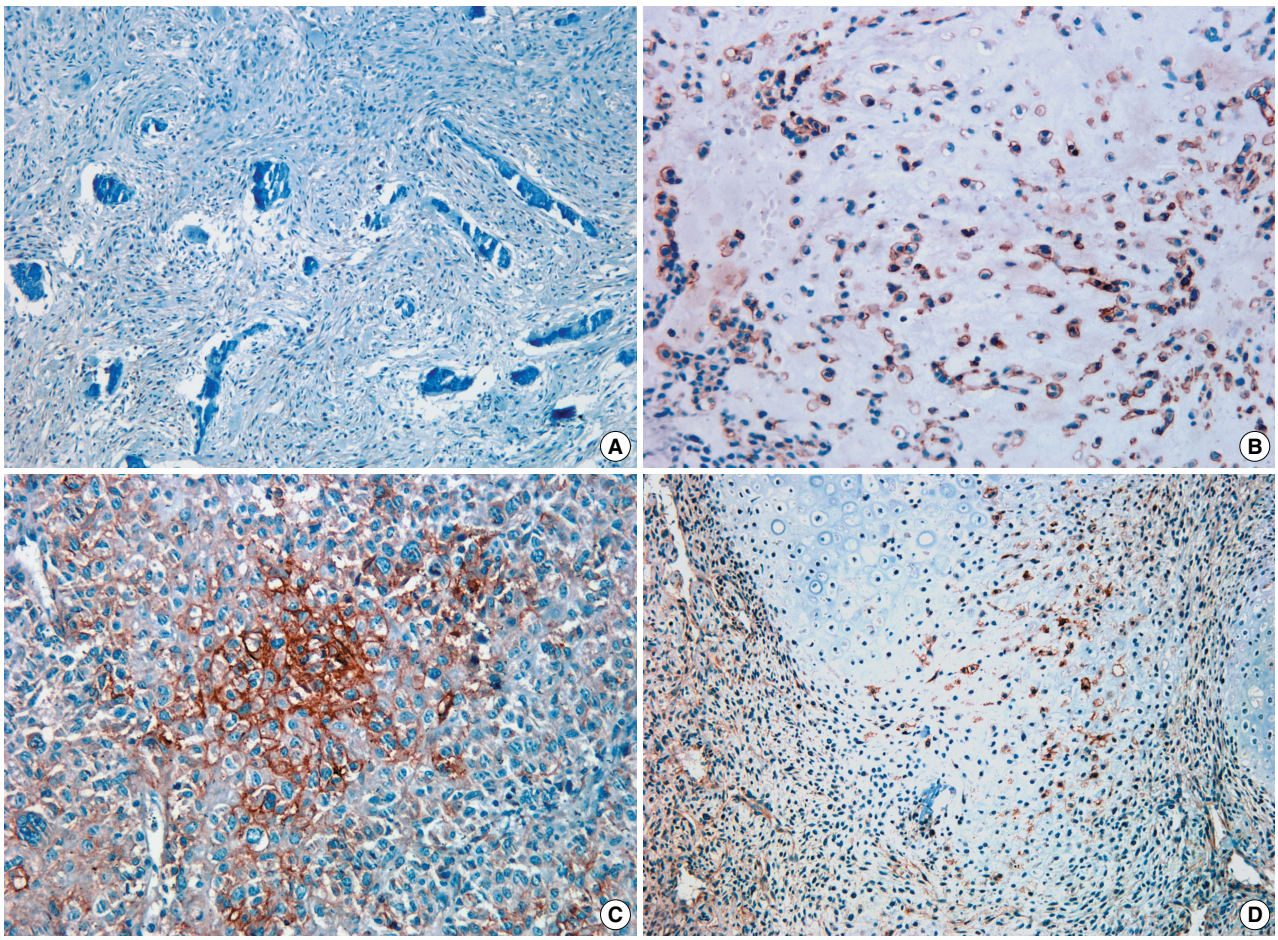

Fig. 1. Immunohistochemistry for carbonic anhydrase IX. (A) Low-grade central osteosarcoma, (B) conventional high-grade osteosarcoma, chondroblastic type, (C) conventional high-grade osteosarcoma, osteoblastic type, (D) metastatic osteosarcoma in a lung.

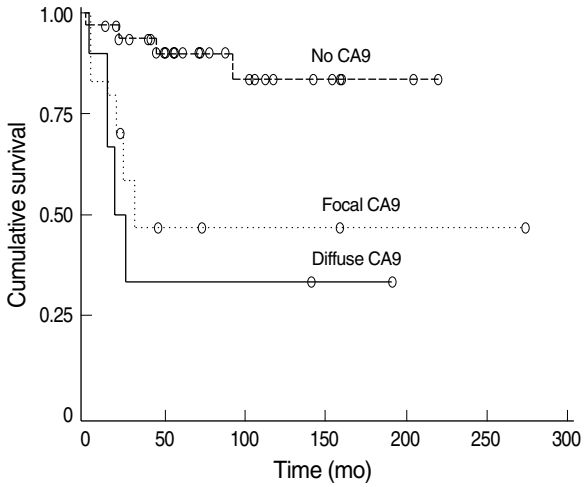

Fig. 2. Kaplan-Meier survival curve according to carbonic anhydrase IX (CA9) expression. Overall survival rate is significantly reduced with increased CA9 expression (p = 0.0012).

DISCUSSION

CA9 is a transmembrane protein that catalyzes the reversible hydration of carbon dioxide into carbonic acid, thus, regulating pH.[12] Through this activity, CA9 helps maintain a normal pH in tumor cells in a hypoxic microenvironment, which may allow tumor cell proliferation.[13] CA9 was originally identified in HeLa cells, where its expression was correlated with cell density.[14] Several authors showed that CA9 is expressed in several types of human carcinomas while being absent from the corresponding normal tissues and suggested that CA9 might be involved in cell proliferation and transformation.[4,5] We found that 57.9% of conventional high-grade osteosarcoma tissues expressed CA9, whereas corresponding normal tissues did not. This expression pattern lends support to the theory that CA9 is a tumor-associated protein of osteosarcoma. We applied immunohistochemical methods using a commercially available antibody, clone NB-

100-417. This antibody was reported to have excellent agreement with membrane-predominant expression of conventional clone M75.[15]

The aim of the study was to explore the relationship between CA9 and metastasis in a number of osteosarcoma patients. We did a retrospective study in which we investigated the potential prognostic value of CA9 in archival paraffin-embedded tissue. Patients were selected who had not received preoperative irradiation or chemotherapy because this might affect the levels of hypoxia and hence the CA9 expression levels being measured in the tumors. The major finding of this study was that there was a significant correlation between CA9 expression and higher histologic grade and the presence of metastasis in patients with osteosarcoma, showing that CA9 may have additional prognostic value in this group of patients. In our study, one patient exhibited diffuse CA9 expression in both primary bone and pulmonary metastatic lesions. These results are consistent with previous reports and represent the first such finding in osteosarcomas.[3,6-8] In terms of overall survival, CA9 expression was found to be prognostically relevant in addition to histologic grade and patient age in our study. Unfortunately, CA9 expression was not correlated with AJCC stage.

Besides renal cell carcinoma, CA9 protein has been described in a variety of other tumors including carcinomas of the cervix, esophagus, nasopharynx, breast, ovary, lung, colorectum, and urothelium, as well as soft tissue sarcoma.[3,4,10,16-23] Contrary to what has been reported in renal cell carcinoma, the expression of CA9 was found to correlate with poor outcome for disease-free and overall survival in a variety of these tumors. In most of these tumors the CA9 expression exhibited a perinecrotic distribution. Tumor hypoxia has been shown to be strongly associated with tumor propagation, malignant progression, and resistance to therapy.[24] The hypoxia-inducible gene *CA9* is an established and validated poor prognostic factor in breast cancer.[18] However, in one series of 945 high-risk premenopausal and postmenopausal women, positivity for CA9 was not an overall independent prognostic marker for survival; only in subgroup analyses especially in postmenopausal women, women with one to three positive nodes, and hormone receptor positive women, was it found to have prognostic value.[19] CA9 overexpression in breast cancer was reported to be associated with overexpression of HER2.[25] In high grade and invasive bladder cancer, although the hypoxia related factor CA9 was significantly associated with necrosis, the presence of necrosis was the independent prognostic factor. Areas of necrosis are thought to represent areas of chronic severe hypoxia. Thus, the development of chronic, severe hypoxia in bladder cancer is independently associated with a worse prognosis.[22] In superficial bladder cancer, CA9 was also coexpressed with vascular endothelial growth factor (VEGF).[26] This colocalization of CA9 and VEGF near the luminal surface and in perinecrotic areas in bladder cancer suggests that the trigger for VEGF upregulation is hypoxia.

For soft tissue sarcoma, positive membranous CA9 staining was reported in 66% of tumors.[10] Patients with CA9-positive tumors had a significantly lower disease-specific and overall survival rate than patients with CA9-negative tumors. These data suggest that CA9, a potential intrinsic marker of hypoxia, is associated with a poor prognosis in patients with deep, large, high-grade soft tissue sarcoma.[10] CA9 may predict 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 been found to correlate weakly with lowered oxygenation in soft tissue sarcoma.[27] Also, a correlation between CA9-positive cells and the Ki-67 proliferation index was found in colorectal cancer.[21] CA9 can be a target of antitumoral therapy in human solid tumors through treatment with CA9 inhibitors, including sulfonamide or sulfamate classes.[9] The determination of tumoral CA9 overexpression may be an important diagnostic tool and a basis for tailored treatment in human malignancy.

A limitation of our study is that the number of samples analyzed was not sufficient, especially the one patient with stage III osteosarcoma. Another limitation is the incomplete information about neo- and adjuvant chemotherapy for high-grade osteosarcoma patients. Larger studies are required to determine whether CA9 has independent prognostic value in this group of tumors. Its precise function remains inadequately determined and so the underlying biological explanation for its independent prognostic value remains to be fully explained.

In summary, we are the first group to demonstrate CA9 overexpression in a series of patients with osteosarcoma and to describe its prognostic implication. Our study demonstrated that CA9 expression is a frequent and tumor-related event in osteosarcoma. CA9 expression is associated with higher grade tumor, metastasis and poor overall survival for osteosarcoma patients.

ACKNOWLEDGMENTS

We thank Seeng Sik Cho and the Clinical Research Coordinating Center for statistical analysis consultation.

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