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Original paper

Expression of heat shock protein 27 and proliferating cell nuclear antigen in human retinoblastoma

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Aim of the study: This study aimed to observe the expressions of heat shock protein 27 (HSP27) and proliferating cell nuclear antigen (PCNA) in retinoblastoma (Rb) cells and to explore the relationships of the expression with Rb differentiation and optic nerve infiltration.

Material and methods: Heat shock protein 27 and PCNA expressions in 36 routine Rb paraffin specimens were observed using PV9000 two-stage immunohistochemical staining. The correlations of the HSP27 and PCNA expressions with Rb differentiation and optic nerve infiltration were analyzed.

Results: Heat shock protein 27 was weakly expressed in the normal retina, specifically in the ganglion cell layer. It was extensively expressed in Rb tissues at a positive rate of 69.4%, and the positive substances were primarily located in the cytoplasm. Proliferating cell nuclear antigen was expressed weakly or not at all expressed in the normal retina and was extensively expressed in Rb tissues at a positive rate of 83.3%, and the positive substances were primarily located in the nucleus. The positive expression rates of HSP27 and PCNA in the differentiated group were significantly higher than in the undifferentiated group ($p < 0.05$). The positive expression rates of HSP27 and PCNA in the optic nerve-infiltrated group were significantly higher than in the non-infiltrated group ($p < 0.05$). Heat shock protein 27 expression was positively correlated with PCNA expression in Rb ($p < 0.01$).

Conclusions: Heat shock protein 27 and PCNA expressions are markedly correlated with cell differentiation and optic nerve infiltration in Rb.

Key words: retinoblastoma, HSP27, proliferating cell nuclear antigen, immunohistochemistry.

Introduction

Heat shock proteins (HSPs) are found extensively in prokaryocytes and eukaryocytes. Heat shock proteins participate in protein synthesis, folding, accumulation, assembly, transportation, and degradation, functioning as molecular chaperones. Furthermore, their expressions increase under stressful conditions to protect cells from potential injuries caused by stress and cytotoxic effects, stabilize intracellular protein conformation, and prevent the occurrence of apoptosis [1–3]. According to the relative molecular weight classification, the important members of HSPs include HSP27, HSP60, HSP70, and HSP90, among others. Heat shock protein 27 is a member of the micromolecular HSP family, and its expression increases in numerous kinds of malignancies [4–6]. Furthermore, HSP27 overexpression is also closely correlated with various carcinogenic factors, such as apoptotic inhibition, enhanced cell protection, and multidrug resistance [7–9]. Overexpressed HSP27 binds to associated proteins at apoptotic accommodation points to exert a direct inhibitory effect on apoptosis. Overexpressed HSP27 also prevents the formation of apoptotic bodies either by directly isolating mitochondria-released cytochrome C or by preventing mitochondria from releasing cytochrome C [1, 2, 9]. Heat shock protein 27 is weakly expressed in normal cells but highly expressed in tumors, such as breast cancer, ovarian cancer, endometrial cancer, gastric cancer, colorectal cancer, hepatocellular cancer, bladder cancer, prostate cancer, and leukemia [10–15]. In breast cancer, ovarian cancer, gastric cancer, and prostate cancer, HSP27 overexpression is correlated with tumor infiltration, as well as chemotherapeutic and radiotherapeutic tolerance [7, 16, 17].

Retinoblastoma (Rb) is a common malignancy in children. In recent years, Rb has shown an increasing trend in incidence. The occurrence of Rb originates from a mutation in the Rb gene. Retinoblastoma poses a risk of serious vision and eye impairment, as well as life endangerment to children. However, to the best of our knowledge, no study on the correlation between HSP27 expression and Rb progression has been reported in the literature to date.

In the current study, the correlations of HSP27 expression in Rb cells with PCNA expression, Rb differentiation, and optic nerve infiltration were investigated. The role and pathological significance of HSP27 expression in Rb cell proliferation and migration were also explored.

Material and methods

Samples

Thirty-six post-eye enucleation Rb tissue specimens were investigated. The specimens were collected from the Second Xiangya Hospital of Central South University and Nanhui Central Hospital between 1999 and 2010, with

complete records. The Rb children participants included 17 males and 19 females with a mean age of 2.25 years (ranging from 6 months to 8 years). All patients suffered from single eye Rb, and did not receive radiotherapy or chemotherapy before operation. Of the 36 Rb samples, 24 were undifferentiated (66.7%), 12 were differentiated (33.3%), 15 were optic nerve-infiltrated (41.8%), and 21 were non-infiltrated (58.2%). In addition, two normal retina specimens were used as the control. The procedure of this study was approved by the Ethics Committee of the Nanhui Central Hospital and the Second Xiangya Hospital.

Immunohistochemistry

All specimens were fixed in 10% paraformaldehyde, routinely embedded in paraffin, serially sectioned (4 µm), and stained with hematoxylin and eosin for observation under a light microscope. The Rb specimens were divided into differentiated and non-differentiated groups according to the presence or absence of rosettes. The specimens were also divided into optic nerve-infiltrated and non-infiltrated groups according to tumor infiltration.

PV9000 two-stage staining was done. The paraffin-embedded sections were dewaxed, dehydrated, and incubated with 3% peroxidase for 10 min at room temperature to block endogenous peroxidase activity. The sections were rinsed with distilled water and saturated in phosphate buffered saline (PBS) for 5 min. The sections were incubated overnight at 4°C with a 1 : 100 dilution of mouse anti-monoclonal antibody (primary antibody; Beijing Zhongshan, China). The sections were incubated with Polymer Helper reagent for 20 min at 37°C and rinsed with PBS. Afterwards, the samples were incubated with poly peroxidase-anti-rabbit IgG for 20 min at room temperature. After PBS washing, the sections were stained with diaminobenzidine solution (Beijing Zhongshan, China), counterstained with hematoxylin, routinely dehydrated, and then mounted. Phosphate buffered saline replaced the antibodies as a negative control, and a known positive section was used as a positive control.

Observation indices and result determination

Cells with buffy-stained cytoplasms or buffy-stained nuclear particles were determined as HSP27-positive cells. PCNA-positive cells were those with specific buffy particles in the nucleus. Result determination was performed using the color morphometric image analysis system designed by the Beijing University of Aeronautics & Astronautics, China. In this system, five high-power visual fields were randomly selected for each section under light microscopy, and a mean cell count was obtained. A positive cell count ≤ 20% was assigned a (–), whereas > 20% was assigned a (+).

Statistical analysis

Data were analyzed using the SPSS10.0 software. χ^2 tests (2 × 2 table exact probability analysis), rank sum tests, and Spearman correlation analysis were performed. $P < 0.05$ was considered statistically significant.

Results

Clinical data

As shown in Table 1, the patients had no history of chemotherapy.

Immunohistochemical staining for HSP27 and PCNA

Heat shock protein 27 was weakly expressed in the normal retinal nerve fiber layer (Fig. 1). In contrast, positive HSP27 was strongly expressed in 25 of 36 Rb tissues (69.4%), and the positive substances were primarily located in the cytoplasm and partly in the nucleus and membrane. Proliferating cell nuclear antigen was expressed weakly or not expressed in the normal retina, whereas positive PCNA was strongly expressed in 30 Rb tissues with a positive rate of 83.3%. The stained site was located in the nucleus rather than in the cytoplasm.

Correlations of HSP27 and PCNA with Rb differentiation

The positive expression rates of HSP27 and PCNA in the undifferentiated group were significantly higher than in the differentiated group ($p < 0.05$; Table 2). Heat shock protein 27 expressions in the Rb differentiated and undifferentiated groups are shown in Fig. 2, and the PCNA expressions in these two groups are shown in Fig. 3.

Correlations of HSP27 and PCNA with Rb optic nerve infiltration

The positive expression rates of HSP27 and PCNA in the infiltrated group were significantly higher than in the non-infiltrated group ($p < 0.05$; Table 3).

Correlation between HSP27 and PCNA in Rb tissues

The linear correlation analysis showed that HSP27 expression was positively correlated with PCNA expression in the Rb tissues ($r = 0.4785$, $p < 0.01$).

Discussion

Heat shock proteins are groups of intracellular proteins synthesized under physiological, pathological, or stressful conditions. They bind with highly conservative amino acid sequences, as well as genes encoded by these sequences, and serve as molecular chaperones to participate in the synthesis, folding, accumulation, assembly, transportation, and degradation of proteins. Additionally, under stressful conditions, HSPs increase cellular resistance to stress injuries [1, 18]. On one hand, increased HSP expression under stressful conditions protects cells against the potential injuries caused by stress and cytotoxicity. On the other hand, HSPs exert the functions of molecular chaperones to stabilize protein conformations in cells, thus preventing apoptosis [19]. Heat shock protein 27 is a member of the micromolecular HSP family. Under a normal state, HSP27 is weakly expressed in cells; however, once stress occurs, HSP27 expression increases, exerting an anti-oxidative damage function [20].

Table 1. Clinicopathological profiles in retinoblastoma cases examined in this study

No.	Age	Gender	Side	Differentiation	Optic nerve invasion	HSP27	PCNA
1	0.5	F	R	Un	+	+	+
2	1	F	R	Un	+	+	?
3	0.5	M	L	well	?	?	+
4	4	F	R	Un	+	+	+
5	0.5	M	R	Un	?	+	+
6	5	M	L	Well	+	+	+
7	4	M	R	Un	+	+	+
8	1	F	R	Well	?	?	?
9	0.5	F	R	Well	?	+	+
10	2	M	L	Un	+	?	+
11	4	F	R	Un	?	+	+
12	0.5	F	L	Well	+	?	+
13	8	M	R	Un	+	+	+
14	3	F	L	Well	?	?	+
15	1	F	R	Un	+	+	+
16	2	M	L	Un	?	+	+
17	3	F	R	Un	?	?	+
18	2	M	R	Well	+	+	+
19	2	F	L	Un	?	?	+
20	1	M	R	Un	+	+	+
21	5	M	L	Well	?	+	?
22	2	M	R	Un	+	+	+
23	1	F	L	Un	?	?	?
24	3	F	R	Well	?	+	+
25	1	M	L	Un	?	+	?
26	3	F	R	Un	?	+	+
27	1	M	R	Un	?	+	+
28	3	F	R	Well	+	?	?
29	2	M	L	Un	?	+	+
30	4	M	L	Un	?	+	+
31	2	M	R	Well	?	+	+
32	1	F	L	Un	+	?	+
33	2	F	L	Un	?	+	?
34	3	F	L	Un	?	?	+
35	2	F	L	Well	?	+	+
36	1	M	R	Un	+	+	+

Un – undifferentiated, Well – well-differentiated

The correlations of HSP27 with clinical diseases, especially with tumors, have attracted increasing attention. Heat shock protein 27 is overexpressed in numerous types of tumors such as breast and ovarian cancers, which promotes tumor development. Heat shock protein 27 expression reflects the malignant potentials and prognoses of tumors [21–23]. Heat shock protein 27 overexpression is associated with tumor infiltration in breast cancer, such that HSP27 antibody treatment can increase the survival rate of patients with breast cancer [12]. Therefore, further investigation into

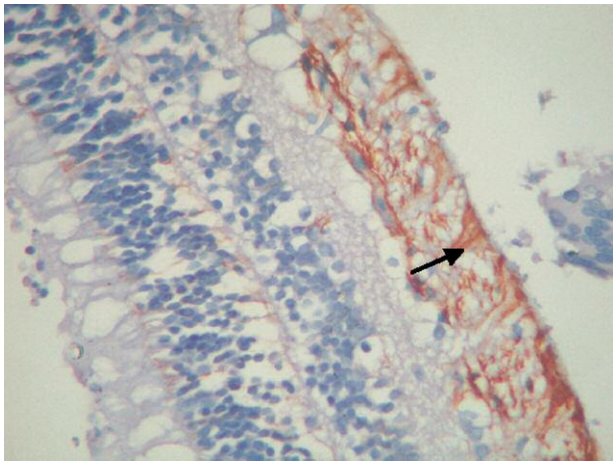

Fig. 1. HSP27 expression in the ganglion cell layer of the normal retina (DAB, magnification 200×)

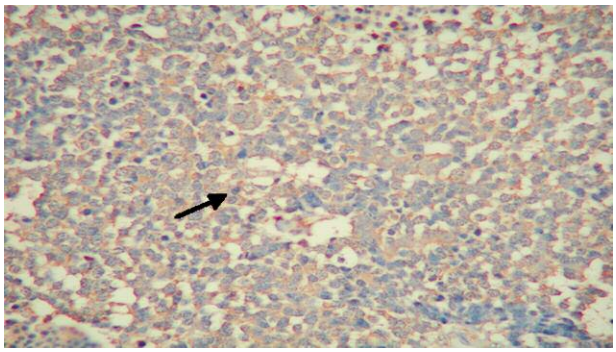

Fig. 2. HSP27 positive expression in Rb tissues: The positive particles are mainly located in cytoplasm (DAB, magnification 200×)

Table 2. Relationship of the positive expression of HSP27 and PCNA with the differentiation degrees of Rb

Groups	*n*	HSP27		PCNA	
		+	−	+	−
undifferentiated	24	16	8	20	4
differentiated	12	9	3	10	2
P		0.0342		0.0246	

Table 3. Relationships of the positive expression of HSP27 and PCNA with nerve infiltration of Rb

Groups	*n*	HSP27		PCNA	
		+	−	+	−
A	15	12	3	14	1
B	21	13	8	16	5
P		0.0295		0.0205	

Group A – the optic nerve infiltrated group, group B – non-infiltrated group

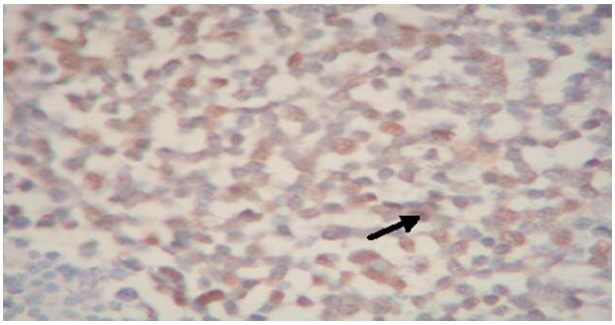

Fig. 3. PCNA positive expression in Rb tissues: The positive particles are mainly located in the nucleus (DAB, magnification 400×)

HSP27 expression in patients with tumors and the regulatory mechanism of HSP27 expression is of great clinical significance in the treatment of tumors.

Retinoblastoma is one of the most common malignancies in children. Increasing the early diagnosis rate and improving the cure for Rb are the keys to Rb treatment. However, although extensive studies on the biological characteristics, role, and mechanism of action of HSP27, as well as its correlations with clinical diseases, especially with tumors, have already been conducted, reports on the roles of HSP27 in the development of Rb are rare in the literature.

The present study shows that positive HSP27 was mainly located in the cytoplasm and extensively expressed in the Rb tissues. Furthermore, the positive expression rate of HSP27 was noticeably higher in the undifferentiated group than in the differentiated group ($p < 0.05$). Abnormal HSP27 expression presumably arises from DNA and/or cell injuries caused by stress, which cannot be cleared up completely. When injuries accumulate in cells to a certain level, abnormal HSP27 expression consequently leads to mutation or apoptosis. This presumption further suggests that HSP27 can play a role in the differentiation of Rb cells and promote the development of Rb. The presence of HSP27 may be an early event in the development of tumors. Heat shock protein 27 expression increases with disease progression. Thus, HSP27 expression has a certain indicative significance in determining the malignant potentials and differentiation levels of tumors [24].

In addition, evaluating cell proliferation is also of great significance in clarifying the biological characteristics of tumors. Proliferating cell nuclear antigen is a type of intranuclear polypeptide, which can only be synthesized and expressed in proliferating cells. It binds with DNA polymerase accessory proteins to participate in cellular DNA replication. Proliferating cell nuclear antigen expression is associated with the cell cycle and is primarily expressed in the S, G1, and early G2 phases of a cell proliferation cycle. A previous study has indicated that PCNA is correlated with the development, differentiation, metastasis, and prognosis of tumors. Thus, PCNA expression can be used as an index for evaluating the proliferation of malignant tumor cells, as well as the malignant potential and prognosis of a tumor [25]. The correlation analysis in this study shows that PCNA expression is correlated with the differentiation and optic nerve infiltration of Rb cells. Positive PCNA was more extensively expressed in the poorly differentiated and optic nerve-infiltrated Rb tissues. This finding is consistent with those reported in the literature [26, 27].

Furthermore, the present study shows a positive correlation between HSP27 and PCNA expressions in Rb. The expression rates of HSP70 and HSP90 in Rb tissues are 65.12% and 86.05%, respectively. HSP90 is positively correlated with Ki-67 (its expression has been used as an index for tumor cell

proliferation evaluation), whereas HSP70 has no close correlation with Ki-67 [28]. These findings indicate that although the HSP family is correlated with the initiation, development, differentiation, and metastasis of various malignancies, its members demonstrate different specificity to tumors located at different sites. Chemical reduction surgery can lead to high HSP27 expression in Rb. Heat shock protein 27 is closely correlated with the drug resistance of tumor cells by inhibiting their apoptosis [29]. In the present study, the results show that PCNA expression was positively correlated with HSP27 expression in Rb tissues ($p < 0.01$), and HSP27 was more extensively expressed in the undifferentiated and optic nerve-infiltrated Rb tissues. The reason may be that HSP27 correlates with the initiation, development, and metastasis of tumor cells by inhibiting numerous apoptotic pathways of tumor cells. Heat shock protein 27 was previously confirmed to promote the occurrence of drug resistance by regulating apoptosis-associated proteins, particularly as follows:

- it reduces the release of cytochrome C and the activation of cysteine-containing aspartate-specific protease 9 by stabilizing the cytoskeleton and the translocation of apoptosis-promoting proteins toward mitochondrial membranes [30];
- HSP27 binds with and activates the serine/threonine protein kinase Akt, which deactivates the Akt substrates, such as Bad and caspase-9 (apoptosis-promoting proteins), thereby inhibiting apoptosis [31];
- it inhibits death domain-associated proteins from exerting inhibitory effects on the apoptosis of signal-regulating kinase-1 associated apoptotic pathways [32]; and
- although HSP27 has no influence on the release of cytochrome C or on the activity of Apaf-1 and caspase-9, it binds with caspase-3 and inhibits its activity, thereby functioning as a downregulatory factor inhibiting the apoptosis of tumor cells [30, 33].

Retinoblastoma is one of the most common malignancies in children. However, although extensive studies on HSP27 have already been conducted, several issues concerning its mechanism of action in Rb still need further investigation.

The authors declare no conflict of interest.

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