# Autoantibodies to the inhibitor of apoptosis protein survivin in patients with brain tumors

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**Abstract.** Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is frequently expressed in cancers, including meningiomas and gliomas. Survivin may be associated with tumor progression and poor prognosis of patients with brain tumors. Using ELISA and immunoblot analysis we asked whether survivin is capable of eliciting a humoral immune response in patients with meningiomas and gliomas. Survivin-specific antibodies were detected in 5 of 42 (11.9%) patients with meningiomas and 3 of 35 (8.6%) patients with malignant gliomas of the WHO grades 3 and 4, but not in healthy controls. Tumors of patients with detectable anti-survivin antibodies demonstrated survivin expression in at least 20% of the tumor cells as assessed by immunohistochemistry. We conclude that patients with meningiomas and malignant gliomas can mount a high-titer IgG immune response against the 'universal' tumor-associated antigen survivin. Anti-survivin antibodies may represent attractive tools for diagnosis and follow-up of brain tumors.

## Introduction

Meningiomas arise from the meninges of the brain and spine and represent the most common benign intracranial tumors with an annual incidence of 6 per 100,000 (1,2). The incidence is the highest in the 5th to 8th decades with a peak at age 60 years. The prevalence of meningiomas in females is twice that of men. Meningiomas generally grow slowly and around 90% of these tumors are classified as benign, World Health Organization (WHO) grade 1. Around one fifth of

meningiomas show aggressive behavior associated with an increased risk of recurrence and a poorer prognosis (3). Standard treatment of meningiomas is surgical resection, but radiotherapy may be used in cases of recurrent atypical and malignant tumors. Long-term prognosis of treated patients is good, although recurrence rates of 20% at 10 years have been seen even after excision of the tumor, surrounding dura, and affected bone (4).

Gliomas are the most frequent intrinsic brain tumors in adults and are characterized by diffuse and invasive growth. They typically arise in the lobar white matter or in the deep grey matter of the brain (5). Malignant gliomas are tumors of the histological WHO grades 3 and 4, which consist of highly proliferative and migratory tumor cells (6-8). Median survival time for patients with malignant gliomas WHO grade 3 is about 18 months and 12 months for patients with WHO grade 4 tumors (5,9). Radical surgical resection alone is unable to cure high grade gliomas, and adjuvant treatments such as fractionated radiation and chemotherapy are currently considered standard of care (10,11).

Inhibition of apoptosis is a key process in oncogenesis. The 16.5 kDa protein survivin is a member of the inhibitor of apoptosis (IAP) protein family, which is known to inhibit the proteases caspase-3, -7, and -9, but not caspase-8. Four alternative survivin splice variants have been described, but their relevance for oncogenesis is not yet clear (12,13). Survivin blocks death-receptor- as well as mitochondria-mediated apoptosis signal cascades and is involved in control of cell cycling during mitosis. It is expressed in the G2/M phase of the cell cycle and was found to interact with the mitotic spindle during cell division. Upregulation of survivin is thought to overcome intracellular surveillance mechanisms at certain 'checkpoints', thereby allowing for extended viability of transformed or otherwise damaged cells and promoting resistance to anticancer drugs and irradiation (14).

Survivin is upregulated in fetal tissues and various human cancers such as breast, lung, colon, ovarian, or prostate cancer, but is almost undetectable in normal differentiated tissues (14). It may thus represent a promising 'universal' target for anti-tumor vaccines. According to an extensive analysis of different cancer transcriptomes in humans, including brain

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tumor transcriptomes, survivin was identified as the fourth most abundant tumor-specific transcript (15). Survivin expression is associated with poor prognosis in some cancers such as lung (16), breast (17), and colorectal (18) cancer.

The majority of meningiomas stain intensely for survivin. Staining is restricted to the cytoplasm and no correlation with clinicopathological features such as recurrence could be demonstrated (19-21). Survivin is also abundantly expressed in gliomas but its prognostic value for glioma patients is discussed controversially (22-28).

Proteins involved in oncogenesis or cell cycle dysregulation, e.g. p53 (29), HER-2/neu (30), or cyclin B1 (31) are known to induce an IgG immune response in cancer patients. Survivin has been shown to elicit humoral as well as cellular immune responses in patients with a variety of cancers such as lung, colorectal, breast, and gastric cancer (32-36). Monitoring these immune responses in tumor patients over time may be a valuable tool for tumor diagnosis and follow-up studies. In this study, we analyze the humoral immuno-reactivity to survivin in sera of patients with benign and malignant brain tumors.

**Materials and methods**

*Patients, tumor tissue and sera*. This study was approved by the Ethics Committee of the Faculty of Medicine, Martin Luther University Halle-Wittenberg. Forty-three patients with meningioma and 35 patients with malignant glioma of the WHO grades 3 and 4 were enrolled in the study. Of the 43 meningioma patients, 36 were diagnosed with WHO grade 1 tumors, 3 with WHO grade 2 tumors, and 4 did not undergo surgery at the time of blood sampling. Median age of all meningioma patients was 61 years (range 32-81 years). Thirty-six patients had primary tumors and 7 presented with recurrent disease. Of the 35 malignant gliomas, 10 were WHO grade 3 and 25 WHO grade 4, including 4 recurrent GBM. Median age of all glioma patients was 61 years (range 30-78 years). Sera from 41 meningioma patients and 35 glioma patients were subjected to survivin ELISA and survivin immunoblot in parallel, while 2 sera from meningioma patients were only tested by ELISA or immunoblot, respectively. Paraffin embedded tumor specimens of 37 meningioma and 26 glioma patients were available for survivin immunohisto-chemistry. Patient sera were compared to sera from healthy controls (n=300 for ELISA and n=56 for immunoblot).

*Purification of recombinant survivin*. Recombinant survivin was expressed in *Escherichia coli* (*E. coli*) using the histidine-tag vector pQE30 (Qiagen, Hilden, Germany). Bacterial cleared lysates were passed over a nickel-nitrilotriacetic acid column and survivin was eluted under denaturing conditions according to the manufacturer's protocol (Qiagen). Purity of eluted protein was determined by SDS-PAGE and Coomassie Blue staining.

*Immunohistochemistry*. Survivin immunohistochemistry was carried out on formalin-fixed, deparaffinized tissue sections (4 $\mu$m) with an affinity-purified polyclonal rabbit anti-survivin antibody (AF 886; R&D, Wiesbaden, Germany or NB 500-201, Acris, Hiddenhausen, Germany). Antigen was retrieved by microwaving in 10 mmol/l citrate buffer. Slides were incubated with primary antibody in a humidified chamber for 1-2 h at 37°C. Bound antibody was detected using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbozole (Dako Cytomation, Ely, UK) as substrate. Negative controls included substitution for the primary antibody with rabbit polyclonal pre-immune serum. Tissue sections were grouped according to percentage of survivin expressing tumor cells as follows: 0, negative; 1, 1-10%; 2, 11-25%; 3, 26-50%; 4, 51-75%; 5, 76-90%; and 6, 91-100% positive.

*ELISA*. The test was essentially performed as described previously (32). Purified recombinant survivin as well as a His-tagged tyrosinase fragment used as control antigen were refolded and diluted in 50 mM sodium bicarbonate buffer. Microtiter plates were coated with 100 $\mu$l of protein solution (5 $\mu$g/ml) and incubated overnight at 4°C. After blocking the wells with 5% skimmed milk in PBS, 100 $\mu$l of serum diluted 1:100 was added to the wells for 1 h. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-human IgG F(ab')$_2$ and the substrate tetramethylbenzidine (Sigma-Aldrich, Steinheim, Germany). After addition of $H_2SO_4$ stop solution absorbance was read at 460 nm. All serum samples were run in duplicate. A positive reaction was defined as an optical density (OD) value of a 1:100 diluted serum that exceeded the mean OD value of sera from healthy controls (n=300) by >3 standard deviations (SD).

*Immunoblot*. Purified survivin was diluted in Laemmli buffer, heated to 95°C for 5 min, separated by SDS-PAGE (5 $\mu$g of protein/lane) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skimmed milk in TBS and incubated overnight at 4°C with sera from patients and healthy controls diluted 1:100. Bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-human IgG F(ab')$_2$ (Dako Cytomation) diluted 1:10,000 and the chemiluminescent substrate West Pico (Pierce, Rockford, IL). Positively reacting sera were also tested at a dilution of 1:1,000 and 1:10,000. The polyclonal rabbit anti-survivin antibody AF 886 (R&D) was used as a positive control. All experiments were carried out at least twice.

*Preabsorption of sera with survivin*. Sera were diluted 1:10-1:100 and incubated with soluble survivin (0.3-1.3 $\mu$g/$\mu$l) in a volume of 200 $\mu$l for 7 h at 4°C. Preabsorbed sera samples were then further diluted and processed as described in the previous section.

*Statistical analysis*. Statistical evaluation was performed using the SPSS software, version 12.0 (SPSS Inc., Chicago, IL). Bivariate correlation of different variables was assessed using the Pearson two-sided $\chi^2$ test. P<0.05 was considered statistically significant.

**Results**

Immunoblotting against purified recombinant survivin detected survivin-specific antibodies in 5 of 42 (11.9%) sera from

Table I. The patients with a positive humoral immune response against survivin.

| No. | Diagnosis | Sex | Age (years) | Survivin positive tumor cells (%)[a] | Maximum serum titer with survivin-positive immune reaction |
|---|---|---|---|---|---|
| 1 | Fibroblastic meningioma WHO G1 | Female | 64 | 80-90 | 1:100 |
| 2 | Meningothelial meningioma WHO G1 | Male | 55 | 51-75 | 1:100 |
| 3 | Psammomatous meningioma WHO G1 | Female | 66 | 60-70 | 1:100 |
| 4 | Meningioma (unknown histology) | Male | 73 | n.a.[b] | 1:100 |
| 5 | Angiomatous meningioma WHO G1 (recurrence) | Male | 81 | 20 | 1:100 |
| 6 | Glioblastoma | Male | 43 | >90 | 1:1000 |
| 7 | Glioblastoma (recurrence) | Female | 60 | 25-30 | 1:100 |
| 8 | Glioma WHO G3 | Female | 74 | n.a.[b] | 1:100 |

[a]Survivin immunohistochemistry was performed in the corresponding tumors resected at the time of blood sampling. [b]n.a., not available.
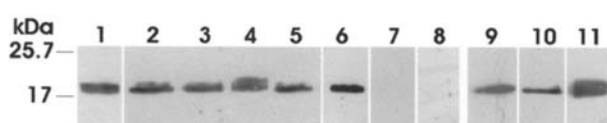


Figure 1. Immunoblotting of 1:100 diluted sera of meningioma and glioma patients against recombinant survivin (5 µg/ lane). 1, positive control = affinity-purified polyclonal rabbit anti-survivin antibody AF 886 (R&D); 2-7, sera of 6 different meningioma patients; 8, healthy control; 9-11, sera of the 3 glioma patients immunoreactive with survivin.
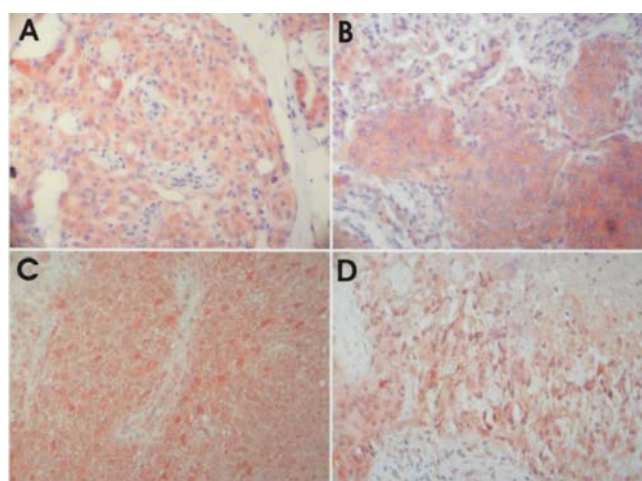


Figure 2. Immunohistochemical detection of survivin expression in meningioma and glioma (magnification x400). (A and B) Two different cases of meningothelial meningioma WHO G1; (C and D), two different cases of glioblastoma. Patients harboring the tumors displayed in (A) and (C) developed autoantibodies to survivin.

meningioma patients and in 3 of 35 (8.6%) patients with malignant glioma at 1:100 dilution, while 56 sera from healthy controls remained negative (Table I, Fig. 1). The same sera, except for one sample from a meningioma patient, were also tested in an ELISA, with which we previously had detected anti-survivin antibodies in patients with lung and colorectal cancer (32). No anti-survivin antibodies were seen in any of these samples using this technique.

When immunoblotting all 8 survivin-immunoreactive sera at a dilution of 1:1,000 against survivin, only one serum from a glioblastoma (GBM) patient still showed a positive reaction. No signal was detected with this serum when diluted further to 1:10,000. Specificity of the immune reaction was demonstrated by preabsorbing the sera with survivin before immunoblotting, which resulted in a marked decrease or a complete disappearance of the signals, respectively (data not shown).

Anti-survivin antibodies were seen in 4 of 35 (11.4%) patients with primary and 1 of 7 (14.3%) patients with recurrent meningioma. The occurrence of autoantibodies specific for survivin was not linked to a certain type of tumor histology. Four of the 5 patients showing humoral immunoreactivity to survivin underwent surgery at the time of blood sampling and their tumors were classified as meningothelial, fibroblastic, psammomatous, and angiomatous, respectively (Table I). None of the 3 patients with meningioma WHO grade 2 developed anti-survivin antibodies.

We also asked whether the occurrence of anti-survivin antibodies was associated with a certain level of survivin expression in the respective meningiomas, but found no correlation (P=0.530). Thirty-one of 37 (83.8%) meningiomas available for immunohistochemistry stained positive for survivin (Fig. 2). In 26 of 37 (70.3%) meningiomas >10% of the tumor cells expressed survivin, in 20 (54%) tumors >50% of the tumor cells were positive for survivin. Expression levels of survivin-positive tumor cells in patients demonstrating humoral survivin immunoreactivity ranged from 20-90% (Table I, Fig. 2). Patients (6 of 37, 16.2%) whose tumors stained negative for survivin did not mount a humoral immune reaction against the protein. On the other hand, 10 of 11 (90.9%) patients with 51-75% and 7 of 8 (87.5%) patients with >75% survivin positive tumor cells also did not develop anti-survivin antibodies.

Antibodies directed against survivin were also detected in 1 of 10 (10%) patients with malignant glioma WHO grade 3, 1 of 21 (4.8%) patients with primary GBM, and 1 of 4 (25%) patients with recurrent GBM (Table I, Fig. 1). Twenty-six of the corresponding tumors were available for survivin immuno-histochemistry. All tumors demonstrated survivin expression, only one malignant glioma had <10% survivin-positive tumor cells. More than 50% survivin-positive tumor cells were seen in 17 (65.3%) of the gliomas. Two tumors of the 3 patients with detectable anti-survivin antibodies could be stained for survivin (Table I, Fig. 2). Interestingly, the tumor of the GBM patient with the strongest anti-survivin antibody response demonstrated >90% survivin-positive tumor cells, while in the other tumor, a recurrent GBM, 25-30% of the tumor cells stained survivin-positive.

## Discussion

We report herein for the first time that survivin can elicit a tumor-restricted high-titer IgG immune response in patients with meningiomas and malignant gliomas. This was demonstrated by a sensitive immunoblotting technique using an enhanced chemiluminescence detection system and purified but denatured recombinant survivin as the antigen. Employing an ELISA, in which polysterene microtiter plates were coated with survivin, the same sera did not demonstrate immuno-reactivity. One possible explanation for this is that different survivin epitopes are presented depending on the immunological test in use. While survivin purified under denaturing conditions was at least partially refolded before coating ELISA plates with this antigen, denatured survivin was used for the immunoblot procedure. Similar findings were reported by Reymond *et al* (37): using purified verotoxin 1 as the antigen they compared immunoblot and ELISA for measuring anti-verotoxin 1 antibodies in patient sera and found that the immunoblot procedure was inherently more specific and sensitive than the ELISA, which prompted them to call the immunoblot assay 'the gold standard'. Gekeler *et al* (38) used the same glycoprotein preparation purified from *Taenia solium* metacestodes in immunoblot and ELISA for the serological diagnosis of neurocysticercosis. Immunoblotting proved to be slightly more sensitive (81.7% versus 80% for ELISA) and clearly more specific (99.4%) than the ELISA (75.3%).

The fact that anti-survivin antibodies in the tested brain tumor patients were not detected by our ELISA may also in part be ascribed to our assay procedure and the stringent cut-off (normal mean +3 SD) we chose. It should be emphasized that this cut-off value was higher than any signal seen with the 300 sera from healthy controls (32), so that positive values could be definitely counted as a tumor-restricted immune response. Thus, relatively low titers of survivin-specific antibodies in the brain tumor patients may not have been detected. Employing a multiple antigen ELISA, with which antibodies to a panel of 7 tumor-associated antigens (including survivin) were examined, Tan and colleagues compared the two cut-off standards 'normal mean +2 SD' versus 'normal mean +3 SD' (39). They clearly demonstrated that sensitivity tends to decrease while specificity rather increases as the assay cut-offs increase (31,39).

It has been demonstrated by several groups that survivin is an immunogenic protein eliciting both humoral and cellular immune responses (31-35,39-45). Autoantibodies to survivin have been shown to occur in patients with different cancers such as lung (31,32,39,40), colorectal (31,32,39), breast (31,39,44), prostate (31,39), bladder (43), and head-and-neck (33) cancer. Depending on the ELISA in use and the chosen cut-off, antibody frequencies varied between 1.2% in patients with hepatocellular carcinoma (42), and exceptionally high 58.1% in lung cancer patients (40). Notably, high antibody frequencies such as 55.8% in bladder cancer patients (43) or 58.1% in lung cancer patients (40) were only found when a cut-off of normal mean +2 SD was chosen and the number of healthy controls used to define the normal mean was rather low [n=7 (40) and n=18 (43), respectively]. Using a cut-off of normal mean +3 SD and including sera from 346 healthy controls Zhang *et al* (31) reported antibody frequencies of 2.9%, 4.4%, 7.8%, 9.9%, 10.7% and 12.5% for patients with prostate, colorectal, breast, gastric, and lung cancer and hepatocellular carcinoma, respectively.

Our results regarding the prevalence of anti-survivin antibodies in meningioma and glioma patients (11.9% and 8.6%, respectively) are in line with these reports. Interestingly, although we demonstrate the presence of anti-survivin IgGs in sera from patients with meningioma and malignant glioma and although survivin is known to be abundantly expressed in these tumors (19-28,45,46) extensive immunoscreening of meningioma and glioma cDNA libraries expressed in *E. coli* with sera from meningioma or glioma patients (47-50) never detected survivin as a tumor-associated antigen reacting with human IgGs. Although this immunoscreening method also relies on immunoblotting, it is conceivable that the antigens expressed by phage-infected *E. coli* still retain some conformation-dependent epitopes as compared to the purified and denatured survivin loaded on a polyacrylamide gel after heating to 95°C. Alternatively, the amount of survivin protein expressed by a single *E. coli* plaque might be too low to be detected by patient sera as compared to the 5 $\mu$g of purified survivin per lane loaded on to the gel.

Overexpression of a wild-type protein rather than expression of a mutated protein is thought to be one of the main reasons for induction of a humoral immune response in cancer patients (51). We found that all meningioma and glioma patients with detectable anti-survivin antibodies had tumors that expressed survivin above a 'threshold level' of ~20% survivin-positive tumor cells. On the other hand, most meningioma and glioma patients with survivin-positive tumors did not mount a survivin-specific immune response. These findings indicate that increased antigenic load may be a prerequisite for induction of an immune response, but is certainly not the main factor responsible for B cell stimulation and consecutive antibody production. The fact that anti-survivin antibodies were not found in brain tumor patients with an ELISA with which we previously had detected such antibodies in 8.2% of patients with colorectal cancer and 21.6% of patients with lung cancer (32) most likely reflects the known decrease in serum IgG levels seen in patients with benign and malignant brain tumors, which seems to be due to lack of B cell stimulation by CD4^{+} T cells (52). Of note, we also did not detect anti-p53 antibodies in sera from 70

patients with malignant glioma by ELISA in an earlier study (53), while high frequencies of these antibodies have been reported for patients with other malignancies (29).

The prevalence of anti-survivin antibodies in brain tumor patients is in line with our previous findings on the presence of autoantibodies to the tumor-associated nuclear antigen minichromosome maintenance protein 3 (MCM3) in patients with malignant glioma (50): in this study, 7 of 74 (9.5%) patients had anti-MCM3 antibodies. However, the biological and prognostic relevance of anti-survivin antibodies in patients with brain tumors can only be assessed after analysis of further cases. Using a multi-antigen panel including several tumor-associated antigens such as survivin and MCM3 may increase the sensitivity to detect tumor-related autoantibodies and thus enhance their diagnostic value (31).

In conclusion, we show herein that patients with meningiomas and malignant gliomas can mount a humoral immune response to the 'universal' tumor-associated antigen survivin. These antibodies may represent attractive novel markers for the diagnosis and follow-up of brain tumors.

## Acknowledgements

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