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Heat shock proteins and survivin: Relationship and effects on proliferation index of retinoblastoma cells

L.-B. Jiang[1]*, X.-Q. Liu[1]*, B. Li[2], X.-J. He[3], Y.-L. Jin[3], L.-Q. Li[2], F. Gao[2] and N.-L. Wang[1]

[1]Capital Medical University Affiliated Eye Center of Beijing Tongren Hospital, [2]Beijing Institute of Ophthalmology and [3]Pathology Department of Capital Medical University Affiliated Beijing Tongren Hospital, China

*The two authors have contributed equally and qualify as the first author.

Summary. Survivin and HSPs (heat shock proteins) are important anti-apoptotic proteins. However, limited research has been done regarding the collective effects of HSPs and survivin on the proliferative activities of RB cells. The purpose of this study was to narrow this gap by focusing on the expression of HSP70 and HSP90 and the interaction of these proteins with survivin. The proliferative activities of RB cells were analyzed by assessing the Ki-67 labeling index. Ki-67 recognizes a nuclear antigen expressed in all phases of the cell cycle except G(0) and early G(1), which makes it an excellent marker of cells in the proliferative phase. Immunohistochemical procedures were performed on retinal tissues from 43 RB patients who had undergone enucleation. Expression of HSP70, HSP90 and survivin was found in 65.12%, 86.05% and 62.79% of the cases respectively. No expression of any of these markers was found in normal retinal tissues. Expression of survivin was more frequent when HSP90 was detected than when HSP90 was not detected ($P<0.05$). The Ki-67 labeling index was higher in cases in which HSP90 or survivin was found than in cases in which neither protein was found ($P<0.05$). The Ki-67 labeling index was higher in cases positive for both HSP90 and survivin than in cases in which neither protein or only one protein was found ($P<0.05$). Expression of HSP70 neither correlated with that of survivin, nor had any significant effect on the Ki-67 labeling index ($P>0.05$). Although expression of HSPs and survivin and the Ki-67 labeling index did not correlate with histopathologic typing of RB ($P>0.05$), our findings demonstrate that expression of HSP90 correlates with that of survivin in RB and the co-existence of survivin and HSP90 probably plays an important role in cellular proliferation in RB. Further work is indicated to clarify the role of these processes in progression of RB.

Key words: Retinoblastoma, Immunohistochemical analysis, Heat shock protein, Survivin, Ki-67

Offprint requests to: Ning-Li Wang or Bin Li, Capital Medical University Affiliated Eye Center of Beijing Tongren Hospital, No 1, Dong-Jiao-Min-Xiang, Dong-Cheng District, 100730, Beijing, China. e-mail: jlbjlb@sina.com

Introduction

Retinoblastoma (RB) is a malignant tumor of the retina that most often occurs in younger children. In order to find possible novel targets for therapy, the detailed molecular mechanisms contributing to the proliferation of RB need to be determined. According to recent studies, heat shock protein (HSP) and survivin, two important categories of proteins inhibiting apoptosis of tumor cells, interact to form the survivin-HSP complex, which suppresses mitochondrial apoptosis and promotes cell proliferation (Fortugno et al., 2003; Suriawinata, 2004). However, limited research has been done regarding the expression of HSPs, the relationship between HSP expression and the expression of survivin in RB, and the mechanism by which the HSP-survivin interaction affects the proliferative activities of RB cells. Proliferation of cells is commonly assessed by analyzing the expression of the Ki-67 antigen, which is located in the nucleus during S, G2 and M phases of the cell cycle. The Ki-67 labeling index is also a significant prognostic indicator for children with RB (Kim et al, 1999). Utilizing immunohistochemical procedures, this study analyzes the expression of HSP70 and HSP90, the interaction of HSP70 or HSP90 expression and the expression of survivin in RB cells, and the impact of HSP-survivin upregulation on the Ki-67 labeling index.

Materials and methods

Subjects

Paraffin-embedded tissues from 43 RB patients who had undergone enucleation were analyzed with

immunohistochemical procedures and compared to normal retinal tissues from the six eyes of three healthy donors. The patients, 27 males and 16 females, were recruited between March 2002 and July 2006. Average age at the time of the surgery was 2.5 years, with the youngest being 3 months and the oldest 8 years. The patients had received no chemotherapy or radiotherapy before the surgery. Depending on whether rosette had formed and the degree of histopathologic differentiation, the tumors were classified as differentiated (15 cases) or undifferentiated (28 cases).

Agents and methods

Mouse monoclonal anti-HSP70, anti-HSP90 and anti-survivin antibodies were purchased from Abcam (U.K.), and diluted in the ratio 1:400, 1:400 and 1:100 respectively. Rabbit anti-Ki-67, histochemical stain-SP kit and DAB kit were purchased from Zhongshan Jinqiao (Beijing, China). Immunohistochemical staining was performed using the labeled streptavidin-biotin peroxidase method. Paraffin-embedded tissues were sectioned at 3 μm, dewaxed and subjected to antigen repair for 2.5 minutes in 10 mmol/L citrate buffer at pH 6.0 in an autoclave sterilizer, followed by incubation in 3% H_2O_2 for 10 minutes to inactivate the endogenous peroxidase. The slides were then incubated with a specific first antibody (anti-HSP70, anti-HSP90, anti-survivin or anti-Ki-67) and the corresponding second antibody according to the instructions in the SP kit, with Haematoxylin-DAB used for staining, resulting in a brown or dark brown signal. Specimens from breast cancer tumors were used as a positive control and substitution of PBS for the first antibody was used as a negative control. Measurements were performed with the HPIAS-1000 image analysis system.

Evaluative criteria

All the sections were evaluated by two independent histopathology experts in a blinded fashion. HSP70 (Fig. 1), HSP90 (Fig. 2) and survivin (Fig. 3) staining was detected in the cytoplasm or/and nucleus. The criteria established by Kawasaki et al. (2001) was applied to distinguish positive cases, and mean percentage of positive tumor cells was determined in 10 fields of view at 400-fold magnification, and only cases with greater than 5% positively stained tumor cells were defined as positive. Ki-67 signal was located in nucleus. The Ki-67 labeling index was obtained by averaging the stained-versus-total ratio in 10 randomly selected fields of view at 400-fold magnification. In each case, a minimum of 2000 cells was counted.

Statistical analysis

To evaluate the relationships between expression of HSPs, survivin, and Ki-67, χ^2 or *t*-tests were conducted and statistical significance was obtained when $P<0.05$. The relationship between expression of these markers and histopathologic type of RB was also evaluated using the χ^2 test.

Results

HSP70, HSP90 and survivin

Among the 43 cases, 28 (65.12%) were positive for HSP70, 37 (86.05%) were positive for HSP90, and 27 (62.79%) were positive for survivin. No HSP70, HSP90 or survivin was detected in the normal retinal tissues. Statistically, expression of survivin was more frequent in cases positive for HSP90 than in cases negative for

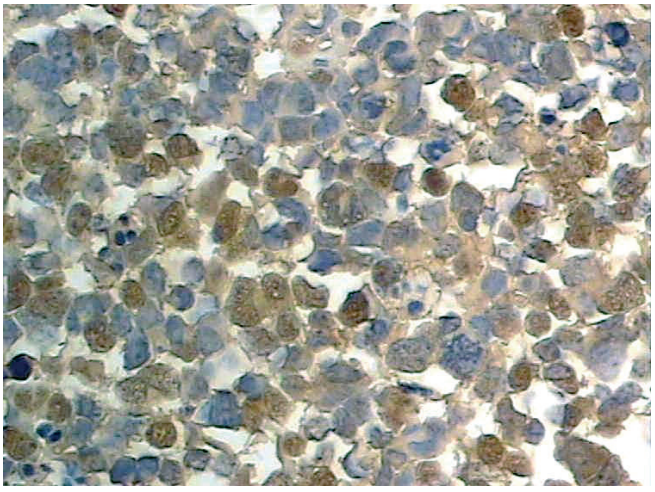

Fig. 1. Staining of HSP70 appeared in nucleus and cytoplasm showing brown (immunohistochemistry, x 400).

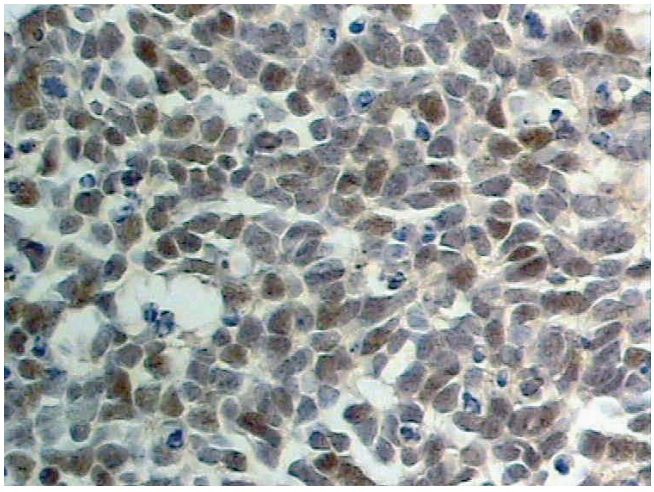

Fig. 2. Staining of HSP90 appeared in nucleus and cytoplasm showing dark brown (immunohistochemistry, x 400).

HSP90 (χ^2=8.851, P<0.05). Expression of HSP70 did not correlate with that of survivin (χ^2=2.563, P>0.05) (Table 1).

Table 1. HSP70, HSP90 and survivin.

	Expression of survivin		χ^2	P
	+	-		
Expression of HSP70				
+	20	8	2.563	0.109
-	7	8		
Expression of HSP90				
+	27	10	8.851	0.003
-	0	6		

Table 2. HSP70, HSP90, survivin and Ki-67 labeling index.

	n	Ki-67 Labeling index (%) ($x \pm s_x$)	t	P
Expression of HSP70				
+	28	22.61±14.41	1.541	0.131
-	15	15.35±15.36		
Expression of HSP90				
+	37	22.56±14.54	5.253	0.000
-	6	4.75±5.89		
Expression of Survivin				
+	27	23.93±15.41	2.298	0.027
-	16	13.58±12.06		
HSP70 and Survivin				
+/+	20	24.54±14.78	1.873	0.068
-/- or +/- or -/+	23	16.20±14.35		
HSP90 and Survivin				
+/+	27	23.93±15.41	2.298	0.027
-/- or +/- or -/+	16	13.58±12.06		

HSP70, HSP90, survivin and the Ki-67 labeling index

The Ki-67 labeling index was significantly higher in cases positive for HSP90 (t=5.253, P<0.05) or survivin (t=2.298, P<0.05) than in cases negative for both proteins. Expression of HSP70 did not significantly correlate with the Ki-67 labeling index (t=1.541, P>0.05) (Table 2).

Expression of HSPs and the Ki-67 labeling index in survivin-positive samples

The Ki-67 labeling index was significantly higher in cases positive for both HSP90 and survivin than in cases negative for one or both (t=2.298, P<0.05), but expression of HSP70 did not correlate with the Ki-67 labeling index regardless of survivin expression (t=1.873, P>0.05) (Table 2).

Table 3. HSP70, HSP90, survivin, Ki-67 labeling index and histopathologic type of RB.

	Histopathologic Type		χ^2	P
	Differentiated	Undifferentiated		
Expression of HSP70				
+	9	19	0.265	0.606
-	6	9		
Expression of HSP90				
+	13	24	0.000	1.000
-	2	4		
Expression of Survivin				
+	9	18	0.077	0.782
-	6	10		
Ki-67 Labeling index (%)	18.09±12.40	21.15±16.31	-0.634	0.530

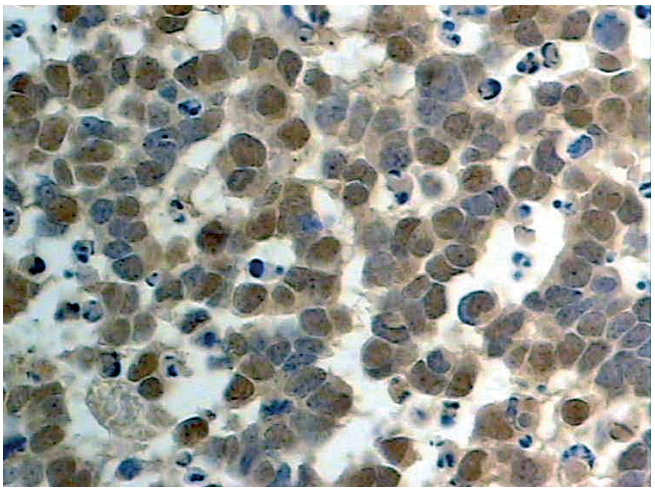

Fig. 3. Staining of survivin appeared in nucleus and cytoplasm showing brown (immunohistochemistry, x 400).

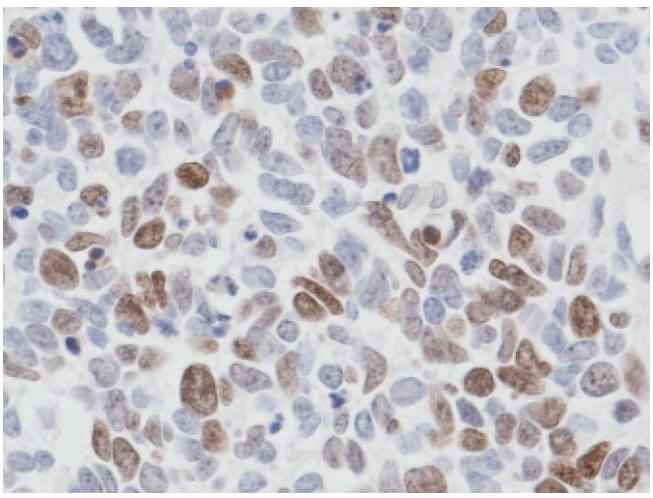

Fig. 4. Staining of Ki-67 was located in nucleus showing brown (immunohistochemistry, x 400).

HSP70, HSP90, survivin, Ki-67 and histopathologic typing of RB

Statistically, no significant difference was identified between the Ki-67 labeling index or expression of HSP70, HSP90 and survivin in differentiated vs. undifferentiated RB cases (χ^2=0.634, 0.265, 0.000, 0.077 respectively, all P>0.05) (Table 3).

Discussion

HSPs are a family of evolutionarily conserved ATPase-directed molecular chaperones that guide proper protein folding, assembly, intracellular disposition and proteolytic turnover. HSPs are required for the stability and functioning of signalling proteins that have been implicated in cancer cell survival. While HSPs are controlled by cell cycle and levels of cellular stress in normal cells, mutated or abnormal proteins can induce the production of HSP in the absence of stress in tumor cells (Mosser and Morimoto, 2004). Some recent studies (Sarto et al., 2000; Ciocca and Calderwood, 2005) have found expression of HSPs, e.g., HSP70 and HSP90, in cancers of body organs such as breast, lung, stomach, prostate and bladder and have shown HSP expression to be associated with the prognosis of patients. The anti-apoptotic function of HSPs can be mediated by the Fas death receptor, JNK/SAPK and the caspase pathway, with differential activity by different members of the HSP family. For example, HSP70 blocks apoptosis by binding the recruitment domain of apoptosis protease activating factor-1 (Apaf-1) and preventing the apoptotic function of caspase-3. HSP90, on the other hand, binds to Apaf-1, RIP and kinase domain of IKKalpha/beta to play the anti-apoptotic role. HSP90 can also suppress TNFα-induced apoptosis (Parcellier et al., 2003; Li et al., 2004).

Nonetheless, the anti-apoptotic role of HSPs in RB has not been well investigated. In this study, expression of HSP70 or HSP90 was found in 65.12% and 86.05% of the cancerous retinas respectively, and no expression of either protein was detected in normal retina. This finding coincides with findings in other malignant tumors, suggesting that HSP70 and HSP90 might inhibit apoptosis in RB via the above mentioned mechanisms. Furthermore, effects of HSPs on the proliferation of tumor cells were evaluated, with Ki-67 used as a biomarker for cell proliferation. Ki-67 recognizes a nuclear antigen expressed in all phases of the cell cycle except G(0) and early G(1), which makes it an excellent marker for determining the growth fraction of a given cell population and a better marker than proliferative cell nuclear antigen (PCNA) (Duchrow et al., 1995; Scholzen and Gerdes, 2000). The Ki-67 labeling index has been widely used in various tumors in the search for a prognostic indictor (Kim et al., 1999; Pollack et al., 2002; Kjellman et al., 2003). Analyzing the proliferative and apoptotic indices of children with RB, Kim et al. (1999) found that the Ki-67 labeling index was a relevant histopathological parameter capable of predicting the clinical outcome of RB. In this study, expression of HSP90 showed a positive correlation with the Ki-67 labeling index, whereas no correlation was detected between expression of HSP70 and the Ki-67 labeling index. This implies that HSP90 might affect the proliferation of RB more than HSP70 does, suggesting that anti-apoptotic pathways activated by HSP90 might be more crucial for the prognosis of children with RB.

Taking into account previous findings (Fortugno et al., 2003) indicating that the survivin-HSP complex involves HSP90 rather than HSP70, we put forth the hypothesis that the above mentioned differential impact on cell proliferation by HSP90 and HSP70 is mediated by survivin. Survivin is a unique member of the IAP (inhibitor of apoptosis protein) family in that it contains a single BIR (baculovirus IAP repeat) domain combined with a COOH-terminal alpha-helix coiled-coil domain rather than the more common zinc-binding RING finger. As one of the most powerful inhibitors of apoptosis, survivin directly weakens the activities of caspase-3 and caspase-7 and is expressed in the G2/M phase of cell cycle in a cycle-regulated manner. Because survivin selectively binds microtubules of the mitotic spindle, it had been commonly believed to be a checkpoint for tumor cell mitosis (Shin et al., 2001; Altieri, 2003). However, a recent study by Fortugno et al. (2003) revealed that survivin interacted with HSP90 through both its own BIR domain and the ATPase domain of HSP90. This and other data indicate that the HSP90-survivin complex resulting from cellular stress responses was the real checkpoint for tumor cell mitosis. The same study also found that HSP90, a molecular chaperone, was required for maintaining the stability and biological activity of survivin.

We detected the expression of survivin in RB tissue, confirming the research by Huang et al. (2004). The significant correlation between survivin and the Ki-67 labeling index suggests that survivin significantly affects the proliferation of RB cells. In further support of this hypothesis, the immunohistochemical analysis demonstrated that the expression of survivin positively correlates with the expression of HSP90 and the Ki-67 labeling index is significantly higher in RB tumors in which both HSP90 and survivin are present than in RB tumors in which neither or only one protein is present. Based on the results, a synergistic action seems to exist between survivin and HSP90, which probably contributes to the proliferation of RB cells. No statistical correlation was found between expression of survivin and HSP70. However, given that the correlation between survivin-HSP70 co-expression and the Ki-67 labeling index approached marginal statistical significance (P=0.068), further investigation is probably needed to decide whether a synergistic action exists between survivin and HSP70. Finally, this study found that the histopathologic typing of RB did not correlate with expression of any of the markers (HSP70, HSP90, or survivin) or with the Ki-67 labeling index.

In summary, our results suggest that expression of HSP90 and survivin are correlated and the expression of both markers as well as their co-expression could affect the Ki-67 labeling index. Future investigations should be directed toward examining the mechanisms by which co-expression of survivin and HSP90 affects the prognosis of children with RB, and evaluation of this co-expression in regard to its value as a predictive indicator. The significance of these markers as therapy targets for RB also deserves further study.

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References

Altieri D.C. (2003). Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 22, 8581-8589.

Ciocca D.R. and Calderwood S.K. (2005). Heat shock proteins in cancer: Diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10, 86-103.

Duchrow M., Schluter C., Key G., Kubbutat M.H., Wohlenberg C., Flad H.D. and Gerdes J. (1995). Cell proliferation-associated nuclear antigen defined by antibody Ki-67: A new kind of cell cycle-maintaining proteins. Arch. Immunol. Ther. Exp. 43, 117-121.

Fortugno P., Beltrami E., Plescia J., Fontana J., Pradhan D., Marchisio P.C., Sessa W.C. and Altieri D.C. (2003). Regulation of survivin function by Hsp90. Proc Natl. Acad. Sci. USA 100, 13791-13796.

Huang M., Ou X. and Liang Y. (2004). Detection of multiple apoptosis related proteins in retinoblastoma. Chin. J. Ocular Fundus Dis. 20, 215-217.

Kawasaki H., Toyoda M., Shinohara H., Okuda J., Watanabe I., Yamamoto T., Tanaka K., Tenjo T. and Tanigawa N.(2001). Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 91, 2026-2032.

Kim C.J., Chi J.G., Choi H.S., Shin H.Y., Ahn H.S., Yoo Y.S. and Chang K.Y. (1999). Proliferation not apoptosis as a prognostic indicator in retinoblastoma. Virchows Arch. 434, 301-305.

Kjellman P., Wallin G., Hoog A., Auer G., Larsson C. and Zedenius J. (2003). MIB-1 index in thyroid tumors: a predictor of the clinical course in papillary thyroid carcinoma. Thyroid 13, 371-380.

Li Z., Zhao X. and Wei Y. (2004). Regulation of apoptotic signal transduction pathways by the heat shock proteins. Sci. China C Life Sci. 47, 107-114.

Mosser D.D. and Morimoto R.I. (2004). Molecular chaperones and the stress of oncogenesis. Oncogene 23, 2907-2918.

Parcellier A., Gurbuxani S., Schmitt E., Solary E. and Garrido C. (2003). Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem. Biophys. Res. Commun. 304, 505-512.

Pollack I.F., Hamilton R.L., Burnham J., Holmes E.J., Finkelstein S.D., Sposto R., Yates A.J., Boyett J.M. and Finlay J.L. (2002). Impact of proliferation index on outcome in childhood malignant gliomas: results in a multi-institutional cohort. Neurosurgery 50,1238-1245.

Sarto C., Binz P.A. and Mocarelli P. (2000). Heat shock proteins in human cancer. Electrophoresis 21, 1218-1226.

Scholzen T. and Gerdes J. (2000). The Ki-67 protein: from the known and the unknown. J. Cell Physiol. 182, 311-322

Shin S., Sung B. and Cho Y. (2001). An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and-7. Biochemistry 40, 1117-1123.

Suriawinata A. (2004). Survivin: Apoptosis inhibitor and its regulation by Hsp90. Lab. Invest. 84, 395.

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