Carbonic anhydrases in meningiomas: association of endothelial carbonic anhydrase II with aggressive tumor features

Laboratory investigation

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Object. Carbonic anhydrase (CA) II and IX are enzymes involved in pH homeostasis and have been shown to be upregulated in several types of cancer. In this study, the authors evaluate the expression of CA II and IX in meningiomas and assess their relationship to patient age, tumor type and grade, tumor sex hormone receptor status, tumor cell proliferation, and tumor recurrence.

Methods. This study was conducted in consecutive patients who underwent meningioma surgeries at Tampere University Hospital between 1989 and 1999. The expression of CA II and IX was studied immunohistochemically using a tissue microarray technique and specific antibodies.

Results. Immunohistological staining with CA II and IX was assessed in 443 primary and 67 recurrent tumor specimens. Of these samples, 455 were benign (WHO Grade I), 49 atypical (Grade II), and 6 malignant (Grade III). Endothelial cells in 14.8% of the tumors stained positively for CA II. Tumor cells were positive for CA IX in 11.6% of the cases. Endothelial CA II expression correlated with increasing histological grade (p = 0.002), and tumor proliferation rates were higher in CA II+ versus CA II− cases (p = 0.002). Androgen receptor–negative tumors were found to be CA II+ significantly more often than androgen receptor–positive tumors (p = 0.001). No associations were found with the CA IX enzyme.

Conclusions. Carbonic anhydrase II positivity in the endothelium was associated with cell proliferation and malignancy grade. These results suggest that CA II expression is associated with malignant progression of meningiomas and could thus be a target molecule for anticancer therapy. (DOI: 10.3171/2008.10.17672)

Key Words • cancer • carbonic anhydrase • immunohistochemistry • meningioma • tissue microarray

Carbonic anhydrases belong to a family of metalloenzymes that catalyze the reversible hydration of carbon dioxide. Carbonic anhydrases participate in numerous biological processes such as acid-base balance, carbon dioxide and ion transport, respiration, and mucosal protection. Distribution patterns of different CA isoforms differ within normal human tissues as well as in tumors. Particular interest has been focused on isoforms CA IX and XII, and to a lesser degree, CA II. The authors of several studies have demonstrated their expression in a wide variety of malignant neoplasms.1,2,6,15,19,21,31

Carbonic anhydrase II is widely expressed in normal organs and is considered a very important enzyme in a number of physiological reactions. In addition, it is also found in malignant brain tumors,25 leukemia,19 lung cancer,2 pancreatic cancer,27 and colorectal cancer.2 Carbonic anhydrase II was recently shown to be expressed in tumor vessel endothelia,39 and is the most widely expressed CA isoenzyme in the CNS. Parkkila et al.25 found CA II in myelin, oligodendrocytes, astrocytes, and the choroid plexus. They also demonstrated expression of CA II in several brain tumors, but the few meningiomas tested did not express this enzyme.

Hypoxia in tumors is associated with aggressive growth and poor response to cancer treatments.4-11 Carbonic anhydrase IX has been proposed as a new hypoxia biomarker because it is strongly induced by hypoxia and is found in hypoxic areas of many cancers, such as those originating from the kidneys,23 lungs,6,7 breasts,5 cervix,21,24 brain,9,31 bladder,12 and ovaries.13 Hypoxia-in-
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ducible expression of CA IX in tumors suggests that the enzyme has an important role in tumor cell survival in hypoxic environments. It has been proposed that CA IX helps maintain a neutral intracellular pH and contributes to the acidification of extracellular space around tumor cells, thereby facilitating tumor growth and invasion. The feasibility of CA IX as a potential hypoxia biomarker has been realized by its very limited expression in the alimentary tract in normal human tissue. Expression of CA IX is very low in the normal brain. It is confined to the cells lining the ventricles and the choroid plexus. Induced expression has been found in high-grade gliomas. Ivanov et al. studied a small number of meningiomas and found enhanced CA IX expression in hypoxic/necrotic areas. A correlation between CA IX expression and poor prognosis has been shown in several cancers.

In the present study, we assessed the expression of CA II and IX in a large series of primary and recurrent meningiomas to evaluate the relationship between their expression and clinicopathological features such as tumor grade, patient age, sex hormone expression, and tumor cell proliferation rate.

Methods

Tumor Specimens

All tumor samples were obtained in patients who underwent surgery for intracranial or intraspinal meningiomas at the Tampere University Hospital between 1989 and 1999. Two patients were excluded because of their young age (4 and 15 years). A total of 443 primary and 67 tumors of second operations were chosen for the study. The study protocol was approved by the Ethical Committee of Tampere University Hospital.

Tumors were classified and graded using the WHO scheme (Grades I–III). Of the 443 primary tumors, 145 (32.7%) were meningothelial, 129 (29.1%) were transition al, 93 (21.0%) were fibroblastic, 44 (10.5%) were other benign tumors, 29 (6.5%) were atypical, and 3 (0.7%) were malignant. Four atypical and malignant meningiomas were irradiated after recurrence. Recurrence was defined as new tumor growth after Simpson Grade I or II removal. Of the 29 recurrent meningiomas, 22 (75.9%) were benign, 5 (17.2%) were atypical, and 2 (6.9%) were malignant. The second operation group consisted of all tumors that required a repeated operation despite the extent of the primary operation. Of these 67 meningiomas, 48 (71.6%) were benign, 16 (23.9%) were atypical, and 3 (4.5%) were malignant. Seven meningiomas in the second operative group were embolized. For statistical analysis, atypical and malignant tumors were grouped together.

Immunohistochemical Analysis

Tissue microarray techniques were used for immunohistochemical analysis. The tumor samples were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks using standard methods. Histologically representative tumor regions (highest grade and highest proliferation) of H & E–stained slides were selected by a neuropathologist (H.H.) and the corresponding areas were sampled in tissue microarray blocks using a custom-built instrument (Beecher Instruments). The sample diameter of the tissue core in the microarray block was 600 µm.

For proliferation analysis, sections cut from the tissue microarray blocks were stained with MIB-1 (Ki 67) antibody (DakoCytomation). Heat-induced epitope retrieval, in Tris-ethylenediamine tetraacetic acid buffer (pH 9.0; samples were microwaved twice for 7 minutes each time) and an automated immunostaining protocol (TechMate immunostainer) were used. The tissue sections were counterstained with methyl green. The proliferation was evaluated by analyzing all tumor cells in the tumor core of the multitissue block with an image analysis system (CAS-200 Software, Becton Dickinson & Co.) as previously described. The MIB-1 PI was reported as the percentage of immunopositive nuclei.

The hormone receptor analyses (AR, estrogen receptor, and progesterone receptor) were performed as previously described. Automated immunostaining for CA II and IX was performed using Power Vision+ Poly-HRP IHC Kit (ImmunoVision Technologies, Co.) reagents and CA II– and CA IX–specific antibodies. The immunostaining method included the following steps: 1) rinsing in Tris-buffered saline and 0.05% TBST; 2) treatment in 3% H2O2 in double-distilled H2O for 5 minutes and rinsing in TBST; 3) blocking with cow Colostrum for 20 minutes; 4) rinsing in TBST; 5) incubation with rabbit anti–human CA II serum (produced and characterized by Parkkila et al.), M75 antibody against human CA IX, or normal rabbit serum. Anti–CA II serum and normal rabbit serum were diluted 1:2000 and M75 1:200, respectively, in Universal IHC diluent (ImmunoVision Technologies, Co.) for 30 minutes; 6) washing with TBST 3 times for 5 minutes each time; 7) blocking with postblocking solution for 20 minutes (only in M75 staining); 8) rinsing in TBST 3 times for 5 minutes; 9) incubation in poly-horseradish peroxidase–conjugated anti–rabbit/mouse IgG for 30 minutes; 10) washing again in TBST 3 times for 5 minutes; 11) incubation in DAB solution (1 drop DAB solution A and 1 drop DAB solution B with 1-ml double-distilled H2O) for 6 minutes; 12) rinsing with double-distilled H2O; 13) CuSO4 treatment for 5 minutes to enhance the signal; and 14) rinsing with TBST and counterstaining with hematoxylin. All procedures were performed at room temperature. The sections were mounted in Entellan Neu (Merck) and examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss).

The reactivity of staining for endothelial CA II and cytoplasmic CA IX was scored from the multitissue blocks on a scale of 0–3. In terms of staining reactivity, the scores were evaluated as follows: 0, no reaction; +, weak reaction, < 10% of cells stained; ++, moderate reaction, 10–50% of cells stained; and ++++, strong reaction, > 50% of cells stained. The scoring was performed by 4 observers (H.H., K.K., R.V., and S.P.) who were blinded to tumor histological characteristics. In the statistical analyses, the specimens were grouped into 2 categories based on the staining reactivity. The positive group (CA II+ or CA IX+) included tumors with moderate or strong reactions, and the negative group (CA II– or CA IX–) included tumors with weak or no reaction.
Out of 510 cases, immunostaining for CA II was successful in 486 cases and for CA IX in 475 cases. Specimens with inadequate samples were excluded from the analysis. The hormone receptor status of meningioma specimens has been described earlier.18

Statistical Analysis

Associations between hormone receptor expression, tumor grade, patient age group, and CA expression were evaluated with the chi-square test. The relationship between CA staining and cell proliferation assessed by MIB-1 staining was tested with the Mann-Whitney U-test. All reported probability values were 2-sided, and values < 0.05 were considered statistically significant. Mean values are presented ± SDs.

Results

Carbonic Anhydrase II Expression

Endothelial CA II immunostaining in blood vessels was strong in 8, moderate in 64, and weak in 175 cases, while in 239 cases there was no reaction. Tumor cells were negative for cytoplasmic CA II. When tumor specimens were grouped into categories based on the endothelial staining reactivity, 414 (85.2%) cases were CA II− (no or weak reaction) and 72 (14.8%) CA II+ (moderate or strong reaction). Carbonic anhydrase positivity was found in 15.9 and 15.2% of meningothelial and transitional meningiomas, respectively, compared to only 4.3% of the fibrous subtype. No significant difference in CA immunopositivity was found between those from primary operations, second operations, and recurrent meningiomas. Carbonic anhydrase II staining reactivity correlated with tumor grade: 13.0% of benign tumor specimens were CA II+ compared with 30.6% of Grade II–III tumor specimens (p = 0.002). Carbonic anhydrase II positivity was more frequent in AR-negative tumors than in AR-positive tumors. Moderate or strong CA II reactivity was found in 19.1% of AR-negative specimens and in 7.7% of AR-positive specimens (p = 0.001). No such association was found for progesterone or estrogen receptor status (Table 1). Results of CA II immunostaining did not show significant correlation with age or sex. The mean MIB-1 PI for CA II+ tumors was 4.82 ± 3.96 and for CA II− tumors was 3.48 ± 3.79. The difference between these groups was statistically significant (p = 0.002).

Carbonic Anhydrase IX Expression

Carbonic anhydrase IX immunostaining was strong in 24 (5.1%), moderate in 31 (6.5%), and weak in 63 (13.3%) specimens. Staining of the plasma membrane remained completely negative in 357 specimens (75.2%). Thus, 420 (88.4%) of 475 specimens were CA IX− (no or weak staining), and 55 (11.6%) were CA IX+ (moderate or strong staining) when the specimens were grouped into 2 categories. The frequencies of CA IX+ tumors in meningothelial, transitional, and fibrous meningioma specimens were 7.1, 9.9, and 9.2%, respectively. Primary operation, second operation, and recurrent tumors did not differ in CA IX immunopositivity. Carbonic anhydrase IX immunostaining did not correlate with the tumor grade—11.1% of Grade I meningiomas and 16.0% of Grade II–III tumors were CA IX+ (p > 0.05). There was no association between CA IX immunopositivity and the sex hormone status of the tumors. Comparison between age groups was not significant, but there was a trend toward more frequent CA IX positivity with increasing age (p = 0.092). Sex differences had no effect on CA IX expression, nor did PI correlate with CA IX reactivity. The mean PI values for CA IX+ tumor samples were 3.87 ± 4.06 and 3.66 ± 3.78 for CA IX− samples (p > 0.05). There was no endothelial immunopositivity for CA IX in the tumor vessels. Endothelial CA II expression did not correlate with cytoplasmic CA IX positivity in tumor cells.

Discussion

Meningiomas are the most common benign tumors of the CNS. Despite their mostly benign nature, their rate of recurrence is high.22 Total resection is often the goal for treatment although it can be laborious because these tumors are often found in places where surgery is most challenging. Carbonic anhydrase IX and II are possible target molecules for new therapeutic interventions in several types of tumors. To our knowledge, this is the first study to evaluate the expression of both CA II and IX in a large series of meningiomas.

Cytoplasmic CA II is a very efficient enzyme. It has been proposed to interact with CA IX and an anion exchanger protein to produce acidosis in the extracellular space of tumors.29 Cytoplasmic CA II expression has

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CA II+ (%)</th>
<th>CA IX+ (%)</th>
</tr>
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<tbody>
<tr>
<td>primary</td>
<td>65 (15.3)</td>
<td>48 (11.6)</td>
</tr>
<tr>
<td>recurrent</td>
<td>3 (10.3)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>2nd op</td>
<td>7 (11.5)</td>
<td>7 (11.7)</td>
</tr>
<tr>
<td>tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>benign</td>
<td>57 (13.0)</td>
<td>47 (11.1)</td>
</tr>
<tr>
<td>atypical/malignant</td>
<td>15 (30.6)†</td>
<td>8 (16.0)</td>
</tr>
<tr>
<td>patient age (yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20–49</td>
<td>18 (15.0)</td>
<td>8 (7.1)</td>
</tr>
<tr>
<td>50–59</td>
<td>19 (14.3)</td>
<td>16 (12.0)</td>
</tr>
<tr>
<td>60–84</td>
<td>35 (15.0)</td>
<td>31 (13.5)</td>
</tr>
<tr>
<td>hormone receptor status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR+</td>
<td>61 (15.1)</td>
<td>44 (11.1)</td>
</tr>
<tr>
<td>PR−</td>
<td>6 (11.5)</td>
<td>6 (11.5)</td>
</tr>
<tr>
<td>ER+</td>
<td>23 (13.3)</td>
<td>13 (7.8)</td>
</tr>
<tr>
<td>ER−</td>
<td>42 (16.0)</td>
<td>35 (13.5)</td>
</tr>
<tr>
<td>AR+</td>
<td>13 (7.7)‡</td>
<td>19 (11.5)</td>
</tr>
<tr>
<td>AR−</td>
<td>49 (19.1)</td>
<td>34 (13.6)</td>
</tr>
</tbody>
</table>

* Values represent number of tumors. Abbreviations: ER = estrogen receptor; PR = progesterone receptor.
† p = 0.002.
‡ p = 0.001; chi-square test.
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been found in some tumor cells such as pancreatic and renal cancer cells.\textsuperscript{27,28} In addition, expression of CA II has been recently discovered in tumor vessel endothelia, suggesting a role for this enzyme in tumor angiogenesis\textsuperscript{39} (Fig. 1). Yoshiura and colleagues\textsuperscript{39} found that the expression of endothelial CA II increased in hypoxic conditions. Hypoxic changes lead to necrosis of tumor tissue, a common feature of higher grade meningiomas. In line with this, one-third of atypical and malignant meningiomas in our study stained positively for CA II, compared to 13% in low-grade tumors. Based on our results, CA II expression also correlated with cell proliferation, while CA II and AR showed reciprocal staining reactivities. Although the reason for more abundant expression of CA II in AR-negative tumors is unclear, it is known that androgens do regulate CA II expression at least in the reproductive system.\textsuperscript{14} The regulatory mechanisms are complex, however, because androgens induce CA II expression in some organs and inhibit it in others.\textsuperscript{10} Expression of CA II has recently been found to be common in the endothelial cells of high-grade oligodendrogliomas and diffuse infiltrating astrocytomas.\textsuperscript{8} Carbonic anhydrase II positivity also correlated with tumor grade and poor prognosis; CA II reactivity was highest in high-grade astrocytomas, which are vascularized tumors containing necrotic areas.

Our findings suggest that CA II may be important in the development of atypical/malignant meningiomas, which are often highly vascular. If the role of CA II in tumor angiogenesis proves to be important, CA inhibitors may present new possibilities for tumor treatment in combination with other therapies such as surgery. Carbonic anhydrase inhibitors have been shown to inhibit growth and invasion of cancers.\textsuperscript{28,34} Teicher et al.\textsuperscript{36} showed that the CA inhibitor acetazolamide was a beneficial adjunct to chemotherapy because it extended tumor growth delay with anticancer drugs in vivo. It is also notable that some CA inhibitors are widely used drugs for neurological and ophthalmological diseases such as brain edema and glaucoma. Because CA II expression seems to be quite common in the worst cases of meningiomas, it could be beneficial to add a CA inhibitor to the treatment regimen, especially in cases with incomplete tumor resection.

The normal human brain shows only slight or no expression of CA IX,\textsuperscript{15} but ectopic expression of CA IX in malignant brain tumors has recently been confirmed.\textsuperscript{9,15,31} The transmembrane protein CA IX is strongly induced by hypoxia, and expression of CA IX is predominantly found in poorly perfused, perinecrotic areas of tumors.\textsuperscript{15,37} It has been suggested that the transmembrane location of CA IX allows it to participate in converting carbon dioxide (which diffuses in from the intracellular space) to bicarbonate and hydrogen ions. Because bicarbonate is exchanged for intracellular chloride, extracellular acidosis is maintained.\textsuperscript{29} Extracellular acidosis and intracellular alkalosis facilitate tumor growth; this is also in line with several previous observations that high expression of CA IX often correlates with a higher malignancy grade and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Photomicrographs. A: Strong positivity for CA IX in shown in meningioma cells. The inset showing the connective tissue around a blood vessel also stains positively for CA IX, but the adjacent tumor cells are CA IX− (asterisks). B: Another CA IX− meningioma is shown. C: Strong endothelial immunostaining for CA II is shown (arrows). D: Blood vessel is shown completely negative for CA II (arrows). Original magnification × 400.}
\end{figure}
poor prognosis.\textsuperscript{7,9} According to our findings, CA IX is expressed in only a minority of meningiomas (11.6%). This low level of expression in the present study is probably due to the mostly benign nature of meningiomas. Malignant and necrotic tumors only represented 1–2% of meningiomas. Grade II and III meningiomas showed only slightly higher expression of CA IX (16 vs 11%) than the benign specimens. Atypical meningiomas constituted the majority of meningioma specimens in the group of higher-grade meningiomas. According to our results, CA IX is not significantly upregulated in atypical meningiomas nor did its expression correlate significantly with the malignancy grade. Yoo and colleagues\textsuperscript{38} recently studied CA IX immunopositivity in 32, 24, and 85% of Grade I, II, and III tumors, respectively. Although the exact reason for the higher staining frequency in their study is unclear, the observed discrepancy may reflect differences in their staining protocol compared to ours in the present study. In breast cancer, CA IX expression correlates negatively with estrogen and progesterone receptor expression;\textsuperscript{39} however, we found no such association with meningiomas.

Conclusions

Our findings indicate that CA II and IX are expressed in a minority of meningiomas. Endothelial CA II positivity was slightly more common than CA IX positivity in these tumor cells. One-third of atypical and malignant meningiomas expressed CA II, which was significantly more than in benign tumors. Carbonic anhydrase II positivity was also associated with a higher cell proliferation rate, which suggests that CA II expression is associated with the malignant progression of meningiomas. Thus, CA II could be a potential target molecule for antitumor therapy, especially of recurrent meningiomas. Our results also suggest that CA IX is not likely to be a suitable target in the search for alternative treatments of meningiomas because its expression did not differ between malignancy categories or correlate with cell proliferation.

Disclosure

This work was supported by grants from the Cancer Society of Finland, Academy of Finland, EU 6th framework programme (DeZnIT), and the Medical Research Fund of Tampere University Hospital.

Acknowledgments

The authors thank Professor Jorma Isola for collaboration, and Paula Kaukoranta and Salla Kolmihaara for their highly skilled technical assistance.

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Accepted October 24, 2008.
Please include this information when citing this paper: published online February 13, 2009; DOI: 10.3171/2008.10.17672.
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