Expression of Carbonic Anhydrase IX in Astrocytic Tumors Predicts Poor Prognosis

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Abstract

Purpose: Carbonic anhydrase IX (CA IX) is a hypoxia-inducible enzyme, which is associated with neoplastic growth. Ectopic CA IX expression has been observed in several tumors, whose normal counterparts do not express this enzyme. Normal human brain tissue shows only slight or no expression of CA IX.

Experimental Design: We describe CA IX expression in human diffusely infiltrating astrocytomas. The association of CA IX is evaluated with clinicopathologic and molecular factors including cell proliferation and apoptosis as well as the expression of p53 and epidermal growth factor receptor.

Results: CA IX immunopositivity was observed in 284 cases of 362 (78%) tumors. The positive areas were often located in close proximity to necrotic regions (P < 0.001). The CA IX immuno-reactivity showed strong association with tumor malignancy grades (P < 0.0001). CA IX showed no association with p53 expression nor did it correlate with epidermal growth factor receptor—amplification, apoptosis, or cell proliferation. CA IX intensity had significant prognostic value in univariate (P=0.0011, log-rank test) and multivariate survival analysis (P = 0.038, Cox analysis).

Conclusions: CA IX expression is common in diffusely infiltrating high-grade astrocytomas. Our results suggest that CA IX is a useful biomarker for predicting poor prognosis of astrocytic tumors. It may also be a promising target molecule for the improvement of therapeutic interventions in astrocytomas.

Carbonic anhydrases (CA) are zinc-containing metalloenzymes that catalyze reversible hydration of carbon dioxide in a reaction CO2 + H2O ⇌ HCO3− + H+. CAs are produced in a variety of tissues where they participate in several biological processes such as acid-base balance, carbon dioxide, and ion transport, respiration, bone resorption, ureagenesis, gluconeogenesis, and formation of cerebrospinal fluid (1–3). The mammalian α-CA family consists of at least 12 active isoenzymes with different structural and catalytic properties (4). In addition to their essential biochemical functions in pH regulation, the expression of some CA family members is also associated with neoplastic process (2, 3, 5–7).

CA IX is a catalytically active plasma membrane isoform containing a central catalytic CA domain, a transmembrane part, and a short COOH-terminal cytoplasmic tail. The structure that makes CA IX different from other isozymes is a proteoglycan-like region in the NH2-terminal side suggested to be capable of acting in cell adhesion (8, 9). CA IX plays a dual role in normal tissues: in the stomach, it supports differentiation, and in the intestine, it is connected to the proliferation of epithelial cells (5). In a mouse model, CA IX seems to be involved in the control of differentiation and proliferation of epithelial cell lineages of the gastric mucosa during morphogenesis of the stomach (10). CA IX is present in few normal tissues but is ectopically expressed in a wide spectrum of human tumors. Expression is observed, e.g., in tumors of kidney, breast, head and neck, lung, bladder, and cervix uteri, i.e., in tissues where CA IX expression is normally absent (5). In a recent study, expression of CA IX was reported in the mouse brain (11). The normal human brain tissue shows only a slight or no expression of CA IX except the epithelial cells of the choroid plexus (6).

As to brain tumors, there is no large study to describe the expression of CA IX in human diffusely infiltrating astrocytomas, which are highly malignant tumors derived from glial cells. In these tumors, the surgical treatment is often impossible or inadequate, and thus, the prognosis of patients is poor. Here, we evaluated the association between CA IX expression and patient survival.
and several clinicopathologic and molecular factors including cell proliferation, apoptosis, p53, and epidermal growth factor receptor (EGFR).

**Materials and Methods**

**Study material.** The study material consisted of 362 diffusely infiltrating astrocytoma samples, which were obtained from surgically operated patients in Tampere University Hospital, Tampere, Finland, during 1983 to 2001. First, the brain tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. On the basis of H&E-stained slides, a neuropathologist (H. Haapasalo) did an evaluation of the tumors according to the WHO 2000 criteria (12). These criteria divide diffusely infiltrating astrocytomas into three grades (2-4) according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The neuropathologist pinpointed one histologically representative tumor region in each sample specimen. From this region, a sample was included to multitissue blocks. The multitissue blocks were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) and the sample diameter of the tissue cores was 600 μm/L. The multitissue blocks were composed of 362 astrocytic tumors (grade 2 (52), grade 3 (45), and grade 4 (265)) and consisted of 281 primary tumors and 81 recurrences. The age of patients with primary tumors varied from 15 to 89 (median ± SD, 64 ± 13) and recurrent tumors from 15 to 89 (median ± SD, 52 ± 12). Overall survival was known for 186 patients [grade 2 (26), grade 3 (21), and grade 4 (139)]. The median follow-up time for 40 survivors was 809 days, and 146 patients died during the follow-up. The tumors were radically resected if possible and most patients with high-grade gliomas also received radiotherapy.

**Immunohistochemistry.** The monoclonal M75 antibody against human CA IX has been described previously (13). The antibody has been characterized for specificity and it has shown no cross-reactivity with other CAs (14). The CA IX enzyme is immunostained by the biotin-streptavidin complex method from the multitissue blocks by following this procedure: (a) pretreatment of the sections with undiluted cow colostrum whey (Biotop Oy, Oulu, Finland) for 30 minutes and washed in PBS, (b) incubation for 1 hour with M75 antibody (1:10) in 1% bovine serum albumin-PBS, (c) incubation for 1 hour in biotinylated goat anti-mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) diluted 1:300 in 1% bovine serum albumin-PBS, and then (d) incubation for 30 minutes with peroxidase-conjugated streptavidin (Zymed) diluted 1:500 in PBS; (e) theretofore, incubation was done for 2 minutes in DAB solution containing 9 mg 3,3’-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 mL PBS and 5 μL 30% H₂O₂. The sections were rinsed thrice for 10 minutes in PBS after incubation steps (b) and (c), and four times for 5 minutes in PBS after step (d). The tumor sections were counterstained with hematoxylin after the immunostaining.

The extent and the intensity of the staining reaction for CA IX were scored from the multitissue blocks on a scale from 0 to 3. In terms of the staining intensity, the scores were evaluated as follows: 0, no reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction. The extent of the signal was also scored by a four-step assessment: 0, no positive cells; +, <25% positive cells; ++, 25% to 50% positive cells; +++, >50% positive cells. The tumors were also divided into two groups based on the nuclear staining of the CA IX as follows: −, no nuclear reaction; +, positive nuclear reaction. Gastric mucosa served as a positive control (15).

Proliferation by Ki-67 (MIB-1), apoptosis by terminal nucleotidyl transferase–mediated nick end labeling and p53 immunohistochemistry were done as previously described (16). EGFR amplification was detected with chromogenic in situ hybridization (17).

**mRNA analysis.** mRNA was isolated from seven brain tumors and one normal brain sample with RNeasy Mini-Kit (Qiagen, Hilden, Germany). Reverse transcription was done with Mo-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random primers (400 μg/mL). The primers for the PCR reaction were designed by using the published information on CA IX mRNA in GenBank (accession no. NM_001216). To produce an amplification product of 457 bp, the forward primer (F1) was 5’TGTCGTCCTGCTGAGGAAAGAA-3’ (nucleotides 915-937) and the reverse primer (R1) 5’GGGGTACGTACACCCCCCTTT-3’ (nucleotides 1392-1372). The control PCR reaction was done with the following primers for human β2-microglobulin (accession no. NM_004048): the forward primer was 5’TATCGGCTATCTCCAAAAGATCA-3’ (nucleotides 120-143) and the reverse primer was 5’GAAGAACAGCTGGAAGTGTCCCA-3’ (nucleotides 288-265). The reagents for the PCR reaction were from BD Biosciences (Palo Alto, CA), except for the deoxynucleotide triphosphate mix which was from Finnzymes. Twenty nanograms of CDNA were used as the template. The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA), and the protocol consisted of a 94 °C denaturation step for 1 minute followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 90 seconds, followed by final extension at 72°C for 3 minutes. The results of the PCR reaction were analyzed using 1.5% agarose gel containing 0.1 μg/mL ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA).

**Statistical analysis.** All statistical analyses were done using SPSS for Windows (Chicago, IL). The significance of associations was defined using χ² test, Mann-Whitney test, and Kruskal-Wallis test. The log rank test, Kaplan-Meier curves, and Cox multivariate regression analysis was used in the survival analysis.

**Results**

**CA IX and clinicopathologic features.** Cellular CA IX immunopositivity was observed in 284 of 362 cases (78%) of diffusely infiltrating astrocytomas. CA IX expression was detected in tissue sections as follows: 57 (16%) were strongly, 84 (23%) moderately, 143 (39%) weakly stained, and 78 (22%) were negative (Fig. 1). Within WHO grade 2 astrocytomas, there were 65% CA IX-positive cases (15% moderately, 50% weakly). Similarly, 73% of grade 3 astrocytomas (9% strongly, 33% moderately, and 31% weakly) and 82% of grade 4 astrocytomas (20% strongly, 23% moderately, 39% weakly) were CA IX-positive. The strongly positive areas were often located close to the necrotic regions (Fig. 1E) and CA IX intensity correlated significantly with the presence of necrosis in the same tissue section (P < 0.001, χ² test). Weak, mainly cytoplasmic, staining was occasionally seen in the neoplastic cells located in the infiltrative zone of lower grade tumors (Fig. 1C). The positive staining was usually unevenly distributed within the tumor. The positive immunostaining did not correlate clearly with the distribution of blood vessels, nor did it associate with endothelial cell proliferation in vessels (volume percentage of endothelial cells, P = n.s., χ² test). It also seemed that the cell cytoplasm was more intensively stained in the tumors with anaplastic features (Fig. 1D). The statistical comparison of cytoplasmic CA IX intensity and tumor grade also revealed significantly higher CA IX intensity in tumors of higher malignancy grade (P = 0.000, χ² test; Table 1).

Nuclear staining for CA IX was observed in 211 (58%) tumors, whereas 151 (42%) cases remained negative. There was more frequent nuclear staining in gliomas of better differentiation in contrast with the grade 4 glioblastomas, in which the nuclear staining was rarely discovered (P < 0.001, χ² test). CA IX extent, which describes the relative area of the highest intensity, was found to be high in 184 (51%), moderate in

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**Imaging, Diagnosis, Prognosis**

*Clin Cancer Res 2006;12(2) January 15, 2006* 474  www.aacrjournals.org

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72 (20%), scant in 28 (8%), and negative in 78 (22%) cases. A significant association was found between the increasing CA IX extent and increasing histological grade ($P = 0.027$, $\chi^2$).

The associations of the patient age and CA IX intensity were studied by dividing the group of patients by the age of 50 into two subgroups (<50 years, $n = 164$; $\geq 50$, $n = 200$). The $\chi^2$ test showed significant correlations between increasing CA IX intensity and patient age ($P = 0.003$) as well as increasing CA IX nuclear staining and patient age ($P = 0.004$). Similarly, CA IX intensity correlated with patient age within grade 2 ($P = 0.010$) and grade 3 ($P = 0.013$) but not in grade 4 astrocytomas.

**CA IX and molecular pathologic features.** When molecular pathologic features typical of astrocytomas were compared with CA IX immunohistochemistry, no association was seen between proliferation by Ki-67/MIB-1 (329 cases studied: grade 2, median 3.1, range 0-35.8; grade 3, median 5.0, range 0-50.9; grade 4, median 13.6, range 0-55.6) or apoptosis by terminal nucleotidyl transferase–mediated nick end labeling index (29 cases studied: grade 2, median 0.35, range 0-0.80; grade 3, median 0.15, range 0-0.3; grade 4, median 0.3, range 0-2.5; Mann-Whitney test). CA IX intensity showed no significant association with p53 (141 cases studied, 69 positive and 72 negative) nor did it correlate with EGFR amplification (183 cases studied, 83 positive and 100 negative; $\chi^2$ test).

**CA IX and survival.** Overall survival data was known for 186 patients. Patient survival was tested by log-rank test in relation to CA IX intensity. Interestingly, CA IX intensity divided the tumors into four significantly differing prognostic subsets ($P = 0.0011$; Fig. 2). The survival difference was even significant within grade 2 ($P = 0.0331$) and grade 4 ($P = 0.0163$), but not in grade 3 tumors. The extent of CA IX immunostaining also had a significant prognostic value ($P = 0.030$, log-rank test).

Most importantly, statistical analysis of the data revealed that the patient age, tumor grade, and CA IX intensity all had independent prognostic value (Cox multivariate analysis; Table 2). However, MIB-1 index did not seem to be an independent prognostic factor although it correlated with overall survival ($P = 0.0047$, log-rank test; see cut points; Table 2) and WHO grade ($P < 0.001$, Kruskal-Wallis test). There was no correlation between p53 status, apoptotic rate, and survival.

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**Fig. 1.** Negative CA IX immunostaining of white matter in normal human brain (A) and grade 2 astrocytoma (B; magnification, $\times 630$). C, weakly positive cytoplasm (arrow) in grade 2 astrocytoma (magnification, $\times 630$). D, moderate cytoplasmic (arrow) and nuclear (arrowhead) CA IX immunostaining in an anaplastic astrocytoma (grade 3; magnification, $\times 630$). E, strongly CA IX–immunopositive border of necrotic area (*) of glioblastoma (grade 4). Tumor cells and especially their plasma membrane stain positively, whereas most nuclei are negative (magnification, $\times 630$).
mRNA analysis. Immunohistochemical results of seven tumor samples (grades 2-4, two samples of each grade and hemangioblastoma) were verified by means of RT-PCR. A strong band for CA IX mRNA was found from one sample of grade 4 astrocytoma. Very weak positive signals were detected in two grade 2 specimens as well as in one grade 3 sample (Fig. 3).

Discussion

According to our study, CA IX expression is common in diffusely infiltrating astrocytomas, especially in high-grade tumors. The CA IX expression was associated with older patient age and poor prognosis. Importantly, CA IX expression was found to be an independent prognostic factor in addition to the patient age and histologic grade of astrocytomas. Even though the highest expression was observed in perinecrotic areas, it was not significantly associated with cell proliferation or apoptotic activity.

CA IX is ectopically expressed at relatively high levels and with high prevalence in tumors whose normal counterparts do not contain this protein (5). In addition to carcinomas of the cervix uteri, esophagus, kidney, lung and breast (18–23), this also seems to be true in human astrocytomas. Similar to carcinomas of the breast, cervix uteri, and lung, CA IX expression is localized to the perinecrotic regions of astrocytic tumors, suggesting that CA IX expression in brain tumors is induced by hypoxic conditions. Hypoxia triggers architectural and phenotypic rearrangements of tumor tissue that results in the development of necrotic areas surrounded by the zones of surviving hypoxic cells. It is these cells which could particularly acquire the most aggressive behavior. When a cell is exposed to hypoxic or anoxic conditions, transcriptionally active HIF-1 is bound to the hypoxia response element in the promoter area of the CA9 gene, thus, inducing CA IX expression (24). HIF-1-regulated pathway is modulated by VHL protein (pVHL), which, under normoxic conditions, targets the α subunits of HIF for proteosomal destruction (25, 26). Because CA9 is a target gene of HIF-1, its expression is increased in tumors associated with von Hippel-Lindau disease in which pVHL is defective due to mutations or deletions in VHL gene. von Hippel-Lindau disease is characterized clinically by vascular tumors, including central nervous system hemangioblastomas located in the cerebellum, spinal cord, or brain stem (27). Interestingly, somatic mutations in the VHL gene have also been reported in other central nervous system tumors including gliomas (28).

Table 1. Association between CA IX immunointensity with WHO grade (P < 0.001, χ² test)

<table>
<thead>
<tr>
<th>CA IX intensity</th>
<th>WHO grade</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
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<td>3</td>
</tr>
<tr>
<td>0</td>
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<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
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<td>45</td>
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</table>

Table 2. The independent prognostic indicators of astrocytomas as evaluated by Cox’s stepwise regression model

<table>
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<tr>
<th>Significance Hazard Ratio</th>
<th>95.0% CI for Hazard Ratio</th>
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<tr>
<td>Age</td>
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</tr>
<tr>
<td>Grade</td>
<td>0.000</td>
</tr>
<tr>
<td>CA IX intensity</td>
<td>0.038</td>
</tr>
</tbody>
</table>

NOTE: Patient age (cut points, 45 and 60 years), histologic grade (2-4), CA IX intensity (0-3), CA IX extension (0-3), CA IX nuclear staining (0.1), and MIB-1 (cut points 3%, 10%, and 20%) were included into the analysis (enter limit P < 0.05).

Fig. 2. Overall patient survival by CA IX intensity. Kaplan-Meier curves are shown (P = 0.0011, log-rank test).

Fig. 3. mRNA analysis. Immunohistochemical results of six tumor samples (grades 2-4, two samples of each grade) were verified by means of RT-PCR. A strong band for CA IX mRNA was found from one sample of astrocytoma grade 4. Only weak bands were observed in two grade 2, one grade 3, and another grade 4 astrocytoma samples. A hemangioblastoma was included in the analysis as a positive control.
Expression of Carbonic Anhydrase IX in Astrocytic Tumors

A recent study has indicated that hypoxia has a dual role in the regulation of CA IX (29). In addition to transcriptional induction, hypoxia also activates the kinetically properties of CA IX enzyme in hypoxic conditions. This may have important implications for tumor progression, because the enzymatic activation probably enhances extracellular acidification in tumors. In addition, experimental data suggests that increased cell density could influence CA IX expression. Both hypoxia and high cell density may contribute to increased CA IX expression in astrocytomas because the most malignant type (glioblastoma multiforme) represents a category of highly hypoxic and cellular tumors.

Coexpression of CA IX with c-ErbB2 and EGFR has been suggested to be regulated by the same oncogenic pathways, e.g., in non–small cell lung cancer (22, 30). However, we did not find an association between CA IX expression and EGFR amplifications which are commonly associated with astrocytic tumors. The differences in oncogenic pathways between different tumors may explain the observed discrepancy.

An infiltrative growth pattern is typical for astrocytic tumors. Instability and disorganization of cadherin-mediated junctions are required to promote migration and invasiveness in glioblastoma cell lines (31). CA IX has also been suggested to play a role in intercellular adhesion. In polarized epithelial Madin-Darby canine kidney cells transfected with the human CA9 cDNA, CA IX protein colocalizes with a key adhesion molecule, E-cadherin, and destabilizes E-cadherin-mediated cell-cell contacts via a mechanism that involves competitive interaction with β-catenin (9). This capability of CA IX is reminiscent of some oncoproteins such as EGFR and c-ErbB2, and makes CA IX a candidate contributor to tumor invasion. Our results suggest that this phenomenon might also take place in diffusely infiltrating astrocytomas. For example, cytoplasmic CA IX staining was also seen in the infiltrative border of lower grade tumors. In terms of cancer therapy, the aggressive character of astrocytoma cells is probably the most problematic feature. This might also explain the strong association between increased CA IX expression and poor survival in these tumors.

In summary, CA IX expression is a common phenomenon in high-grade astrocytomas. Our results suggest that CA IX is a useful biomarker for predicting poor prognosis in these tumors. It may also be a promising target molecule for the improvement of therapeutic interventions.

Acknowledgments
We thank Reija Randen and Aulikki Lehms for skilful technical assistance.

References
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