Low-Molecular Weight Caldesmon as a Potential Serum Marker for Glioma

Ping-Pin Zheng, Wim C. Hop, Peter A. E. Sillevis Smitt, Martin J. van den Bent, Cees J. J. Avezaat, Theodorus M. Luider, and Johan M. Kros

Abstract

Purpose: Testing the feasibility of using the low-molecular weight caldesmon (l-CaD) level as a serum marker for the presence of glioma.

Experimental Design: Within a total of 230 serum samples, the l-CaD level was measured in healthy volunteers (30), patients with gliomas (57), non-glial intracranial tumors (107), and non-neurologic diseases (36) by ELISA. The specificity of the assay was monitored by combination of immunoprecipitation and immunoblotting.

Results: The serum level of l-CaD is significantly higher in the group of glioma patients as compared with any of the other groups (P < 0.001). The cutoff value of 45 yields optimal sensitivity and specificity of the assay (91% and 84%, respectively; area under the curve score = 0.91). The specificity of ELISA was confirmed by the immunoprecipitation/immunoblotting control experiments. There were no significant differences in serum l-CaD levels between patients with low- or high-grade gliomas.

Conclusions: The serum l-CaD level as determined by ELISA is a good discriminator between glioma patients versus patients with other intracranial tumors, other neurologic diseases, and healthy people. Prospective studies are required to test the contribution of the assay in making the diagnosis of glioma, or its feasibility for monitoring the tumor during treatment.

Over the last decade, significant progress has been made in unraveling genetic pathways underlying the large variety of glial neoplasms. In addition to histologic variables for biological aggressiveness of gliomas, lineage-specific genetic markers have been identified which by now play a role in therapeutic decision-making (1, 2). However, for genetic typing, grading, and genetic profiling, tumor tissue samples from a craniotomy or brain biopsy are required, making its clinical usefulness in patient surveillance both cumbersome and difficult. Currently, neuroradiology is still the sole method for the surveillance of glioma progression or regression as response to therapeutic intervention. A drawback of such neuroradiological evaluation is the limited ability to delineate the tumor size and there is substantial subjectivity in the assessment of the images, particularly when only minor changes in tumor sizes have occurred. A serum marker of glioma would be easier to collect, quantify, reproduce, and use in clinical practice as alternative means of measuring response to treatment or making diagnosis.

Thus far, there is no glioma marker established for guiding clinicians in making the diagnosis, or aiding neurooncologists in monitoring the progress of glioma or measuring the effects of irradiation or chemotherapy.

Low-molecular weight caldesmon (l-CaD) has not previously been mentioned as a potential marker for glioma. Caldesmon is a major actomyosin-binding protein distributed in smooth muscle cells [high-molecular-weight isoforms (h-CaD), 120-150 kDa] and non-muscle cells (l-CaD, 70-80 kDa; ref. 6). There are at least four protein isoforms of the l-CaD class generated by alternative splicing (HeLa and WI-38 types; refs. 7, 8). The conserved regions of all isoforms of l-CaD contain their ability of binding to actin, tropomyosin, Ca(2+)-calmodulin, myosin, and phospholipids (9). The l-CaD protein is crucially involved in the assembly and stabilization of the microfilament network in non-muscle cells and therefore, l-CaD is an important regulator of various cell functions, including cell motility (8, 10–12). In previous studies, we identified l-CaD in the cerebrospinal fluid of glioma patients but not in normal controls by using two-dimensional PAGE, followed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry analysis, and by specific immunohistochemical staining of tissue slides of the corresponding tumors. We showed that expression of this protein is restricted to vessels of the glioma microvasculature (13). Gliomas are the most highly
vascularized tumor type in humans, and therefore, serve as an excellent model to elucidate the process of tumor neo-
vascularization. Following RT-PCR and immunoblotting to microdissected blood vessels from gliomas and normal brain tissue, we discovered that the differential expression of this protein is mainly a sequel of abnormal splicing of the human caldesmon gene (CALD1) in glioma vasculature, which functionally result in up-regulation of the l-CaD whole class (14). It was further found that overexpression of this protein class in glioma vasculature is connected to the activation of endothelial cell motility (15), an essential step for neovascularization-dependent glioma progression. Because l-CaD was differentially present in the cerebrospinal fluid samples of the glioma patients investigated, we have tested the feasibility of using l-CaD as a serum marker for glioma.

Patients and Methods

Serum samples and tissue materials. Blood samples were collected from patients admitted to the Departments of Neurosurgery and Neurology of the Erasmus Medical Center, Rotterdam. Four different groups of individuals were included in this study. The first group (group A) consisted of 57 patients with gliomas (15 low-grade gliomas and 42 anaplastic gliomas). The second group (group B) consisted of 107 patients with nonglial intracranial tumors. The third group (group C) consisted of 36 patients with variety of neurologic diseases without tumors. Within this group, 15 patients who had suffered from stroke were included. Blood samples from 30 healthy people (group D) were obtained from the Sanguin Blood Bank, Rotterdam, the Netherlands. Informed consent was obtained from all patients and healthy controls prior to study. The use of human blood samples was approved by the Institutional Review Board of the Erasmus Medical Center. The blood samples of the tumor patients were all taken prior to surgery or any other treatment modalities. The blood samples were allowed to clot at room temperature for 30 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatant was aliquoted and stored at −80°C until further analysis. The pathologic diagnosis of the tumors was made by microscopic examination of the biopsy or resection tissues (Table 1).

Low-molecular weight caldesmon ELISA. In brief, ELISA plates (Nunc Maxisorp Bioscience, Inc., Naperville, IL) were coated overnight at 4°C with 100 µL per well of the diluted sera (4-fold dilution) by coating buffer [0.1 mol/L carbonate/bicarbonate buffer (pH 9.6)]. Following blocking with 1% bovine serum albumin in PBS + 0.05% Tween (PBST) for 1 hour at room temperature, three washes were done in PBST (5 minutes each) at room temperature. Plates were incubated with 100 µL per well of the mouse anti-l-CaD (Sigma-Aldrich, St. Louis, MO) at 1:250 dilution for 2 hours at room temperature and washed thrice. The plates were further incubated with AP-conjugated swine anti-mouse IgG (Dako, Glostrup, Denmark) at 1:1,000 dilution for 2 hours at room temperature, followed by three washes in PBST. The wells were developed using p-nitrophenyl phosphate substrate (Sigma-Aldrich) at room temperature for 20 minutes and the reaction was stopped by 3 mol/L NaOH as the manufacturer indicated. The yellow reaction product was read at 405 nm on a microtiter plate reader (Model 450 microplate reader, Bio-Rad, Hercules, CA). Each serum was tested in duplicate. A positive control (PC) from a glioma patient and a negative control (NC) from health subject verified by immunoprecipitation/immunoblotting were the same on each plate. The index value was defined by the following formula (16): index value = ([ODsample – ODNC] / [ODPC – ODNC]) × 100. The negative controls revealed the nonspecific background noise of the system, which was subtracted from all values on the plate.

The precision of the assay was assessed using five serum samples. Each sample was tested in 10 different wells in the same assay plate and the coefficient of variation of 10 duplicates was used to determine intra-assay precision. Furthermore, each sample was subjected to five independent assays using five different plates to assess inter-assay precision. The intra-assay coefficient of variation and the inter-assay coefficient of variation were 6.66% and 10.86%, respectively.

Immunoprecipitation/immunoblotting of low-molecular weight caldesmon in human sera. To investigate the specificity of ELISA, all glioma cases (57), all the cases from groups C and D (66) and 40 cases from group B were subjected to combined immunoprecipitation/immunoblotting. A pair of monoclonal anti-l-CaD antibodies recognizing two separate epitopes on the l-CaD molecule were used for immunoprecipitation followed by immunoblotting detection, respectively. Human serum samples (2 mL for each patient) were incubated with monoclonal anti-l-CaD (2 µg/mL; Clone 8, BD Biosciences, San Jose, CA) for 1 hour on ice, followed by adding 10% rec-Protein A-Sepharose 4B Conjugate (Zymed Laboratories, Inc., South San Francisco, CA) for 1 hour on ice, followed by adding 10% rec-Protein A-Sepharose 4B Conjugate (Zymed Laboratories, Inc., South San Francisco, CA) in the lysis buffer containing 0.5% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris-HCl and cocktail protease inhibitors. The samples were incubated for 1 hour at 4°C on a rocking platform. The beads were precipitated by centrifugation at 10,000 × g for 15 seconds at 4°C. The supernatant was removed by gentle aspiration. The immune complexes on the beads were washed thrice with the lysis buffer. After a final wash, 50 µL of 1× SDS gel-loading buffer was added followed by denaturing the proteins on the beads at boiling for 5 minutes, centrifuging at 10,000 × g for 15 seconds at 4°C and loading the supernatant on SDS-PAGE. The gel was blotted onto a nitrocellulose

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### Table 1. Tumor diagnosis and l-CaD serum level values

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>ELISA values</th>
<th>Value &gt; 45 ( % )</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Glioma</td>
<td>57</td>
<td>0-440</td>
<td>120</td>
</tr>
<tr>
<td>Low-grade</td>
<td>15</td>
<td>0-285</td>
<td>100</td>
</tr>
<tr>
<td>High-grade</td>
<td>42</td>
<td>0-440</td>
<td>123</td>
</tr>
<tr>
<td>Nonglial intracranial tumors</td>
<td>107</td>
<td>0-173</td>
<td>0</td>
</tr>
<tr>
<td>Meningioma</td>
<td>46</td>
<td>0-173</td>
<td>0</td>
</tr>
<tr>
<td>Metastasis</td>
<td>14</td>
<td>0-150</td>
<td>15</td>
</tr>
<tr>
<td>Schwannoma(CPA)</td>
<td>12</td>
<td>0-132</td>
<td>0</td>
</tr>
<tr>
<td>Pituitary adenoma</td>
<td>23</td>
<td>0-100</td>
<td>0</td>
</tr>
<tr>
<td>Other nonglial intracranial tumors</td>
<td>12</td>
<td>0-54</td>
<td>0</td>
</tr>
<tr>
<td>Nontumor neurologic disease</td>
<td>36</td>
<td>0-40</td>
<td>0</td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>30</td>
<td>0-53</td>
<td>0</td>
</tr>
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</table>
membrane followed by incubating with monoclonal anti-l-CaD at 1:1,000 dilution (clone C21, Sigma-Aldrich). The blot was incubated with horseradish peroxidase-conjugated anti-mouse IgG at 1:5,000 dilution (Dako). The target protein band was visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). The criteria for evaluation of immunoprecipitation/immunoblotting data were defined as: definite band (+), smear (±), no band (−). The target protein band is located at 70 to 80 kDa.

**Statistical analysis.** Nonparametric tests (Kruskall-Wallis test or Mann-Whitney test) were used to compare median levels between groups. Comparisons of the fraction of cases with 0 values was done using Fisher’s Exact test. Two-sided P values < 0.05 were considered to be statistically significant. Sensitivities and specificities were calculated for all cutoff levels of the serum l-CaD values and graphically displayed in receiver-operating characteristic curves.

**Results**

**Significantly elevated low-molecular weight caldesmon levels in sera from glioma patients.** In Table 1, the ranges, mean, and median values of the serum l-CaD values of the 57 patients with glioma and the three other groups B, C, and D (i.e., other intracranial tumors, neurologic disease without tumors, and healthy individuals) are listed. The distribution of the serum values for each group is shown in Fig. 1. Only 7% of sera from glioma patients had a l-CaD value of 0, whereas these percentages were 59%, 72% and 53% for groups B, C, and D, respectively. The median serum l-CaD values for the low- and high-grade gliomas were 100 and 123, respectively. The median values were 0 in the subgroup of meningiomas, schwannomas, pituitary adenomas, and 15 in the group of 14 metastases. The differences of percentages of 0 values between the four groups was highly significant (P < 0.001; χ² test). The comparisons of these percentages of the glioma group versus each of the other groups yielded highly significant differences (P < 0.001; Fisher’s exact test). Pairwise testing of the percentages of 0 values between the other three groups B, C, and D yielded nonsignificant P values for each comparison (P = 0.170, 0.677, and 0.131, respectively; Fisher’s Exact test). The results prompted us to consider these three groups as one control group. Because one may speculate that the serum l-CaD values of stroke patients is elevated because of effects of reactive neovascularization following infarction, the group of 15 stroke patients within group C was separately investigated. The values of serum l-CaD of the stroke patients were well within the range of group C, the highest value being 31 and in 13 of 15 patients, the value was 0. There was no statistical difference between the serum l-CaD value of the stroke patients and the other patients with neurologic diseases without tumor (P = 0.45). The difference between the median values of the l-CaD serum values of the group of glioma patients versus this combined control group (n = 173) was highly significant (P < 0.001). Within the glioma group, there were 15 patients with low-grade tumors and 42 patients with high-grade tumors. The serum l-CaD values of the 15 patients with low-grade glioma ranged from 0 to 285 with a mean of 124 and a median of 100. The serum values of the 42 patients with high-grade gliomas ranged from 0 to 440 with a mean of 140 and a median of 123. There was no statistically significant difference in median serum l-CaD values between the two groups of different glioma malignancy grade (P = 0.329).

**Determination of cutoff values.** A receiver-operating characteristic analysis was done to determine the cutoff value for differentiating between patients with or without a glioma. The serum l-CaD value which gave the highest sum of sensitivity (%) and specificity (%) was defined as the cutoff value. The serum l-CaD value as determined by ELISA has a very good discriminative power for identifying patients with gliomas as shown by the large area under the curve (0.91) of the receiver-operating characteristic curve (Fig. 2). For a cutoff score of 45, the sensitivity and specificity of l-CaD are 91% and 84%, respectively. For comparison, the l-CaD cutoff value of 40 yields a sensitivity of 91% and specificity of 82%, and a cutoff value of 50 yields a sensitivity of 88% and a specificity of 85%.

**Immunoprecipitation/immunoblotting of low-molecular weight caldesmon in human sera.** To determine the specificity of ELISA, combination of immunoprecipitation/immunoblotting was done in a large portion of the serum samples (Table 2). Representative cases are shown in Fig. 3. The largest discrepancy is found in the group of healthy individuals. No l-CaD was detected in 93% of sera of the group. For the other groups, the results of the two techniques showed a good concordance. The immunoprecipitation/immunoblotting results were in agreement with the ELISA measurement of l-CaD in the sera, thus demonstrating the specificity of ELISA.

**Discussion**

We tested whether the serum value of l-CaD can serve as a serum marker for glioma and the results indicate that patients with biopsy-proven gliomas have significantly increased l-CaD serum levels as compared with the control group. For a cutoff score of 45, sensitivity of 91% and specificity of 82% was achieved. The serum l-CaD level was 0 in only 7% of the
glioma patients, as opposed to 59% in patients with other intracranial tumors; 72% in patients with neurologic disease without tumors; and 53% in the group of healthy people. l-CaD was detectable in 44 out of 107 (41%) of serum samples of patients with nonglial intracranial tumors and in this group only 22 out of 107 (21%) of the values were over the index value of 45. The mean serum l-CaD value in the group of patients with neurologic disease without tumor was 5.5 with a median of 0, and of the sera of the healthy people 13.9 and 0, respectively. Therefore, l-CaD is a good candidate serum marker for glioma. We now plan to investigate the contribution of serum l-CaD in making the diagnosis of glioma in prospective studies. For instance, the neuroradiological presentation of a solitary metastasis may be indistinguishable from that of glioma. The median value of serum l-CaD of the 14 patients with metastases included in the present study was only 15 with a mean value of 48.8, whereas that of glioma was 120 with a mean of 129. Therefore, measuring the serum l-CaD level may well become a valuable addition for differentiating gliomas from metastases in patients with ambiguous neuroradiology.

Table 2. Results of immunoprecipitation/immunoblotting

<table>
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<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Immunoprecipitation/immunoblotting results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioma</td>
<td>57</td>
<td>+  51 3 3</td>
</tr>
<tr>
<td>Nonglial intracranial tumors</td>
<td>40</td>
<td>+ 11 8 21</td>
</tr>
<tr>
<td>Nontumor neurologic diseases</td>
<td>36</td>
<td>+ 0 5 31</td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>30</td>
<td>+ 0 2 28</td>
</tr>
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</table>

The source of l-CaD in the serum of glioma patients is from the tumor vasculature, possibly by shedding or secreting of this protein into the bloodstream, or from circulating endothelial precursor cells released from the bone marrow which contribute to neoplastic vasulogenese. Furthermore, there is a scavenging mechanism for balancing the titer of cytoskeletal proteins in serum (17). However, once a protein exceeds the protective scavenging capacity, its level in serum will increase. An explanation for the high l-CaD serum levels in patients with gliomas is that these tumors are among the highest vascularized neoplasms in man, with all different modalities of neoplastic vascularization, in which l-CaD may play its specific role, is encountered (18). In the serum of some patients with nonglial tumors, such as carcinomas of different types, we also found a relatively high l-CaD expression. It may very well be that these tumors were more richly vascularized as compared with others of the same kind—the abnormal l-CaD expression is found in a large variety of cancers. Nevertheless, the serum levels of the majority of the patients with these tumors seemed to be low.

To date, classification of low- and high-grade glioma is still largely based on histology (19). The presence of microvascular proliferation in a biopsy specimen of diffuse glioma prompts to make the histopathologic diagnosis of anaplastic glioma. Because l-CaD is overexpressed in the neoplastic microvasculature, one would expect a higher l-CaD level in the serum of patients with high-grade (anaplastic) gliomas. This difference, however, was not found. There may be several reasons for this: processes of vasculogenesis, sprouting angiogenesis, intussusceptive angiogenesis and cooption of host vessels contribute to neovascularization of glioma. Vasculogenesis and sprouting angiogenesis occur in early stages of glioma neovascularization. Sprouting angiogenesis is discernable in routine histology, as is endothelial proliferation in existing vessels, whereas vasculogenesis, mirrored by the individually dispersed angioblasts/endothelial cell progenitors or noncannelized endothelial chains, is hardly noticeable in routine stains. Furthermore, in more advanced stages of neovascularization, intussusceptive angiogenesis becomes more prominent (20). One important characteristic of intussusceptive angiogenesis is that it develops at an exceedingly low rate of endothelial cell proliferation (21). In a previous study, we observed that l-CaD was predominantly expressed in early stages of glioma neovascularization and significantly tapers off in intussusceptive angiogenesis as observed in glioblastoma (15).

In summary, the study renders clinical support for the value of l-CaD in the prediction of glioma existence, and allows for the indirect assessment of neo-angiogenic activity of gliomas. Whether the l-CaD serum value will prove itself as a marker which will be applied in clinical practice remains to be established in prospective studies.
References


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