Survivin Expression in Glioblastomas Correlates with Proliferation, but not with Apoptosis

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Abstract. *Background: Survivin is expressed in proliferating tissues and in tumors. It is a member of the inhibitory apoptosis protein (IAP) family known to regulate mitosis and to inhibit apoptosis. It has therefore been regarded as a target for therapies. In malignant gliomas it increases with malignancy, even though in glioblastomas it does not seem to correlate with outcome. Materials and Methods: Survivin was immunohistochemically studied in 39 selected viable glioblastoma areas belonging to 20 cases which were assayed for apoptosis, using a TUNEL assay, caspase-3, poly(ADP-ribose)polymerase 1 (PARP-1), Bid (BH3-interacting domain death agonist) and with the proliferation index Ki-67/MIB-1 and mitotic index (MI). Results: A positive linear correlation was found between the survivin labelling index (LI) and the Ki-67/MIB-1 LI and MI. No inverse correlation was found with apoptosis. Conclusion: This double behavior can be attributed to mechanisms mediating survivin activity, either as a mitosis regulator and apoptosis inhibitor, and should be taken into account in therapeutic strategies using survivin.*

The survivin gene is located on human chromosome 17q25.3 and encodes a 16.5 kDa protein comprising 142 amino acids. It is the smallest member of the IAP family, contains only one Baculovirus IAP repeat (BIR) domain and lacks a RING structure. Survivin is highly expressed during the G2-M phase and inhibits caspases 3 and 7 (1-4). As a chromosomal passenger protein it maintains mitosis and promotes cell proliferation (5, 6). Survivin is expressed in embryonic tissues, but not in normal adult tissues. It is highly expressed in malignancies where angiogenetic properties are also promoted (7).

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The subcellular distribution of survivin has been controversial: is it a microtubule-associated protein or a chromosomal passenger protein? It has been observed that the sequence Ala3-Ile19 identifies the nuclear pool of survivin and segregates with nucleoplasmic proteins; based upon fluorescence, it localizes to the kinetochores of metaphase chromosomes and to the central spindle midzone during anaphase. The sequence Cys57-Trp67 characterizes the cytosolic pool associated with microtubules, centrosomes, spindle poles and mitotic spindle microtubules during metaphase and anaphase. A polyclonal antibody recognizes both pools within the same mitotic cells. The predominant survivin pool is associated with microtubules and participates in the assembly of a bipolar mitotic spindle (8).

Survivin initiates cell cycle entry as a result of nuclear translocation followed by an interaction with CDK4. Its overexpression accelerates the S-phase and activates CDK2/cyclin E and Rb phosphorylation. Its translocation to the nucleus depends upon Fas stimulation and cell proliferation. The survivin/CDK4 complex releases p21, which interacts with pro-caspase 3 to suppress Fas-mediated cell death (6, 9, 10). Survivin also functions as a kinetochore passenger protein (6) and is degraded in the ubiquitin-proteasome system (11). The survivin paradigm is based on the existence of different variants of survivin (12) and on its nuclear and cytosolic pools (7). Vascular endothelial cells express survivin (13-14).

Many tumors including gliomas have been investigated for survivin because of its involvement in mitosis regulation and apoptosis. In most studies it is reported as an adverse prognostic factor because its index increases with malignancy (15-19). An increase has also been shown by Western blot analysis (18) and mRNA analysis, which, however, does not correlate with the telomerase level in glioblastomas (13). Survivin expression has been correlated with the absence of (20) or reduced capacity for apoptosis (18). In a series of glioblastomas, it was found to correlate with Ki-67/MIB-1 and the topoisomerase II index, but not with the apoptotic index, and did not influence patient outcome (19).

Any correlation study of survivin with proliferation and mitosis in glioblastomas must take into consideration two characteristics: the regional heterogeneity of proliferation markers and apoptosis, and the existence of diverse pathways to the latter. Most studies dedicated to the above-mentioned correlations had different goals and did not focus on such characteristics. Because of the regional heterogeneity, Ki-67/MIB-1 LI, calculated in histological sections either random or in selected tumor areas, may or not be prognostic. The contributions of researchers are divided on this point (21).

Since survivin has been proposed in tumors as a selective target for therapies as a member of IAP family (22, 23), we wanted to investigate, in a series of glioblastomas, whether any correlation exists between survivin LI and Ki-67/MIB-1 LI, the apoptotic index (AI) and the mitotic index (MI), trying to overcome the bias of regional heterogeneities by comparing corresponding areas in serial sections of all the cases studied.

Materials and Methods

This study was carried out in 39 areas belonging to 20 surgical samples from glioblastomas operated on in the Department of Neuroscience, University of Turin. The samples were fixed in buffered formalin, embedded in paraffin and cut in 5-μm-thick sections. Only non-necrotic areas of solid tumor were evaluated.

All the tumor sections were divided into viable areas, carefully excluding visible necrosis, of 0.075 mm^2, roughly corresponding to at least 7 high power fields (HPF) at x1000 with oil immersion, containing >1000 cells.

Immunohistochemistry (IHC). The following primary antibodies at the indicated dilutions were used for IHC: rabbit polyclonal anti-survivin (NB-500-201 K3, 1:500; Novus Biological Inc., Littleton, CO, USA), mouse monoclonal anti-Ki-67/MIB-1 (M7240, 1:100; Dako, Carpinteria, CA, USA), rabbit polyclonal anti-PARP-1 (9542, 1:200; Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-cleaved PARP-1 (9541, 1:100; Cell Signaling Technology), rabbit polyclonal anti-caspase-3 (AB3623, 1:20; Chemicon International Inc., Temecula, CA, USA) and a rabbit polyclonal anti-BID (AB1730, 1:25; Chemicon International Inc.).

On consecutive sections, besides the H&E method, the immunohistochemical reactions were carried out using a standard labeled avidin-biotin protocol (Dako). Microwave antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0 or 7.4) (3x5 min at 600 W). Negative controls were performed by omission of the primary antibody and positive controls were performed using colon adenocarcinoma, known to have a high survivin immunoreactivity, processed in the same way as glioblastoma sections.

For each antigen, a LI was calculated in each area as the percentage of positively stained cells, after counting at least 1000 cells. Mitoses were counted in the same way and a MI was calculated. In the counts, vessel cells were not considered.

Double-labelling immunofluorescence. For Ki-67/survivin double-immunofluorescence staining, the following protocol was used. Sections from all the cases were incubated overnight at 4˚C with a survivin rabbit anti-human antibody, diluted 1:500 in 1x Tris-buffered saline (TBS) pH 7.4, and a Ki-67/MIB-1 mouse anti-human antibody, diluted 1:100 in 1xTBS. An incubation was then performed for 1 h at room temperature with a goat anti-rabbit IgG conjugated with FITC (Vector Laboratories, Burlingame, CA, USA), diluted 1:200 in 1xTBS, and a rabbit anti-mouse IgG conjugated with TRITC (Sigma-Aldrich Co., St. Louis, MO, USA), diluted 1:50.

Sections were immediately mounted with Vectashield mounting medium (Vector Laboratories) containing 4,6-diamidino-2-phenylindole, dihydrocloride (DAPI) as a DNA counterstain. Controls were performed with the omission of the primary antibodies. Observation was performed on a Zeiss Axioskop fluorescence Microscope (Karl Zeiss, Oberkochen, Germany) equipped with an AxioCam5MR5c coupled to an Imaging system (AxioVision Release 4.5; Zeiss).

TUNEL method. Apoptosis was demonstrated on tissue sections by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay. The analysis was performed with the In situ Cell Death Detection fluorescent Kit (Roche Diagnostic Corporation Indianapolis, IN, USA) according to the manufacturer's instructions.

In addition, apoptosis was assessed by the caspase-3 and cleaved-PARP-1 methods as assessed before. The counts were performed by all the three methods and the figures obtained were similar.

Western-blotting analysis and quantitative evaluation. For protein extraction, paraffin sections were deparaffinized and homogenized in a lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal (Sigma-Aldrich Co.), 2% sodium dodecyl sulfate (SDS), Na deoxycholate 0.5%, 10 mM EDTA and a Protease Inhibitor Cocktail (Sigma-Aldrich Co.)].

One hundred micrograms of protein, quantified by a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA), were loaded onto a 12% sodium dodecyl sulphate-polyacrilamide (SDS-PAGE) gel and then transferred to a nitrocellulose membrane (Biorad, Hercules, CA, USA). Membranes were blocked in blocking buffer (5% BSA/0.2% Tween in 1x phosphate-buffered saline (PBS) pH 7) for 1 h at room temperature.

Blots were incubated with 1 μg/mL of (NB-500-201 K3) anti-survivin antibody in 5% BSA/0.2% Tween in 1xPBS for two hours at room temperature and incubated with the appropriate AP-conjugated secondary antibody (Dako) in 5% BSA/0.2% Tween in 1xPBS for 1 hour at room temperature. A specific anti-vimentin antibody (NeoMarkers, Fremont, CA, USA) was used for normalizing sample integrity, loading and transfer. Bands were visualized by a colorimetric method with BCIP/NBT solution (Roche Diagnostics). Image analysis and signal quantification were performed using NIH Image J (RSB, NIMH, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was carried out using Interactive Statistical Calculation Pages (http://StatPages.org). The Pearson's correlation coefficient was used to examine correlations between variables. The linear correlation coefficient was studied for each pair of antigens considered.

Results

Survivin variably stained nuclei in all viable areas; cytoplasmic staining was inconstantly and irregularly found in some areas only. The two stainings were evaluated

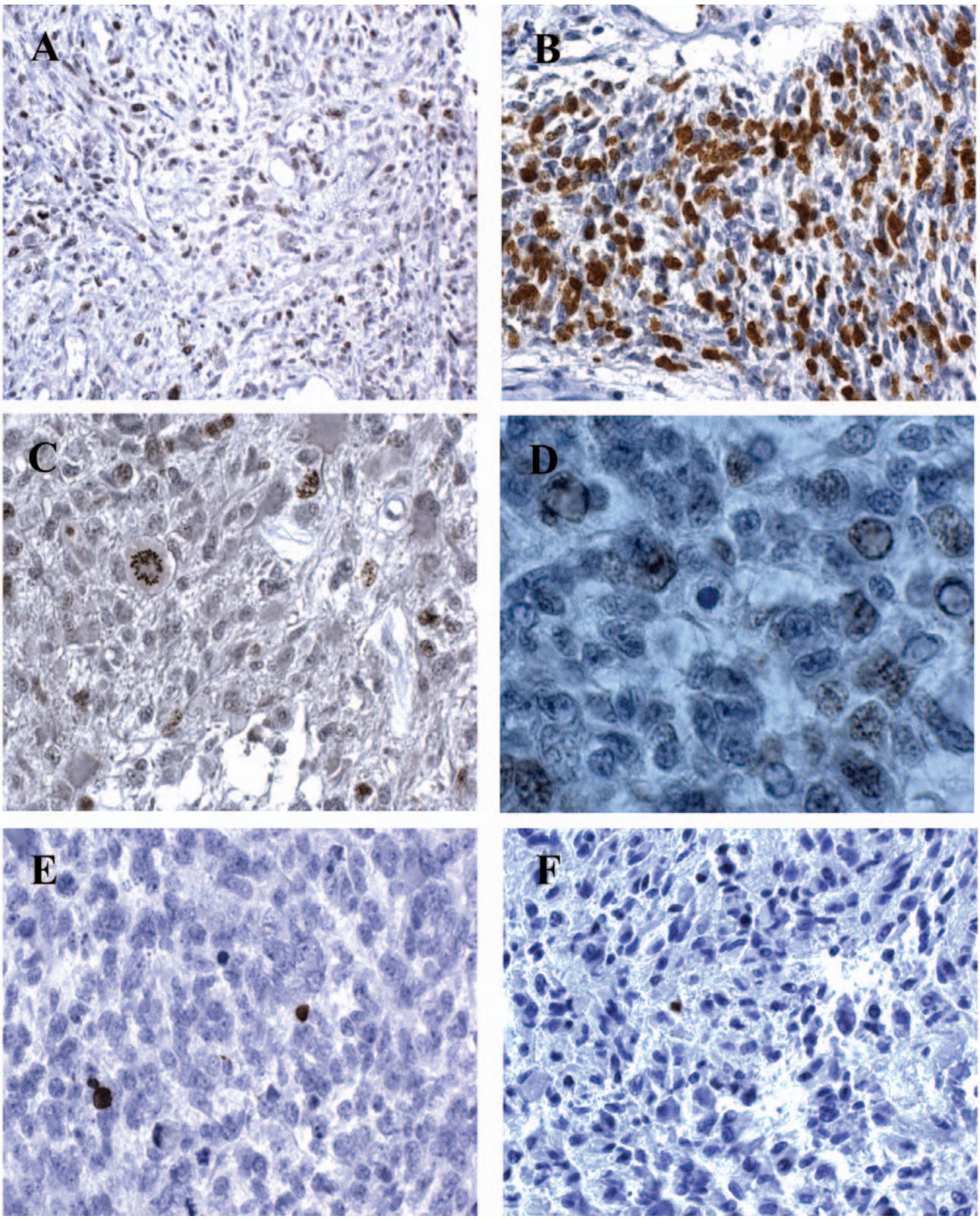

Figure 1. *Immunohistochemistry of survivin, Ki-67/MIB-1, apoptosis, caspase-3 and PARP-1. Case 7 (Table I) A, Survivin-positive nuclei, x40; B, Ki-67/MIB-1-positive nuclei, x40. C, Case 26 (Table I) Survivin-stained mitosis, x40. Case 32 (Table I) D, Apoptotic nucleus, survivin, hematoxylin counterstaining, x1000; E, Cleaved caspase-3 positive nuclei, x40; F, Cleaved PARP-1 positive nuclei, x40 (DAB).*

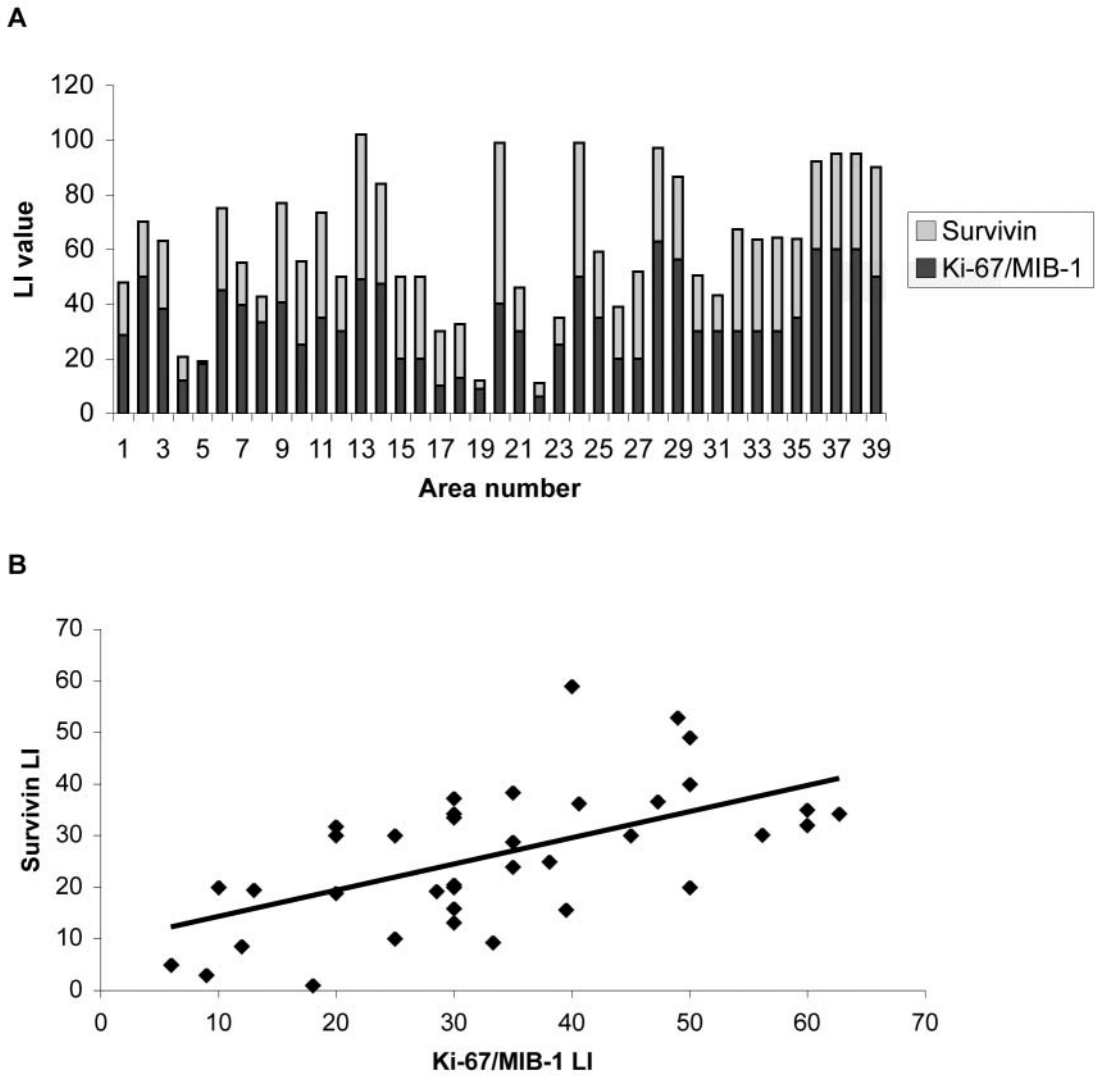

Figure 2. *Survivin distribution and correlation in glioblastoma. A, Ki-67/MIB-1 and survivin distribution. B, Significant correlation between survivin LI and Ki-67/MIB-1 LI (r=0.592, p=0.0001).*

separately and only nuclear staining was considered for comparative evaluation. Survivin-positive nuclei were present in all the tumors (Figure 1A), and were heterogeneously distributed. Some hyperplastic endothelial cells and cells of the microvascular proliferations were also positively stained. The staining intensity and the number of positive cells were much lower than those observed with Ki-67/MIB-1 (Figure 1B).

Table I shows Ki-67/MIB-1 and survivin LIs and the MI and AI found in all the areas. In 69.1% of the areas, the mean Ki-67/MIB-1 LI was greater than the mean survivin LI of 11.2%. The opposite was found in 30.9% of the areas of 6.2%. The two antigens were distributed as shown in Figure 2A. There was a positive linear correlation between the Ki-67/MIB-1 LI and the survivin LI (Figure 2B) and between the latter and MI (Figure 3A), with a Pearson's correlation coefficient of $r=0.592$ ($p=0.0001$) and $r=0.451$ ($p=0.004$), respectively. No significant correlation was found between the survivin LI and the AI (Figure 3B) (Pearson's correlation coefficient $r=0.232$, $p=0.1547$).

Mitoses were much more recognizable by survivin staining than by H&E, even though the counts with the two methods gave approximately the same results (Figure 1C).

No positive signal was obtained with Bid antibody. No significant correlation was found between Ki-67/MIB-1 LI or survivin LI and survival.

By Western blotting, survivin was easily demonstrable in all the samples. Its expression, however, was of different

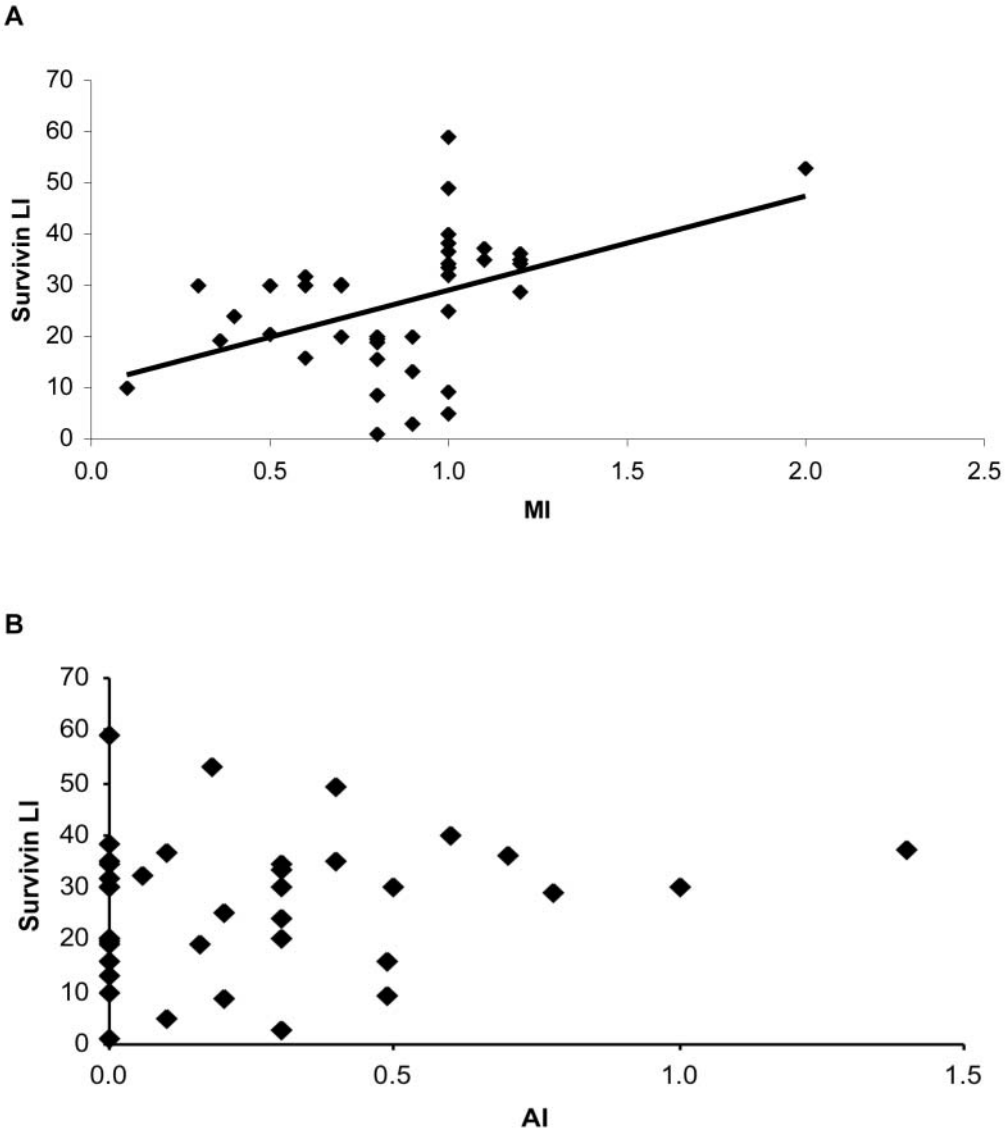

Figure 3. *Survivin correlations in glioblastoma. A, Significant correlation between survivin LI and MI (r=0.451, p=0.004). B, Scatter-plots showing lack of significant correlation of survivin LI with AI.*

intensity and corresponded to the values of the survivin LI (Figure 4, Table I). By double immunofluorescence, it was observed that the number of nuclei stained by survivin and by Ki-67/MIB-1 corresponded approximately to the numbers determined by immunohistochemistry. However, the staining intensity and the number of nuclei stained by survivin was much lower than for Ki-67/MIB-1 (Figure 5A-C). Mitoses were stained with both antibodies, with a greater intensity for survivin, whereas spindle material was stained only for survivin and was well visible during both anaphase and telophase, corresponding to microbodies (Figure 5D).

Discussion

The sub-cellular localization of survivin is the first point to be discussed. Most studies of survivin in brain tumors refer to a nuclear localization. Only two studies also mention a cytoplasmic localization (13) or describe only cytoplasmic staining (20). This problem has not been completely solved, because antigen localization also depends on the type of antibody used. The antibody we used binds to both types of survivin, but predominantly to the nuclear one (24) as other antibodies did (19). However, the problem resides in the existence of two pools of survivin, one nuclear and one

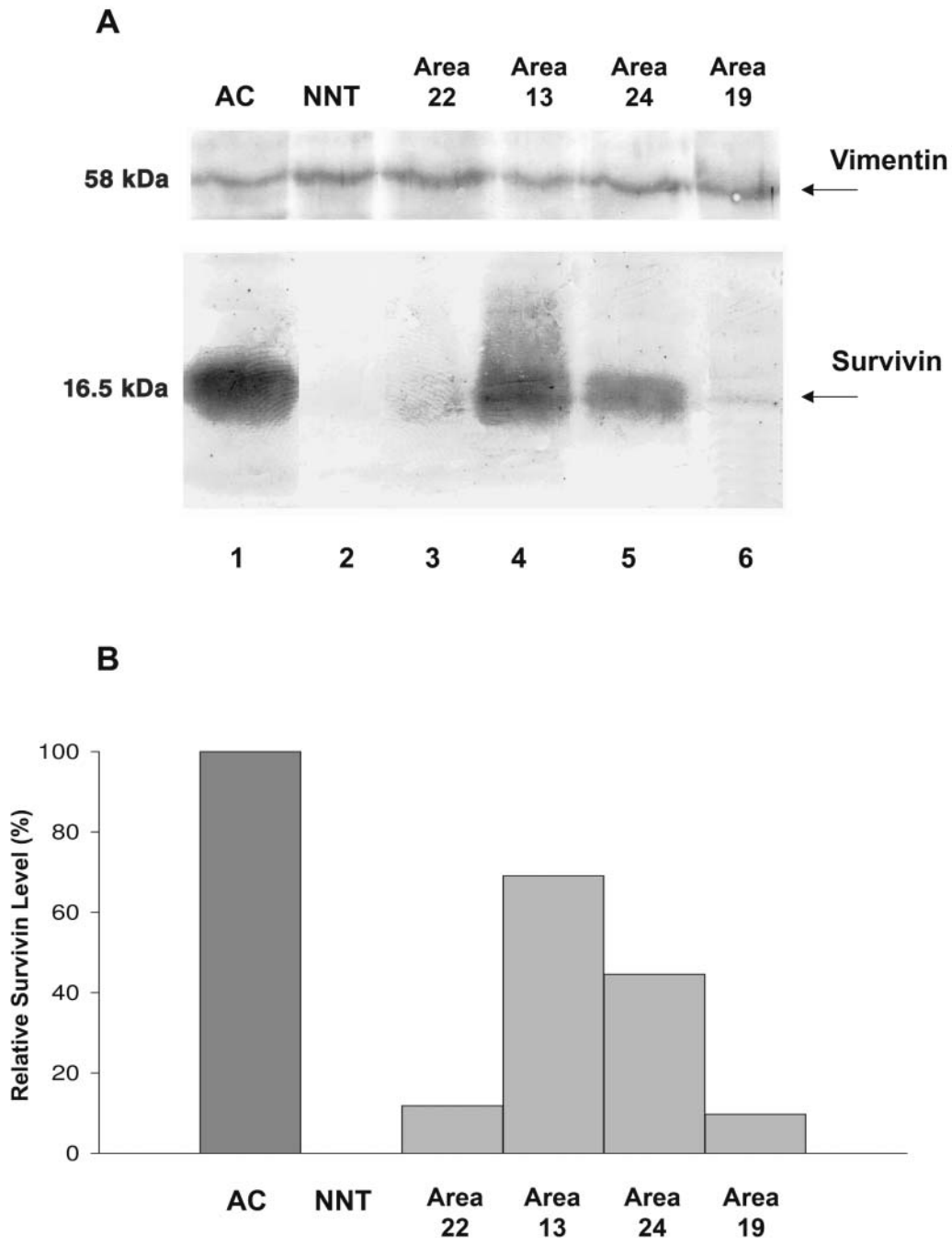

Figure 4. *Western blotting of survivin expression. A, Western blotting of survivin expression: 1, colon adenocarcinoma (AC); 2, normal nervous tissue (NNT); 3, 4, 5, 6 correspond to areas 19, 22, 24, 13. B, Quantitative analysis of survivin levels normalized to vimentin data.*

cytosolic (8), in the traffic between them, with diffusion of survivin to the nucleus or its export from the nucleus (25), and in the different functions of splice variants (12, 26). The spliced variant Δex3 localizes to the nucleus and survivin wild-type and survivin-2B localizes to the cytoplasm (12). The antibody we used recognizes the three isoforms of survivin (12, 27), and our evaluations of survivin expression were carried out at its nuclear location. Cytoplasmic staining was found in few areas; however, it did not show any correlation with mitosis or apoptosis.

Ki-67/MIB-1 is widely used today for the assessment of the proliferating potential of tumors. In gliomas, it increases

Table I. *LIs in the chosen areas.*

Area (N=39)	Patient no.	LI		Mitotic index	Apoptotic index	Survival (months)
		Ki-67/MIB-1	Survivin			
1	7512	28.5	19.2	0.36	0.16	12
2	7466	50.0	20.0	0.90	0.00	11
3	7858	38.1	25.0	1.00	0.20	13
4		12.0	8.6	0.80	0.20	
5	7777	18.0	1.0	0.80	0.00	7
6	7665	45.0	30.0	0.50	0.50	2
7		39.5	15.6	0.80	0.49	
8		33.3	9.3	1.00	0.49	
9		40.6	36.2	1.20	0.70	
10	7691	25.0	30.5	0.60	0.30	7
11		35.0	38.3	1.00	0.00	
12	7514	30.0	20.0	0.80	0.30	11
13		49.0	52.9	2.00	0.18	
14		47.3	36.6	1.00	0.10	
15	7658	20.0	30.0	0.30	0.00	11
16		20.0	30.0	0.70	1.00	
17	7573	10.0	20.0	0.70	0.00	10
18		13.0	19.5	0.80	0.00	
19		9.0	3.0	0.90	0.30	
20	7610	40.0	59.0	1.00	0.00	15
21	7630	30.0	15.9	0.60	0.00	8
22	7878	6.0	5.0	1.00	0.10	4
23	7716	25.0	10.0	0.10	0.00	15
24	7617	50.0	49.0	1.00	0.40	23
25	7666	35.0	24.0	0.40	0.30	16
26		20.0	18.9	0.80	0.00	
27		20.0	31.8	0.60	0.00	
28	7685	62.7	34.3	1.20	0.00	14
29		56.2	30.2	0.70	0.00	
30	7630	30.0	20.5	0.50	0.00	8
31		30.0	13.2	0.90	0.00	
32	7693	30.0	37.2	1.10	1.40	2
33		30.0	33.5	1.00	0.30	
34		30.0	34.2	1.00	0.30	
35		35.0	28.8	1.20	0.78	
36	7631	60.0	32.0	1.00	0.06	10
37		60.0	35.0	1.20	0.40	
38		60.0	35.0	1.10	0.00	
39		50.0	40.0	1.00	0.60	

with malignancy grade and is used as a prognostic factor with an adequate cut-off, even though a high inter-observer variability can limit its usefulness (28). In comparison with its use as a prognostic factor among the three grades of malignancy of astrocytic tumors, its reliability is much less within each tumor type or grade. In glioblastoma, there is a large range of LI values, such that in a single case, the LI can be used for diagnostic purposes only above a certain cut-off. The contributions on this matter in the literature regarding glioblastomas are divided between those supporting the prognostic significance of the LI and those denying it (21). It has even been observed that a high Ki-67/MIB-1 LI may be associated with a more favourable outcome (29) due to a greater sensitivity of tumors with greater proliferation capacity to radiotherapy. Survivin has been extensively studied in gliomas and its LI was found to increase with malignancy and to correlate or not with prognosis, apoptosis, the Ki-67/MIB-1 LI and survival. Recently, it was observed that the survivin LI correlates with the Ki-67/MIB-1 LI but not with survival (19), due to its expression only during the G2/M phase of the cell cycle (5). The lack of correlation of survivin with patients' survival is confirmed by our observations. In spite of this, the protein expressed during the whole cell cycle is a good marker of proliferation, if referred to each tumor area, because of the heterogeneous proliferation potential of the tumors. Ki-67/MIB-1 LI is more reliable than MI, because mitoses are less frequent than positive nuclei and cover only a minor part of the cell cycle.

For different reasons, the studies carried out to date of survivin correlations are deficient or with contrasting results, concerning the problem of heterogeneity of cell proliferation and apoptosis: i) an inverse relationship exists between survivin and apoptosis, but not statistically significant (20); ii) no correlation was investigated (15); iii) the studied areas have not been selected or proliferation was not studied (16); iv) the distribution of the proliferation index in gliomas of the three grades was peculiar (17); v) apoptosis was studied in areas different from those choosen for survivin and proliferation (19) .

We found a linear correlation of the survivin LI with the Ki-67/MIB-1 LI and the MI and same ratio between the two antigens in each examined area. The survivin LI did not correlate with the AI, whereas the Ki-67/MIB-1 LI was found to correlate with the MI. The values of the Ki-67/MIB-1 LI were 11.2% higher than those of the survivin LI in most areas. This corresponds to the weaker staining and to the reduced number of nuclei stained by survivin in comparison with Ki-67/MIB-1 seen on double immunofluorescence analysis. However, in 30.9% of areas, the survivin LI was 6.2% higher than the Ki-67/MIB-1 LI. The occurrence of nuclei positive for Ki-67/MIB-1 and negative for survivin can be explained by the longer time of expression of the former protein during the cell cycle than the latter, whereas the opposite behavior could be based on the prevailing disposition of survivin expression to inhibit apoptosis rather than to regulate mitosis. This may be due to non cell-cycle-dependent mechanisms that drive the survivin gene independently of mitosis (30) .

No correlation was found between the survivin LI and the AI. Very likely, apoptosis of proliferating areas should be triggered by the intrinsic or transcriptional pathway, in opposition to apoptosis of perinecrotic pseudo-palisades triggered by the extrinsic receptorial pathway (31). This is not acceptable in absolute terms, for many reasons, however it

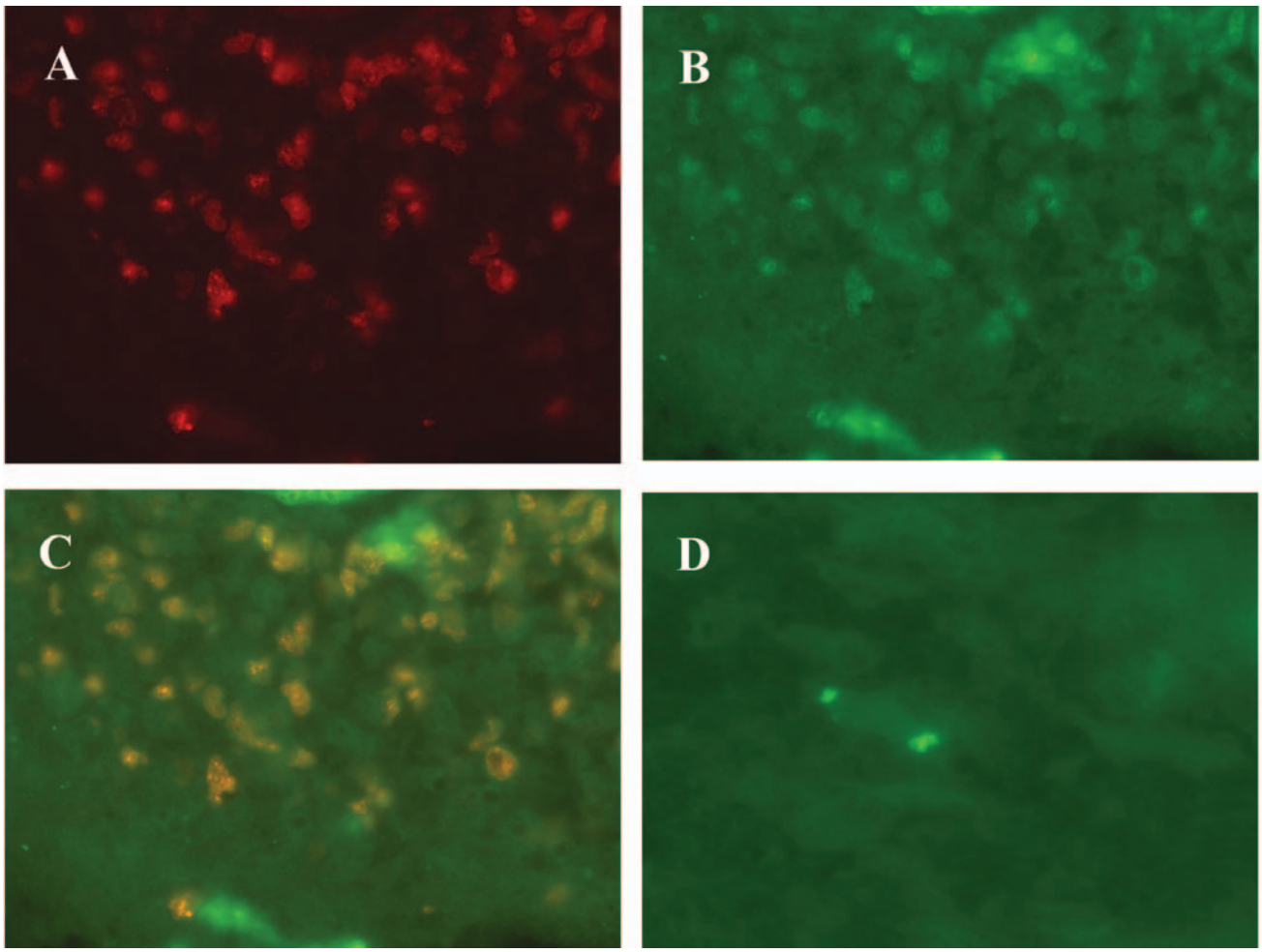

Figure 5. *Double immunofluorescence for Case 24 (Table I). A, Ki-67/MIB-1 TRITC, x40; B, Survivin FITC, x40; C, Merge, x40; D, Anaphase with survivin FITC, x40.*

could indicate different biological significances of the two apoptotic types. The signalling from the extrinsic pathway to the intrinsic one through Bid (32) can be disregarded, because caspase-8 has been demonstrated to be low or absent in many gliomas (33). Our negative results on Bid support this.

The mechanisms by which survivin regulates mitosis and inhibits apoptosis can clarify our results. Survivin is a mitotic protein; the gene undergoes cell-cycle-dependent transcription during cell division (5) and the protein undergoes mitotic post-translational modification, including phosphorylation by p34 (34). It has a role in microtubule behavior and in spindle formation (35). Survivin is a chromosomal passenger protein that localizes to kinetochores at metaphase, transfers to the central spindle midzone at anaphase and accumulates in midbodies at telophase (36). As an IAP, survivin functions in apoptosis inhibition, perhaps not directly inhibiting caspases, but mediating a ubiquitin-dependent destruction of caspase-9 (37), requiring a physical interaction with cofactor molecules (35). Control of mitosis and antagonization of cell death can be mutually integrated; however, there are non cell-cycle-dependent mechanisms that drive the survivin gene independently of mitosis which can become dominant in tumors, where anti-apoptotic function dominates its mitotic properties (35).

There is a lack of correlation with apoptosis both of the survivin LI and the Ki-67/MIB-1 LI. No correlation of the survivin LI with survival was found in our cases, as in our previous series (19). The lack of an inverse correlation of apoptosis with a protein that is specifically believed to inhibit it is somewhat surprising. Among the possible explanations, the difficulty in obtaining a reliable AI in tumor tissues must be taken into account. Direct demonstrations of apoptosis, either by TUNEL or by caspase-3 expression or PARP-1, have a variety of limitations. TUNEL may stain non-apoptotic nuclei with broken DNA, for example necrotic nuclei, and caspase-3 has some limitations in revealing

apoptotic nuclei (32, 38). Another reason is that the intervention of survivin in apoptosis inhibition is rather indirect (37) and mitosis-dependent expression of survivin can elevate its mitochondrial stores and heighten the anti-apoptotic threshold during cell division (37). In agreement with the hypothesis that non cell-cycle-dependent mechanisms drive survivin gene transcription independently of mitosis are citations (39) and personal observations of diffuse expression of STAT3 in malignant gliomas. However, the mechanisms of the relationship between survivin and apoptosis have not yet been fully clarified, also in therapeutic experiments on cell lines of non-nervous tumors (40).

The positive response of nuclei in microvascular proliferations should be interpreted as belonging to survivin as a regulator of mitosis, in line with the extremely rare finding of apoptosis in the pathological vessel walls of glioblastoma.

Acknowledgements

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