Carbonic anhydrase II (CA II) is a cytosolic enzyme that is highly expressed in most organs, including the brain, where it is mainly located in the oligodendrocytes. Recent studies have shown that its expression is induced in the endothelium of neovessels in melanoma and esophageal, renal, and lung cancer. Immunological studies further indicate that CA II represents a major target antigen stimulating an autoantibody response in melanoma patients. These results prompted us to investigate endothelial CA II expression in two types of brain cancer: oligodendrogliomas and astrocytomas. A series of 255 astrocytoma and 71 oligodendroglial tumor specimens was immunostained for CA II. The staining results were correlated with a number of different clinicopathological factors and survival data. CA II showed weak or no expression in low-grade tumors, while grade 3 mixed oligoastrocytoma and glioblastoma multiforme were the most positively stained tumor types. Survival analysis indicated that endothelial CA II staining is significantly associated with a poor prognosis in patients with astrocytomas. About 17% of patients with CA II–negative tumors (weak or no endothelial signal) were still alive at the end of the follow-up period of five years. The presence of CA II in the tumor endothelium suggests that it may play an important functional role in tumor metabolism.


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evident after the identification of two membrane-bound isofoms, CA IX and XII, that are overexpressed in several types of cancer.\(^5\) These two CAs have become attractive research topics, given the hope that an understanding of their roles in tumor physiology could lead to the development of novel strategies for cancer detection, prognosis, and treatment.\(^5\) Only few studies have been conducted on the CA II enzyme in tumors, however, and this isofom has not been considered very attractive because most results regarding epithelial tumors have indicated that malignant cells contain no CA II or only low levels.\(^6\)\(^7\) In contrast, it has been reported that some, but not all, malignant blast cells in leukemia patients express CA II.\(^8\) The expression of CA II in the tumor vessel endothelium had never been investigated, however, until the recent article by Yoshiura et al.\(^1\) The results of that study will probably attract researchers to explore CA II in more detail in connection with various cancers, and it may be that the generation of autoantibodies to it in some patients could represent an important step in the natural defense system against cancer, and, even more important, as suggested recently,\(^1\) CA II associated with the endothelium of neovessels could serve as a potential target for cancer therapy. The present study was designed to analyze endothelial CA II expression in a large series of malignant gliomas. These brain tumors usually represent highly vascularized neoplasias with very poor clinical prognoses. Our results showed that 31% of diffuse astrocytomas and 14% of oligodendrogliomas show moderate or strong CA II expression in the tumor vessel endothelium.

**Materials and Methods**

**Materials**

The materials were obtained from surgical patients at Tampere University Hospital, Tampere, Finland, in 1983–2002. The study was approved by the research ethics committee of Tampere University Hospital. The brain tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. On the basis of hematoxylin and eosin–stained slides, a neuropathologist (H.H.) evaluated the tumors according to the WHO 2000 criteria. One histologically representative tumor region was selected in each specimen, and a sample from this region was included in multipurpose blocks constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The tissue cores were 600 \(\mu\)m in diameter.

**Astrocytic Tumors**

There were 255 astrocytoma samples included in the analyses (27 of grade 2, 39 of grade 3, and 189 of grade 4), of which 189 were primary tumors and 66 were recurrences. The WHO criteria divide diffusely infiltrating astrocytomas into three grades (2–4) according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The ages of the patients with primary tumors varied from 1 to 82 years (mean \(\pm\) SD: 48 \(\pm\) 20), and those of the patients with recurrent tumors varied from 1 to 76 years (mean \(\pm\) SD: 40 \(\pm\) 18). Overall survival was known for 195 patients, of whom 161 had died during the five-year follow-up period. The tumors were resected if possible (95%), and most patients with high-grade gliomas also received radiotherapy (55%) and/or chemotherapy (24%).

**Oligodendrogial Tumors**

There were 71 oligodendrogial tumor samples included in the analyses (pure oligodendrogiomas: 31 of grade 2, and 12 of grade 3; and oligoastrocytomas: 18 of grade 2, and 9 of grade 3), of which 52 were primary tumors and 19 were recurrences. The WHO criteria group oligodendrogial tumors into two main categories: pure oligodendrogiomas and mixed oligoastrocytomas, which are further divided into two grades (2 and 3) according to the presence of increased cellularity, atypia, and mitotic activity. The ages of the patients with primary tumors varied from 8 to 76 years (mean \(\pm\) SD: 43 \(\pm\) 14), and those of the patients with recurrent tumors varied from 12 to 69 years (mean \(\pm\) SD: 41 \(\pm\) 14). Overall survival was known for 52 patients: The median follow-up time for the 26 survivors was 8 years 2 months, and 26 patients died during follow-up. Detailed information about therapies was known for 31 patients. Sixteen patients received radiotherapy, and two received chemotherapy and radiation therapy. 1p/19q analysis by fluorescence in situ hybridization was performed as previously described.\(^9\)

**Immunohistochemistry and In Situ Hybridization**

Proliferation was examined by Ki-67 (MIB-1), apoptosis was assessed by TUNEL, and p53 immunohistochemistry was performed as previously described.\(^10\) Epidermal growth factor receptor (EGFR) amplification in the astrocytic tumors was detected with chromogenic in situ hybridization.\(^9\) The immunostaining for CA IX was performed as previously described.\(^11\)

Automated immunostaining for CA II was performed using Power Vision+ Poly-HRP IHC Kit (polymerized horseradish peroxidase immunohistochemistry kit; ImmunoVision Technologies, Brisbane, CA, USA) reagents. The immunostaining method included the following steps: (a) rinsing in wash buffer; (b) treatment in 3% hydrogen peroxide in double-distilled water (ddH\(_2\)O) for 5 min and rinsing in wash buffer; (c) blocking with Universal IHC Blocking/Diluent (ImmunoVision) for 30 min and rinsing in wash buffer; (d) incubation with rabbit anti-human CA II serum (produced and characterized earlier)\(^13\) or normal rabbit serum diluted 1:2,000 in Universal IHC Blocking/Diluent for 30 min; (e) rinsing in wash buffer for 3 \(\times\) 5 min; (f) incubation in poly-HRP–conjugated anti-rabbit immunoglobulin G for 30 min and rinsing in wash buffer for 3 \(\times\) 5 min; (g) incubation in 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (1 drop of DAB solution A and 1 drop of DAB solution B with 1 ml ddH\(_2\)O) for 6 min; (h) rinsing...
with ddH$_2$O; (i) copper(II) sulfate treatment for 5 min to enhance the signal; and (j) rinsing with ddH$_2$O. All procedures were carried out at room temperature. The sections were mounted on Entellan Neu (Merck, Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

The intensity of the staining reaction for endothelial and cytoplasmic CA II was scored from the multitsiss blocks on a scale from 0 to 3. The scores were evaluated in terms of staining intensity as follows: 0, no reaction; +, weak reaction; ++, moderate reaction; and +++ strong reaction. In the statistical analyses, the specimens were grouped into two categories based on the staining intensity: CA II+ve tumors, including those exhibiting moderate or strong reactions in the endothelium; and CA II−ve tumors, with weak or negative immunostaining results.

Statistical Analysis

All of the statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA). The significances of associations were defined using the chi-square test, Mann-Whitney test, and Kruskal-Wallis test. The log-rank test, Kaplan-Meier curves, and Cox multivariate regression analysis were used in the survival analysis.

Results

Astrocytic Tumors

Some brain tumors clearly appeared to express CA II in the vascular endothelium (Fig. 1), whereas the normal brain tissues showed no endothelial CA II expression (data not shown). Out of 261 diffusely infiltrating astrocytoma cases, endothelial CA II immunopositivity was observed in 117 (45%). The signal was strong in 45 cases, moderate in 36, and weak in 36. A total of 144 tumors (55%) were completely negative. High cytoplasmic CA II expression within the tumor cells appeared to be rare among these tumors, with only six specimens showing a strong signal. Moderate cytoplasmic staining was observed in 35 tumors and weak reactions in 52. A total of 168 cases (64%) showed no CA II expression within the tumor cells. No significant differences in CA II immunostaining were detected between primary tumors and recurrences ($p$ = NS, chi-square test).

When the specimens were grouped into two categories according to the endothelial staining intensity, 81 cases (31%) of 261 diffusively infiltrating astrocytomas were CA II+ve (moderate or strong reaction in the vascular endothelium) and 180 cases (69%) were CA II−ve (weak or no endothelial staining). The endothelial staining was most often seen in the proliferative endothelium and in small neovessels of grade 4 astrocytomas. The positive cells in the proliferative endothelium were usually located near the vascular lumen. The grade 2 astrocytomas did not express CA II at all in the endothelium (Fig. 1).

When the important clinicopathological factors were also evaluated, positive endothelial CA II staining was found to correlate significantly with the presence of EGFR amplification ($n = 172$, $p = 0.008$, chi-square test), whereas it showed no association with p53 expression ($n = 103$, $p$ = NS, chi-square test) or MIB-1/Ki-67 proliferation ($n = 234$, $p$ = NS, Mann-Whitney test). Endothelial CA II expression did not correlate with the area of necrosis in the same tissue section ($n = 37$, $p$ = NS, Mann-Whitney test). Nor did we find significant association between CA II reactivity and necrosis when the cut point of the latter was set to the median value ($p$ = NS, chi-square test). We previously analyzed the expression of CA IX in the same diffuse astrocytomas, and comparison of the immunostaining results indicated...
a significant association between the presence of CA II in the endothelium and CA IX in the tumor cells (p = 0.006, chi-square test).

Positive endothelial staining of CA II was significantly associated with a higher tumor grade (p < 0.001, chi-square test) (Fig. 2A). In a five-year survival analysis of patients with primary tumors, the patients having strong or moderate endothelial CA II staining showed significantly poorer survival (p = 0.032, log-rank test) (Fig. 3). In fact, 80 (99%) of the 81 patients having strong or moderate endothelial staining for CA II (CA II+ve) died during the follow-up period, whereas about 26 (17%) of the 156 patients with weakly stained or negative tumors (CA II–ve) were still alive at the end. Endothelial CA II was not of prognostic significance in univariate survival analysis when grades 2, 3, and 4 were tested separately. When only patients with grade 4 astrocytomas were examined after the five-year follow-up, all the patients having strong or moderate endothelial staining (n = 58) died during the five-year follow-up, whereas 11 (13%) of 87 patients with weakly stained or negative tumors were still alive. However, when endothelial CA II staining (CA–ve versus CA+ve) and conventional prognostic markers (tumor grade; patient age, cutoff points 50 and 65 years; MIB-1, cutoff points 5% and 15%) were included into the Cox multivariate analysis of primary tumors, only tumor grade (p < 0.001; odds ratio, 2.268) and patient age (p < 0.001; odds ratio, 2.011) showed independent prognostic value.

**Oligodendroglial Tumors**

Of the 43 pure oligodendrogliomas, 26 (60%) showed immunoreactions for CA II in the endothelium, and 15 (58%) of the 26 mixed oligoastrocytomas were positive. Figure 2B shows CA II immunostaining intensity in different grade categories. It is significant that the oligodendroglial tumors generally showed weaker endothelial expres-
sion of CA II than the astrocytomas (Figs. 2B and 4), the staining intensities being strong in 9, moderate in 1, and weak in 31 of 71 cases. Cytoplasmic tumor cell–associated CA II expression was detected in only a minority of the specimens. The signals were strong in 4, moderate in 1, and weak in 23 cases. A total of 43 oligodendrogial tumors (61%) showed no immunoreactions in the tumor cells. In oligodendrogial tumors, no significant differences in CA II expression were observed between primary tumors and recurrences (p = NS, chi-square test).

Of the 71 oligodendrogial tumors, 10 (14%) showed strong or moderate immunoreactions (CA II+ve) in the endothelium, while endothelial CA II was more often expressed in high-grade mixed oligoastrocytomas than low-grade tumors (p = 0.018, chi-square test) (Fig. 2B). There was no association between 1p or 19q loss and CA II expression (n = 17, p = NS, chi-square test), and neither endothelial nor cytoplasmic staining for CA II was a significant predictor of survival in this series of primary oligodendroglias (n = 52, p = NS, log-rank test).

**Discussion**

A recent attractive finding has indicated that CA II expression is induced in the endothelial cells of neovessels in melanoma and esophageal, renal, and lung cancers. It was proposed that the presence of CA II in the endothelium could contribute to the generation of an autoantibody response that would in turn be a desired outcome of immune therapy for cancer. The original finding raised the question of whether CA II expression is induced in other cancers, as well. We investigated here the expression of CA II in two major types of brain cancer: astrocytomas and oligodendroglias. A high-grade astrocytoma is typically a highly neovascularized tumor that confers a dismal prognosis with a median survival of only 1–2 years, while the other common adult CNS tumors (i.e., low-grade astrocytomas and oligodendroglias) carry a less ominous, yet still poor, prognosis.

According to our findings, endothelial CA II expression seems to be a common phenomenon among high-grade diffusely infiltrating astrocytomas and is also present to some extent in high-grade mixed oligoastrocytomas and less so in pure oligodendroglias. This finding suggests that the astrocytic component within the tumor associates with higher CA II expression in the endothelium. It would be of interest to investigate possible paracrine or other factors that potentially induce CA II expression in the tumor endothelium. Increased endothelial CA II expression was associated with a higher malignancy grade in both tumor types, and, consequently, endothelial CA II expression was also found to correlate with poor prognosis in diffuse astrocytomas.

The present results indicate an association between endothelial CA II expression and EGFR amplification. There have been suggestions that some CA isozymes may be regulated by similar oncogenic pathways to EGFR. Based on our finding, this could also be the case with CA II expression in malignant brain tumors. The EGFR gene is amplified in more than one-third of glioblastomas and in a few anaplastic astrocytomas, but not in grade 2 astrocytomas. We did not find any correlation between endothelial CA II and p53 protein expression, possibly because all grades of diffuse astrocytomas contain p53 mutations, which is an early event in a major subset of diffuse astrocytomas. It was of interest that
CA II immunoreactivity did not correlate significantly with cell proliferation determined by MIB-1 immunostaining although both showed the highest signals in the grade 4 astrocytomas. Even though this finding cannot be explained at the present stage of the studies, it is notable that CA II has never been reported to associate with cell proliferation. The recent study by Yoshiura et al. suggested that endothelial CA II could be a potential target antigen for dendritic cell therapy. Since all solid brain tumors show high neovascularization, this kind of therapy might be beneficial in patients suffering from glial tumors. Significantly, this strategy has already been used successfully in the treatment of diffuse astrocytomas, although the investigators did not report the antigen(s) responsible for dendritic cell activation. Based on the present findings, it is reasonable to hypothesize that CA II could represent a promising candidate target of dendritic cell therapy for astrocytoma, as was the case for melanoma.

Yoshiura et al. also proposed that anti–CA II antibody status could be a useful marker for monitoring the clinical response to dendritic cell therapy. If CA II were to represent a target for therapy in malignant brain tumors as well, immunohistochemical analysis of the tumor specimen could be used as a guide in assessing the appropriateness of the treatment. Endothelial CA II staining could also be used to help in the evaluation of neovascularization in the histological diagnosis of astrocytic tumors.

The presence of at least two CA isozymes—CA II and CA IX—in diffuse astrocytomas adds another option for therapeutic applications; that is, via inhibition of CA enzymatic activity. Recent drug developments have produced a number of compounds with efficient CA inhibition profiles, and it has been shown that some of these can markedly reduce the invasive capacity of cancer cells and may also disturb neoangiogenesis, thus reducing tumor growth. The present findings suggest that it could be worth testing the effect of some potent and well-tolerated CA inhibitors such as acetazolamide and topiramate on brain tumor angiogenesis. Because of the extremely high mortality rate associated with glial tumors, urgent efforts should be made to discover novel treatment strategies that might prolong the survival time of patients.

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References