Expression of Survivin, a Novel Inhibitor of Apoptosis and Cell Cycle Regulatory Protein, in Human Gliomas

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Abstract **Objective** To investigate the relationship of survivin gene expression level in human gliomas and the malignant degree, cell proliferative activity and apoptosis of the tumors. **Methods** Apoptotic cells and apoptotic bodies were detected by TUNEL. Immunohistochemical stains were performed to examine the expression of Bcl-2, Survivin and PCNA. The software package SPSS 10.0 was used for statistical analysis. **Results** The percentage of Survivin positive expression was 56% in 50 specimens. The Survivin-postive rate was statistically significant difference between high and low grade tumors(73.9% versus 40.7%; $P<0.05$). Survivin expression was correlated with Bcl-2 expression($P<0.01$). Survivin weighted scores were significantly correlated negatively with AI ($P<0.01$), but which were significantly correlated positively with PI ($P<0.01$). There were significant positive linear correlations between AI and PI($P<0.001$). The difference of postoperative one year survival rate between Survivin-positive group and Survivin-negative group was statistically significant(50% versus 78.9%; $P<0.05$). Pathological grade, Survivin, AI, PI and operation were associated with prognosis of the patients with glioma with Logistic regression analysis($P<0.01$). **Conclusions** Over-expression of survivin gene inhibited apoptosis of glioma cells, and promoted the excessive cell proliferation, which may play an important role in the development and malignant progression of gliomas. Identification of prognostic significance of Survivin should be clarified with additional use of these prognostic markers including pathological grade, AI, PI and operation.

Key Words Glioma; Survivin; Apoptosis; Proliferation

Recently, a novel antiapoptosis gene, i.e., survivin, was identified as a structurally unique member of the inhibitor of apoptosis protein family (IAP). It is located on chromosome 17q25[1]. Survivin is a 16.5 kDa protein that is expressed during the G2/M phase of the cell cycle. And Survivin expression is turned off during fetal development and not found in non-neoplastic adult human tissues but is again turned on in the most common human cancers. To our knowledge, Survivin expression in the tumor of central nervous system has been studied by many scholars[2-4], but the relationship between survivin expression and cell apoptosis, proliferation activity has not yet been studied in human gliomas. Here, we investigated the expression of survivin in 50 patients with human gliomas, and determined its association with cell apoptosis, proliferation, and its impact for tumor progression and prognosis.

MATERIALS AND METHODS

Tissue Samples

50 patients with glioma who underwent operation at the Second Affiliated Hospital, Hebei Medical University from 2000 to 2001 were collected, which included 24 cases of Male and 26 cases of Female. The range of age was from 8 years old to 72 years old, and included 13 cases of <30 years old, 29 cases of 30-60 years old and 8 cases of > 60 years old. Preoperative chemiotherapy, radiotherapy and immunosuppressive therapy were not given to the patients. Among the patients underwent total surgical treatment was 27 cases, subtotal surgical treatment was 20 cases, and Partial surgical treatment was 3 cases. The tumors were graded and classified according to the WHO(1999) scheme and consisted of 11 cases of grade I, 16 cases of grade II, 14 cases of grade III, and 9 cases of grade IV. 5 cases of normal brain tissue used as control group were obtained from the patients with brain trauma who underwent inter-decompression. 45 cases (90%) of all patients were followed up in a year, which consisted of 17 cases (37.8%) of death, 28 cases (62.2%) of survival, 20 cases (44.4%) of postoperative radiotherapy, and 30 cases (66.7%) of postoperative chemiotherapy.

Immunohistochemistry

Immunohistochemical stains were performed on paraffin-embedded formalin-fixed tissue sections with a Strept Avidin-Biotin peroxidase complex (SABC) method (SABC Kit, Boster Co.ltd, Wuhan.) using DAB/hydrogen peroxide as a chromogen (DAB Kit, Boster Co.ltd, Wuhan.). The mono- and polyclonal antibodies (Mabs, Pabs) used included Survivin(5E8) (Pab; NeoMarkers, USA; dilution 1: 100), Bcl-2 (100) (Mab; Boster Co.ltd, Wuhan; ready to use), and PCNA (Mab; Boster Co.ltd, Wuhan; ready to use). Antigen retrieval by boiling pretreatment (92-98℃ for 20 min in 10mM citrate buffer, pH 6.0) was performed for all Abs. Incubation with the primary Abs was done at 40℃ overnight. As a negative control, sections were processed in the absence of primary Ab. Tissue sections with known strong expression of Survivin, Bcl-2 and PCNA were used as a positive control.

TUNEL

Apoptotic cells and apoptotic bodies were detected by TdT-mediated-dUTP-X nick end labeling (TUNEL) using an in situ Detection kit (Boster Co. ltd, Wuhan). In brief, deparaffinized and rehydrated sections were digested with proteinase K (dilution 1:200 in TBS) for 20 min at room temperature and washed after quenching in 3% hydrogen peroxide for 10 min, washing with TBS, and adding the equilibration buffer for 10 min, terminal deoxynucleotidyl transferase and digoxigenin-labelled dUTP were pipetted onto the sections, which were then incubated at 37℃ for 2 h. After stopping the reaction by putting sections in stop/wash buffer and washing, anti-digoxigenin- peroxidase was added to the slides. Finally, slides were washed with TBS, stained with DAB/hydrogen peroxide as a chromogen. A specimen known to be positive for apoptotic cells was used as positive control. Substitution of terminal deoxynucleotidyl transferase with TBS was used as negative control.

Evaluation Criteria

Apoptotic index (AI) and proliferative index (PI) was assessed by scoring the percentage of labelled cells in at least five high-power fields (×400). Semi-quantitative assessment of Survivin and Bcl-2 expression was based on the mean percentage of positive tumour cells in at least five high-power fields (×400) assigned to one of five categories: 0, <1%; 1, 1-25%; 2, 25-50%; 3, 50-75%; 4, >75%. The intensity of immunostaining was scored as: 1, weak; 2, moderate; 3, intense. In tumours displaying heterogeneous staining, the predominant pattern was considered for scoring. The percentage of positive tumour cells and staining intensity were multiplied to produce a weighted score for each case. Tumors with a weighted score = 0 were designated as negative; all others were considered positive[5,6].

Statistical Analysis

The software package SPSS 10.0 was used for statistical analysis. Proliferative index and apoptosis index are reported as mean±standard deviation. The χ^2 test, ANOVA and Pearson correlation were used to analysis in this report. Logistic regression analysis was used to examine the predictive values of individual variables. $P<0.05$ was considered statistically significant.

RESULTS

Expression of Survivin

Immunohistochemistry for Survivin revealed buffy granular staining in the cytoplasm of positive glioma cells(Fig.1). But no expression was detected in the normal brain tissues. After multiplying the weighted survivin score, 28 cases of glioma in this series were defined as positive (56%), with a weighted survivin score from 1 to 12. The distributions of weighted survivin scores were 0 for 22 cases, 1 for 6 cases, 2 for 5 cases, 3 for 5 cases, 4 for 4 cases, 6 for 3 cases, 8 for 3 cases, 9 for 1 cases, and 12 for 1 cases. the Survivin-postive rate was statistically significant difference between high and low grade tumors (73.9%, 17/23; 40.7%, 11/27; $P<0.05$).

Relationship Between Expression of Survivin and Bcl-2

Among the 50 gliomas studied, 29 cases (58%) showed positive cytoplasmic immunoreactivity for Bcl-2 (Fig.2). Expression of survivin showed a strong positive correlation with expression of bcl-2, and it was very significantly segregated with bcl-2-negative cases (20 of 29, 69.0% versus 13 of 21, 61.9%).

Apoptosis Index (AI)

Heterogeneous extremely buffy was showed in apoptotic cell nuclei. And positive cells were scattered in glioma tissues. In some cases, positive cells tended to be found in clusters (Fig.3). Every

case of glioma examined showed apoptotic cells and apoptotic bodies that were detected by in situ labeling. The mean AI of 50 cases was 0.94% (SD, 0.541%; range, 0.1%–2.3%), with a median value of 0.900%. AI was associated with the grade of glioma (F=9.028, P<0.001). that is AI increased with the progress of glioma (Table 1). The mean AI in Survivin–positive tumors was 0.800±0.484%, which was significantly lower than the mean AI of 1.118±0.569% observed in Survivin–negative tumors (P<0.05). There were significant negative linear correlations between Survivin weighted scores and AI (r=–0.372, P<0.001).

Proliferative Index (PI)

PCNA positive cells with buffy nuclear were scattered unequally in glioma tissues (Fig.4). PCNA positive cells could be found in every case of glioma. The mean PI of the 50 cases was 16.98% (SD, 10.85%; range, 1%–40%), with a median value of 15.00%. There were significant positive linear correlations between AI and PI (r=0.755, P<0.001;) and between Survivin weighted score and AI (r=–0.372, P<0.001). PI was associated with the grade of glioma (F=51.218, P<0.001), that is PI increased with the progress of glioma.

Table 1 AI and PI of each grade of gliomas

WHO grade	n	AI(%; ±s)	PI(%; ±s)
Grade Ⅰ	11	0.455±0.398	8.36±5.59
Grade Ⅱ	16	0.825±0.340★	12.81±5.58★
Grade Ⅲ	14	1.164±0.481★◇	22.29±5.12★◇
Grade Ⅳ	9	1.389±0.582★◇▲	24.00±5.24★◇▲
Total	50	0.940±0.541	16.50±8.16
F value		9.028	21.980
P value		<0.001	<0.001

★Compared with Grade Ⅰ, P<0.05; ◇Compared with Grade Ⅱ, P<0.05; ▲Compared with Grade Ⅲ, P>0.05.

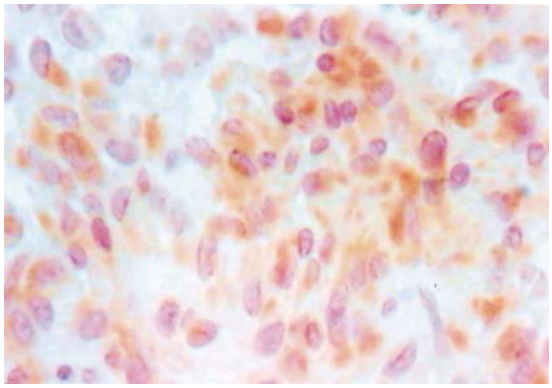

Fig.1 Ependymoma cells (Grade III) were strongly positive for Survivin. SABC ×400

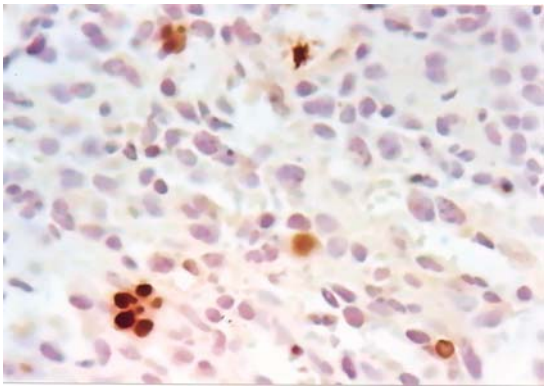

Fig.3 In a medulloblastoma (Grade IV), apoptotic cells were found in clusters. TUNEL ×400

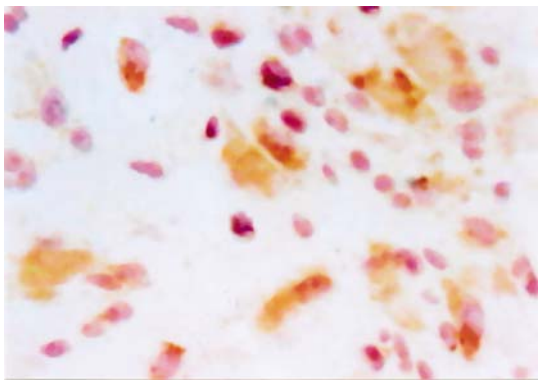

Fig.2 Glioblastoma multiforme (Grade IV) showed strongly and diffusely cytoplasmic reactivity for Bcl–2. SABC ×400

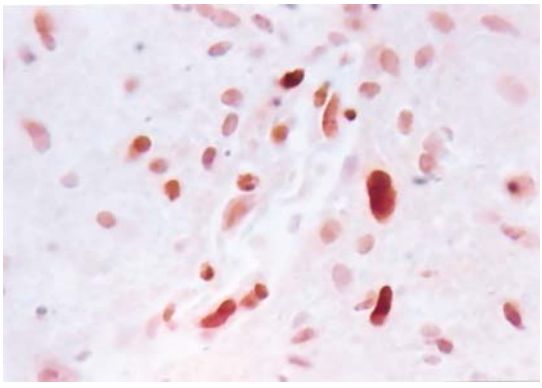

Fig.4 PCNA positive cells with buffy nuclear were scattered unequally in anaplastic astrocytoma (Grade III). SABC ×400

Prognostic Index

The difference of postoperative one year survival rate between Survivin–positive group and Survivin –negative group was statistically significant (50% versus 78.9%; $P<0.05$). A series of prognostic factors such as sex, age, pathological grade, Survivin, bcl–2, AI, PI, chemiotherapy, radiotherapy and operation were analysed with Logistic regression analysis. And the results suggested that pathological grade, Survivin, AI, PI and operation were associated with prognosis of the patients with glioma ($P<0.01$).

DISCUSSION

Among the recently described IAP family, Survivin is characterized by a unique structure with a single BIR and no zinc–binding domain known as the RING finger, and it expressed selectively in common human cancers but not in normal adjacent tissue in vivo[1]. In this study, specific staining for Survivin was detected in 28 cases (56%), and the Survivin –postive rate was statistically significant difference between high–grade and low grade tumors. This result was consistent with Sasaki´s report [4], which indicated that Survivin expression could reflect anaplastic degree and proliferative activity of glial cell.

The imbalance between cell proliferation and apoptosis is the base of malignant progress of tumor. Therefore, it is necessary to implicate proliferation and apoptosis of tumor cell for understanding biological specificity and clinical features of tumor. This data indicated that AI and PI increased with the progression of glioma, and there were significant positive linear correlations between AI and PI. Apoptotic activity was increased in faster proliferative, higher grade tumor, but its increased degree was less than proliferation´s.

We found compelling evidence that the presence of Survivin in human gliomas was strongly associated with expression of Bcl–2 and with reduced AI. Our results were consistent with the findings of previous investigations, which showed a similar association in neuroblastoma, gastric cancer, colorectal cancer, breast carcinoma and high–grade non–Hodgkin´s lymphoma[6–9]. The survivin gene is encoded at chromosome 17q25, whereas the bcl–2 gene is located at chromosome 18q21 and may be involved in the tumorigenic t (14;18) translocation. These data imply that other transcriptional factors may contribute to the coregulation of both gene products in the progression of cancer. Survivin and Bcl–2 genes are regulated by TATA–less, GC–rich promoter sequence in similar manners, and both are markedly transcribed in actively proliferating cell types[10], suggesting common mechanism(s) of transcriptional activation. However, regardless of the pathway of simultaneous coexpression, it appears that Survivin and Bcl –2 proteins may mediate nonoverlapping, antiapoptosis mechanisms. Although Bcl–2 is an integral inner mitochondrial membrane protein implicated in counteracting cytochrome C release from the mitochondria, Survivin potentially prevents apoptosis by targeting the terminal effectors caspase–3 and caspase–7[11]. Expression of Survivin alone or Survivin plus other antiapoptosis genes like Bcl–2 may result in more pronounced antiapoptotic effects[6], but both AI and the level of Survivin expression increased with increasing anaplasia across the spectrum of gliomas. The explanation relevant to this issue was that apoptosis could be induced because of the errors in DNA duplication or cell division, and adjusted as network by a series of related genes including unidentified potential effector. A recent study in vitro demonstrated that antisense survivin RNA down–regulated expression of endogenous Survivin in transformed cells and resulted in increased apoptotic cell death [11]. We suspected that targeted antagonists of Survivin may be beneficial as apoptosis–based therapy for human gliomas.

There was significant positive correlation between weighted scores of Survivin expression and PI. A similar correlation between Survivin expression and PI has also been reported in hepatocellular carcinomas and pancreatic carcinomas[5,12]. These observations are entirely consistent with the regulated expression of Survivin in the G2/M phase of the cell–cycle such that over–expression may overcome an apoptotic checkpoint and favour aberrant progression of malignant transformed cells through mitosis[11]. It is also known that anti–sense targeting of Survivin gene expression results in inhibition of cellular proliferation [1], whereas Survivin over–expression promotes cell cycle entry with an accelerated S phase shift and resistance to G1 arrest[13].

Pathological grade, Survivin, AI, PI and operation were associated with prognosis of the patients with glioma with Logistic regression analysis. Survivin expression was an important poor prognostic factor, prognostic significance of which should be

clarified with additional use of these prognostic markers including pathological grade, AI, PI and operation.

REFERENCES

1. Ambrosini G., Adida C., Sirugo G., et al. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. J Biol Chem, 1998, 273: 11177–11182.
2. Chakravarti A, Noll E, Black PM, et al. Quantitatively determined survivin expression levels are of prognostic value in human gliomas. J Clin Oncol, 2002, 20(4): 1063–1068.
3. Das A, Tan WL, Teo J, et al. Expression of survivin in primary glioblastomas. J Cancer Res Clin Oncol. 2002, 128(6): 302–306.
4. Sasaki T, Lopes MB, Hankins GR, et al. Expression of survivin, an inhibitor of apoptosis protein, in tumors of the nervous system. Acta Neuropathol (Berl), 2002, 104 (1): 105–109.
5. Sarela A I, Verbeke C S, Ramsdale J, et al. Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. Bri J Cancer, 2002, 86(6): 886–892.
6. Tanaka K, Iwamoto S, Gon G, et al. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res, 2000, 6(1): 127–134.
7. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcino mas. Cancer Res, 1998, 58: 1808–1812.
8. Kawasaki H, Altieri DC, Lu CD, et al. Inhibition of apoptosis by survivin predict shorter survival rates in colorectal cancer. Cancer Res, 1998, 58: 5071– 5074.
9. Adida C, Berrebi D, Peuchmaur M, et al. Anti–apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet, 1998, 351: 882–883.
10. Ambrosini G, Adida C, Altieri DC. A novel anti–apop–tosis gene, survivin, expressed in cancer and lymphoma. Nat Med, 1997, 3: 917–921.
11. Li F, Ambrosini G, Chu EY, et al. Control of apopto–sis and mitotic spindle checkpoint by survivin. Nature, 1999, 396: 580–584.
12. Ito T, Shiraki K, Sugimoto K, et al. Survivin promotes cell proliferation in hepatocellular carcinomas. Hepatology, 2000, 31(5): 1080–1085.
13. Suzuki A, Hayashida M, Ito T, et al. Survivin initiates cell cycle entry by the competitive interaction with Cdk4/p16(INK4a) and Cdk2/cyclin E complex activation. Oncogene, 2000, 19: 3225–3234.