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Expression of the Small Heat Shock Protein (hsp) 27 in Human Astrocytomas Correlates with Histologic Grades and Tumor Growth Fractions

Humayun Khalid,[1,3] Keisuke Tsutsumi,[1] Hiromi Yamashita,[1] Masao Kishikawa,[2] Akio Yasunaga,[1] and Shobu Shibata[1]

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SUMMARY

1. Cellular expression and distribution of the stress response small heat shock protein 27 (hsp27) in 39 high-grade astrocytomas (27 glioblastoma multiformes, 12 anaplastic astrocytomas) and in 27 low-grade astrocytomas (grade I–II) were analyzed immunohistochemically.

2. The correlation between hsp27 expression and tumor growth fractions of the astrocytomas was examined following Ki-67 immunostaining.

3. The hsp27 staining was cell cytoplasmic. The hsp27 immunopositive rate was significantly higher in high-grade astrocytomas; the rates were 74% for glioblastomas, 58% for anaplastic astrocytomas, and 37% for low-grade astrocytomas. The small and large tumor cells, especially in glioblastomas, multinucleated tumor giant cells, tumor cells in the pseudopalisading and necrotic areas, cells of the microvascular endothelial proliferations, and tumor vascular smooth muscles were usually hsp27 positive. The mean percentage of hsp27-positive cells was significantly higher in the glioblastomas alone and in the combined high-grade

[1] Department of Neurosurgery, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852, Japan.
[2] Department of Pathology, Scientific Data Center for the Atomic Bomb Disaster, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan.
[3] To whom correspondence should be addressed.

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astrocytomas, compared to the low-grade, and in recurrent rather than in primary high-grade astrocytomas.

4. The high-grade astrocytomas had a highly statistical significant Ki-67 labeling index. The Ki-67 labeling indices were significantly higher in the hsp27-positive than the hsp27-negative astrocytomas, irrespective of the histological grade. In the high-grade astrocytomas with a Ki-67 labeling index of five and above, 81% of those tumors were hsp27 positive.

5. Thus, a large number of human astrocytomas express hsp27, and hsp27 expression correlates with histological grades of astrocytoma and with tumor growth fractions. This being the case, hsp27 is likely to have a role in the growth of human astrocytomas.

INTRODUCTION

Heat shock proteins (hsp), also termed stress proteins, are induced in cells by various combination of stresses in response to environmental challenges and transitions of development. The stress response proteins in general and hsp27 are thought to be induced in cells by thermal and other kinds of physical and chemical stresses, and hsp27 could protect the cells against further stress (Arrigo *et al.*, 1988; Fuqua *et al.*, 1989; Landry *et al.*, 1989). The small heat shock protein hsp27 and others may be expressed under nonstress conditions and they have been described as normal cellular constituents termed "molecular chaperones." They appear to have a variety of functions, including roles in signal transduction (Michishita *et al.*, 1991), regulation of protein synthesis, folding, assembly and degradation, and cell proliferation (Ellis and Van der Vies, 1991; Pechan, 1991). Molecular genetics revealed that at least three related human hsp27 gene sequences are located on chromosomes 3, 9, and 10 (McGuire *et al.*, 1989).

In humans, expression of the stress response hsp27 was seen to occur in the brain of aged persons and also in Alzheimer's disease brain (Renkawek *et al.*, 1994), and they also have been demonstrate in various neoplasms, including human brain tumors (Kato *et al.*, 1992, 1993; Thor *et al.*, 1991; Yokoyama *et al.*, 1993). hsp27 is also related to the state of tumor growth and to the aggressive state of tumor cells (Thor *et al.*, 1991). hsp facilites induction of cell proliferation pathways by influencing regulatory repressor proteins (Pechan, 1991); synthesis of both large and small heat shock proteins is cell cycle dependent (Chambard *et al.*, 1983; Pechan, 1991).

Among brain tumors, human astrocytomas are a most aggressive and hihgly vascular neoplasm, the proliferative activity of which has been reported to correlate with the cell proliferative marker Ki-67 labeling index (Ki-67 LI) (Raghavan *et al.*, 1990; Shibuya *et al.*, 1993). We examined immunohistochemical expression of the small heat shock protein hsp27 in a number of human astrocytomas of different histological grades. We also studied growth fractions of these tumors, as assessed by Ki-67 LI, and we compared the expression of hsp27 in astrocytomas with growth potentials and with other clinicopathological

parameters. We found that the expression of the hsp27 correlated with the histological grade of astrocytomas and with their growth potentials.

MATERIALS AND METHODS

Chemicals

Mouse monoclonal antibody against human hsp27 was purchased from Stress Gen, Victoria, Canada; mouse monoclonal antibody MIB-1 "paraffin Ki-67" was purchased from Immunotech S.A., Marseilie Cedex, France; goat anti-mouse immunoglobulin and avidin–biotin complex were from Vector Laboratories, Burlingame, California, USA; and 3,3′-diamino-benzidine-4 hydrochloride (DAB) was purchased from Dojindo, Kumamoto, Japan.

Patient Population and Tumor Specimens

Forty-eight patients were surgically treated on 66 occasions at the Nagasaki University Hospital, Japan, from 1985 to 1993. The resected tumor samples were fixed in 10% neutral formalin and embedded in paraffin. The 5-μm tumor sections were stained for routine histologic study, and consecutive sections were stained immunohistochemically with anti-hsp27 and -Ki-67 antibodies. Clinicopathological features of the patients are shown in Table I. There were 31 men and 17 women; their ages ranged from 1 to 80 years (median age, 53.5 years). The histological classification and grading were based on the World Health Organization criteria (Zülch, 1979). There were 27 glioblastoma multiformes, 12 anaplastic astrocytomas, and 27 low-grade (grade I–II) astrocytomas. Twelve patients had tumor recurrence once or more, and there were 7 glioblastomas, 3 anaplastic astrocytomas, and 10 low-grade astrocytomas (recurrent) tumor samples. Normal brain tissue obtained during surgical manipulations of the tumor was also immunostained for hsp27.

Table I. Clinicopathology of 66 Human Astrocytomas

		Tumors		
		Primary	Recurrent	Total
Total patients	48			
Sex, male/female	31/17			
Age (yr)				
Range	1–80			
Median	53.5			
Tumor histology				
Low-grade astrocytoma		17	10	27
Anaplastic astrocytoma		9	3	12
Glioblastoma multiforme		20	7	27

Small Heat Shock Protein hsp27 Immunostaining

The deparaffinized tissue sections were placed in methanol containing 0.3% hydrogen peroxide for 30 min and washed in 0.05 *M* phosphate-buffered saline (PBS; pH 7.4) for 15 min. Tissue nonspecific activity was blocked by goat normal serum for 30 min, and sufficient primary antibody mouse monoclonal antibody against human hsp27 diluted 1:50 in PBS was applied and incubated overnight at 4°C. The bridge antibody applied was biotinylated goat anti-mouse immunoglobulin (1:100 in PBS), at room temperature for 1 hr, then the avidin biotin complex was applied for 30 min. After each incubation, sections were washed three times for 5 min each in PBS. The substrate chromogen was DAB. The sections were counterstained in Mayer's hematoxylin, dehydrated, and mounted. The light microscopic visual assessment of the hsp27-positive cells was obtained as the percentage of the total tumor cells in a given section. When at least 5% of the total tumor cells in that section were stained by the primary antibody, the section was considered hsp27 positive.

Ki-67 Antigen Immunostaining

The deparaffinized tissue sections were rinsed in distilled water for rehydration. The sections were then placed in a glass container filled with 10 m*M* citrate buffer (pH 6.0) and processed in a domestic microoven (Hitachi MR-A330) for 15 min at 500 W, then the sections were cooled down to room temperature. After pretreatment of the sections with 0.2% trypsin followed by a rinse in PBS, tissue endogeneous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. The subsequent steps were the same as for hsp27 immunostaining, with the exception that the primary antibody was mouse monoclonal antibody MIB-1 "paraffin Ki-67" diluted 1:50 in PBS and incubated overnight at 4°C. In each section, tumor cells of the 5 to 10 light microscopic high-power fields (varied for available tumor cells) set with a video monitoring screen were counted. The total tumor cells of the visualized fields in that section divided by the total immunostained cells gave the percentage of the Ki-67 labeling index (Ki-67 LI).

Controls and Statistical Analysis

Breast cancer tissues served as the positive controls. Appropriate negative controls were obtained for each immunostain by omitting the primary antibody, and comparisons were made with that of the positive controls. For statistical analysis, mean values and standard deviations (SD) were calculated; and *P* values were obtained by the Mann–Whitney *U* test.

RESULTS

Immunoreactivity of the hsp27 in the 66 Astrocytomas

Thirty-seven (56%) of the 66 astrocytomas showed positive hsp27 immunoreactivity. The staining of the hsp27 was cell cytoplasmic (Figs. 1 and 2). The hsp27 positivity rates were 37% (10/27) for low-grade astrocytomas, 58% (7/12) for anaplastic astrocytomas, and 74% (20/27) for glioblastomas (Table II). There was a statistically significant higher positivity rate in high-grade compared to low-grade astrocytomas ($P = 0.0194$). In the case of high-grade astrocytomas, especially in glioblastomas, tumor cells in the pseudopallisading areas, and isolated tumor cells in the necrotic areas, the small and large multinucleated tumor giant cells were usually positive for the hsp27 (Figs. 1 and 2). Cells of microvascular endothelial proliferations and smooth muscle of the tumor blood vessels also were frequently hsp27 positive (Fig. 2). In four-fifths of the hsp27-positive glioblastomas, more than 50% of the tumor cells strongly stained for hsp27, including seven glioblastomas, with more than 80% tumor cells being hsp27 positive (Fig. 1C).

The mean percentage (mean ± SD) of the hsp27-positive cells was 14.26 ± 24.09 in low-grade astrocytomas; 23.33 ± 24.15 in anaplastic astrocytomas, and in 44.78 ± 34.40 in glioblastomas (Table II). There was a statistically significant higher percentage of hsp27-positive cells in glioblastomas compared to anaplastic and low-grade astrocytomas ($P < 0.05$ and $P < 0.001$ respectively), but the values between anaplastic astrocytomas and low-grade astrocytomas did not differ. The hsp27 positivity rates of primary tumors were not statistically different from those of the recurrent astrocytomas, irrespective of the histological grade, but the mean

Table II. Immunoreactivities of hsp27 in 66 Human Astrocytomas

Tumor type[a]	No. of tumors	hsp27-positive tumors[b]		Significance[c]
		Positive (%)	Positive cells (%)	
LGA	27	10 (37)	14.26 ± 24.09	
AA	12	7 (58)	23.33 ± 24.15	
GB	27	20 (74)	47.78 ± 34.4	[a]P = 0.0006 [b]P = 0.0324
Combined HGA vs LGA				P = 0.0018
HGA				
Primary	29	18 (62)	33.97 ± 32.88	
Recurrent	10	9 (90)	58.50 ± 33.57	P = 0.0392
LGA				
Primary	17	7 (41)	15.88 ± 25.69	P = 0·5819
Recurrent	10	3 (30)	11.50 ± 22.17	

[a] LGA, low-grade astrocytoma; AA, anaplastic astrocytoma; GB, glioblastoma; HGA, high-grade astrocytoma.
[b] Mean ± SD.
[c] *P* value (Mann–Whitney *U* test): [a]*P* LGA and GB; [b]*P* between AA and GB. The significances are expressed for the percentage of hsp27-positive cells.

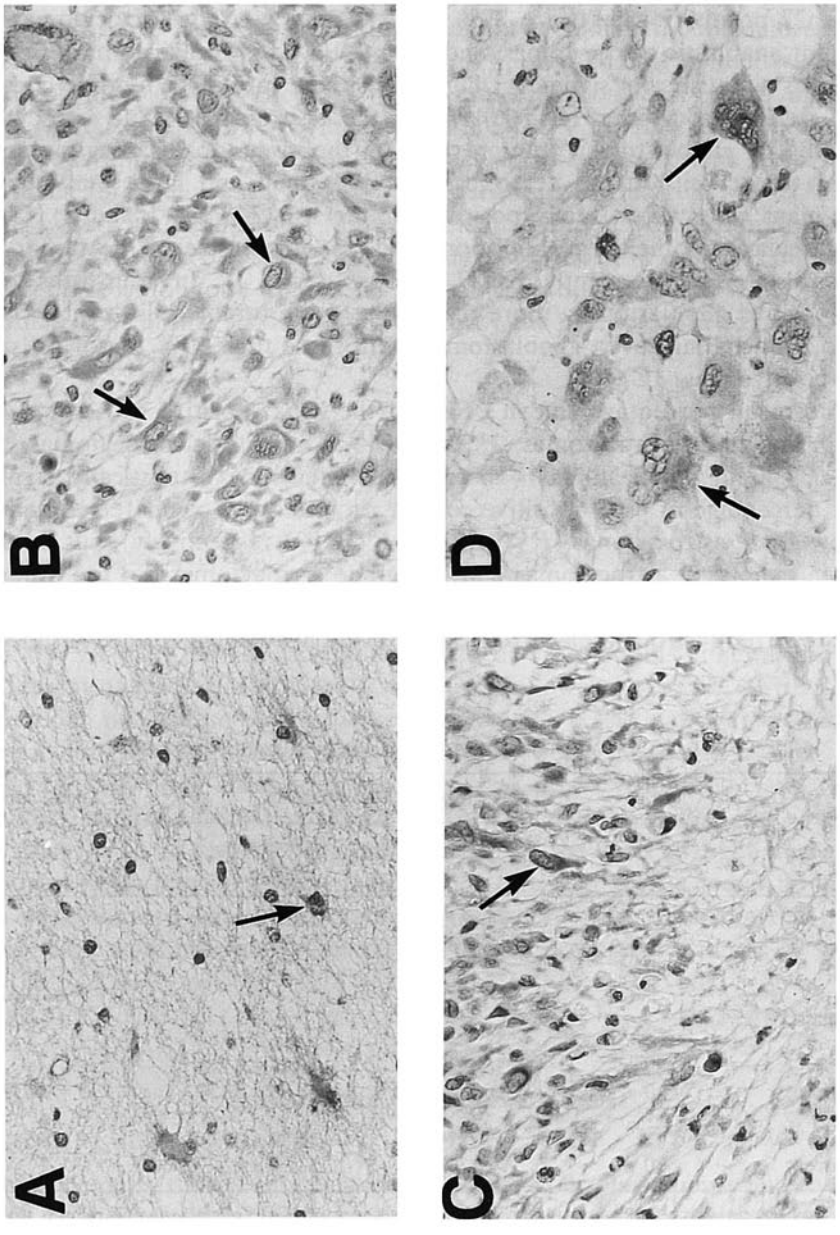

Fig. 1. Photomicrograph of astrocytomas stained for small heat shock protein (hsp) 27. As indicated by arrows, cytoplasmic hsp27 expression is shown in a low-grade astrocytoma (A), an anaplastic astrocytoma (B), glioblastoma tumor cells in the pseudopalisading area (C), and multinucleated tumor giant cells (D). Avidin–biotin complex immunohistochemistry; hematoxylin counterstain. Original magnification, ×400.

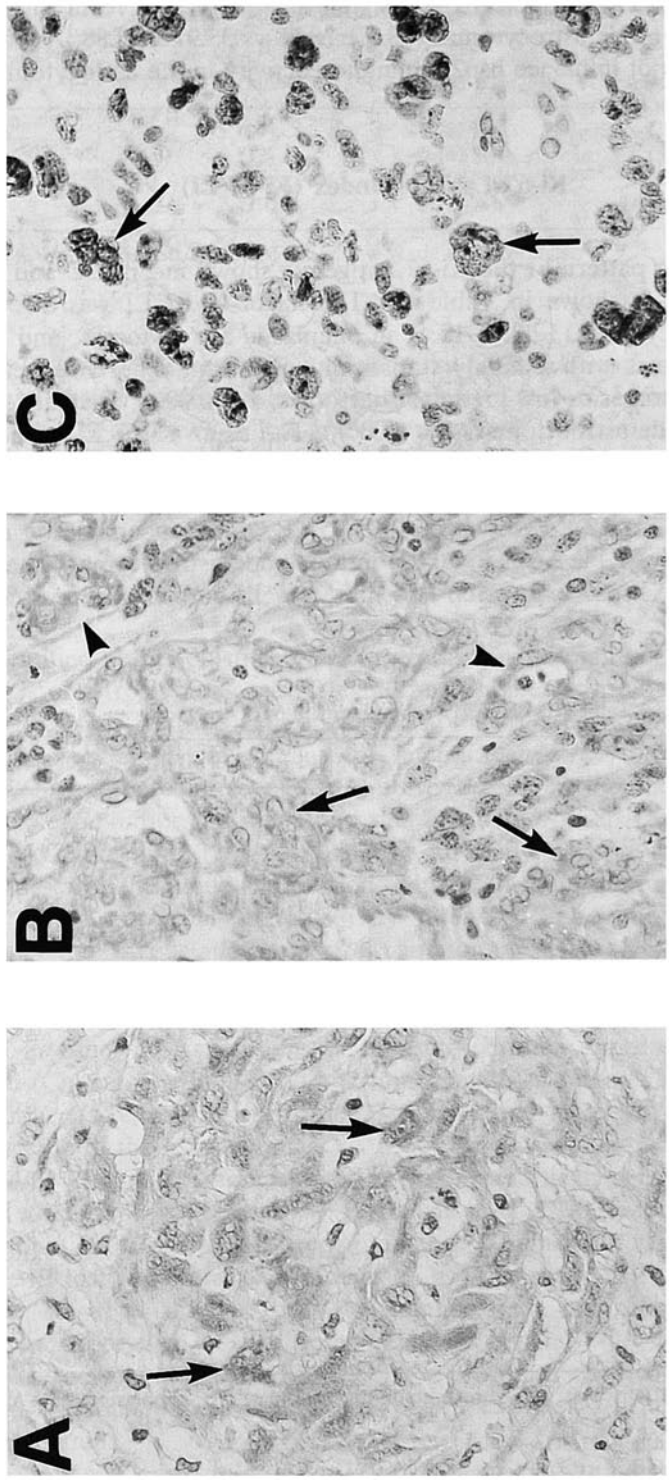

Fig. 2. Photomicrograph of glioblastomas stained for hsp27 and Ki-67 antigen. hsp27 immunoreactivity is shown in glomeruloid endothelial proliferation (A; arrows), endothelial cells (B, arrows), and proliferative vessel (B, arrowheads). Ki-67 antigen immunoreactivity in nuclear positions (C, arrows). Avidin–biotin complex immunohistochemistry; hematoxylin counterstain. Original magnification, ×400.

percentage of hsp27-positive cells was significantly higher in recurrent compared to primary high-grade astrocytomas (58.5 ± 28.68 vs 33.97 ± 32.88; $P < 0.05$). Age and gender did not influence hsp27 immunoreactivity in the astrocytomas.

Ki-67 Labeling Index (Ki-67 LI)

The staining pattern of the Ki-67 antigen is shown in Fig. 2C and the Ki-67 LI (mean ± SD) is shown in Table III. The mean Ki-67 LI was 1.75 ± 2.99 in low-grade astrocytomas, 12.37 ± 12.41 in anaplastic astrocytomas, and in 9.20 ± 10.43 glioblastomas with a highly statistically significant difference between the groups (glioblastomas vs low-grade astrocytomas, $P < 0.0001$; anaplastic astrocytomas vs low-grade astrocytomas, $P < 0.0001$). The mean Ki-67 LI in the primary and recurrent tumors showed no statistical difference. The mean Ki-67 LIs in the hsp27-positive and -negative tumors were 11.03 ± 11.51 vs 3.97 ± 2.99 ($P < 0.05$) in glioblastomas, 18.12 ± 12.89 vs 4.31 ± 5.95 ($P < 0.05$) in anaplastic astrocytomas, 2.84 ± 3.36 vs 1.1 ± 2.65 ($P < 0.02$) in low-grade astrocytomas. The relationship between the hsp27 expression and the Ki-67 LI cutoff value was considered

Table III. Ki-67 Labeling Index in 66 Astrocytomas

Tumors[a]	Ki-67 LI (mean ± SD)	Significance (P)[b]
LGA	1.75 ± 2.99	
AA	9.74 ± 6.51	0.0001
GB	11.85 ± 16.08	0.0001[c]
Combined HGA	11.43 ± 14.59	0.0001
vs LGA	1.75 ± 2.99	
LGA		
Primary	2.03 ± 3.3	0.4229
Recurrent	1.07 ± 2.14	
HGA		
Primary	9.53 ± 10.83	
Recurrent	11.68 ± 12.08	0.3154
hsp27		
Positive (all tumors)	10.16 ± 11.32	0.0001
Negative (all tumors)	2.35 ± 3.64	
hsp27		
Positive LGA	2.84 ± 3.36	0.0172
Negative LGA	1.10 ± 2.65	
hsp27		
Positive GB	11.03 ± 11.51	0.0309
Negative GB	3.97 ± 2.99	
hsp27		
Positive AA	18.12 ± 12.89	0.0473
Negative AA	4.31 ± 5.95	

[a] LGA, low-grade astrocytomas; HGA, high-grade astrocytomas; GB, glioblastomas; AA, anaplastic astrocytomas.
[b] *P* value (Mann–Whitney *U* test).
[c] Between LGA and GB.

Table IV. Relationship Between Ki-67 LI Cutoff Value and hsp27

Tumors[a]	Ki-67 (No. of tumors)	hsp27 positive	hsp27 negative
HGA	≥5 (27)	22 (81%)	5 (19%)
	<5 (12)	5 (41%)	7 (59%)
LGA	≥2 (8)	5 (63%)	3 (37%)
	<2 (19)	5 (26%)	14 (74%)

[a] HGA, high-grade astrocytomas; LGA, low-grade astrocytomas.

(Table IV). In high-grade astrocytomas when the Ki-67 LI was 5 and above, 81% of tumors were hsp27 positive, whereas in the case of a Ki-67 LI below 5, 59% of tumors were hsp27 negative. In low-grade astrocytomas, when the Ki-67 LI was 2 and above, 63% of tumors were hsp27 positive, but when the value was below 2, 74% were hsp27 negative.

DISCUSSION

We identified immunohistochemically expression of small heat shock protein hsp27 in 56% of astrocytomas, with a significantly higher positivity rate and mean percentage of hsp27-positive cells in glioblastomas alone and in the combined high-grade astrocytomas. The recurrent high-grade astrocytomas also had a significantly higher percentage of hsp27-positive cells. While the expression of hsp27 in astrocytic tumors has been reported by other workers (Kato *et al.*, 1992, 1993), the hsp27 positivity rate was higher in our study. The previous authors did not clearly describe the tissue distribution of hsp27 in brain tumors; in the current study, we noted not only the positivity rates, but also the percentage of hsp27-positive tumor cells and areas of distribution in sections of astrocytomas, in which hsp27 expression was remarkably demonstrated in cells and tissue types typical of subtypes of astrocytomas. It has been reported that overexpression of hsp27 and or other stress proteins in breast carcinoma correlated with tumor ploidy level (Chamness *et al.*, 1989) and tumor aggressiveness (Thor *et al.*, 1991). In the current study, expression of hsp27 significantly increased with increasing malignant grade of the astrocytomas.

The Ki-67 LI in astrocytomas noted in the present study is in agreement with reported studies (Montine *et al.*, 1994; Raghavan *et al.*, 1990; Shibuya *et al.*, 1993). We found that hsp27-positive astrocytomas has a significantly higher Ki-67 LI irrespective of histologic grade. Although all hsp27-positive cells were not in the proliferative stage, as assessed by Ki-67 immunostain, our relative comparison suggests that expression of hsp27 increases in parallel with the proliferative activity of astrocytomas and that hsp27 has a profound effect on the growth of astrocytomas. Studies on cell cycle-related hsp27 expression in human astrocytomas are warranted.

The source and exact role of hsp27 in the astrocytic tumor are unknown. It

has been suggested that the balance of interaction between the damaged protein molecules and the stress protein has profound effects that impinge on normal cell growth and differentiation (Morimoto, 1991). In the current study, the overexpression of hsp27 in tumor cells of pseudopalisading areas and in isolated tumor cells of tumor necrotic areas may be an intercellular accumulation of damaged cellular proteins (Morimoto *et al.*, 1991) or tissue hypoxia (Thor *et al.*, 1991) may signal the induction of hsp27 gene transcription in astrocytomas, thereby facilitating rapid entry of tumor cells into the degradative pathway, which would prevent normal cell function. hsp27 was reported to cause rapid stimulation of the *in vitro* growth rate of endothelial cells (Darbon *et al.*, 1986, 1990). In the current study, most of the microvascular endothelial proliferative cells in high-grade astrocytomas overexpressed hsp27, hence endothelial cells may secrete hsp27 during the process of tumor angiogenesis.

The clinical significance of hsp27 expression in human astrocytomas has yet to be studied. It has been suggested that small heat shock protein hsp27 and others can be induced by factors such as fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor (Chambard *et al.*, 1983; Sahai *et al.*, 1986) and by cytokines such as tumor necrosis factor and interleukin (Hepburn *et al.*, 1988; Kaur and Saklatvala, 1988). The growth of human astrocytomas is also highly influenced by the above growth factors and cytokines (Fleming *et al.*, 1992; Rasheed *et al.*, 1994) and may facilitate the synthesis of hsp27 in astrocymas. Thus, it is possible that hsp27 in astrocytomas is crucial as a common postreceptor event after binding of growth factors and cytokines which regulate cellular proliferation and differentiation.

A role for heat shock proteins in general and hsp27 in cellular proliferation has been suggested. As the hsp27 gene contains a sequence that acts as a tumor promoter in *in vitro* transcription systems (Manley *et al.*, 1983), the hsp may inactivate the repressor protein that normally inhibits induction of cell proliferation by disrupting and by preventing the binding of these repressor protein to the DNA target (Chirico *et al.*, 1988; Deshaies *et al.*, 1988); and the hsp increases the level of proteins involved in cell proliferation by inhibiting the synthesis of enzymes required to inactivate the proliferation specific proteins (Key *et al.*, 1981).

In summary, the present study suggests that hsp27 expression correlates highly with the histologic grade of astrocytomas and with tumor growth fractions. hsp27 may participate in the growth of human astrocytic tumors, perhaps functioning as an intercellular signaling molecule in postreceptor events in the common pathway of certain growth factors which facilitate cellular proliferation in human astrocytomas.

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