

Expression and its clinical significance of heat shock protein gp96 in human osteosarcoma

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The aim of the study was to observe the expression of heat shock protein gp96 (HSPgp96) and explore its clinical significance in human osteosarcoma.

The expression of HSPgp96 was studied in 44 osteosarcoma tissues including 24 osteoblastic sarcoma and 20 chondroblastic sarcoma, normal tissues adjacent to the sarcomas were evaluated simultaneously.

The immunoreactivity was found positive in all osteosarcoma tissues (44/44), but 21.5% (9/44) in normal tissues. HSPgp96 was mainly expressed in cytoplasm of osteoblastic sarcoma, while in nucleus of chondroblastic sarcoma. HSPgp96 immunolabelling had significantly correlation with the Price degree ($P < 0.05$), but not with the clinical stages ($P > 0.05$), and histological subtypes ($P > 0.05$).

The HSPgp96 is highly expressed in osteosarcoma and has different immunolocalization during two subtypes of osteosarcoma. The immunopositivity is significantly higher in tumors with lower differentiation. The research implies that HSPgp96 may play a contributive role on the pathogenesis and development in human osteosarcoma, and there is hope in its application in determining the degree of malignancy of cancer and utilization as a target for tumor immunity.

Key Words: heat shock protein gp96, immunoreactivity, osteoblastic sarcoma, chondroblastic sarcoma.

Heat shock proteins (HSPs) were otherwise called molecular chaperones for its function in preventing misfolding polypeptides and facilitating protein folding [1]. Recently, it has been further reported that HSPs play important role in antigen presentation, activation of macrophages and lymphocytes, maturation of dendritic cells [2, 3], which are indispensable for tumor immunity and its dysfunction may help the tumor cells escape the immune surveillance with the result of advancing the pathogenesis and development in human sarcoma. Recent research reported that human colonocyte gp96 serves as a plasma membrane binding protein that enhances cellular entry of TxA, participates in cellular signaling events in the inflammatory cascade, and facilitates cytotoxicity [4].

HSPgp96 (heat shock protein glycoprotein of 96 KD) is one member of the HSPs family, it is ubiquitously resided in cells [5]. Just as its other family members, HSPgp96 exists as important biochemical regulators in mediating cell growth, apoptosis, protein homeostasis and cellular targets of peptides [6, 7]. It is commonly accepted that HSPgp96 is expressed on the endoplasmic reticulum. Recent researches pointed out that it is expressed on the tumor cell cytoplasmic as well, and enhanced with the increased tumor immunogenicity [8].

Recent studies indicate that HSPgp96 is highly expressed in cancer tissues and may be used as prognostic marker in some tumors [9–11]. Osteosarcoma is the most common primary malignancy of the bone, with the characteristics of highly malignancy, highly metastasis, and insensitive to radiotherapy and chemotherapy, which affects mainly adolescents and young adults, and the survival and prognosis of the patient is poor during the last 30 years.

Our research used immunohistochemistry to determine the expression of HSPgp96 in osteosarcoma tissues, as well as the normal tissues adjacent to the sarcoma, in order to explore the distribution of these molecules and its clinical significance.

Materials and methods

Obtainment of osteosarcoma tissues. The informed consent from the patients or their relatives was obtained. As well, this investigation was performed according to the guidelines approved by the Institutional Human Care and Use Committee. Paraffin wax embedded specimens from 44 patients with osteosarcoma undergoing radical resection were collected from the Renmin Hospital of Wuhan University, Wuhan, Hubei,

China between April, 2003 and August, 2008. Normal tissues adjacent to the sarcomas were obtained simultaneously to form the control group.

The patients included 18 males and 26 females, with a mean age of 23, ranging from 11 to 39. Routine pathological diagnosis confirmed that all cases were osteosarcoma. 24 cases were osteoblastic sarcoma, 20 cases were chondroblastic sarcoma. According to pathological of the Price grade, there were 11 cases of Grade I, 18 of Grade II and 15 of Grade III. According to the classification of Enneking stages, there were 12 cases of stage IA, 18 cases of stage IIA, 10 cases of stage IIB and 4 cases of stage III. All the patients had not accepted any pre-operative radiotherapy, chemotherapy, and were not involved in viral hepatitis, immunological diseases and so on.

Observing the expression of HSPgp96 in human osteosarcoma tissues. Immunohistochemical labeling methods. The specimens were fixed in 10% buffered formalin and processed to embedding in paraffin wax by routine protocol. Serial sections of 5 μ m thickness were cut by microtome and mounted on silane-coated glass slides. All sections were deparaffinised and rehydrated through graded alcohols (100%, 95%, 80%) by routine protocols. Endogenous peroxidase was then blocked with 3% H₂O₂ diluted in methanol for 30 min at room temperature. Antigen retrieval was performed by heating the slides in citrate buffer in a microwave oven for 10 min. The slides were then incubated in a humid chamber with the rabbit anti-human gp96 polyclonal antibody (Wuhan Boster Biological Technology, Wuhan, Hubei, China), at a dilution of 1:100, at 4 °C, overnight. The slides were then incubated with horseradish peroxidase-labelled goat anti-rabbit antibody (Wuhan Boster Biological Technology, Wuhan, Hubei, China), diluted 1:100, for 45 min at 37 °C. Slides were then developed with

DAB color kit (Wuhan Boster Biological Technology, Wuhan, Hubei, China) with 0.03% hydrogen peroxide for 8 min, and then counterstained with hematoxylin, dehydrated through graded alcohols (80%, 95%, 100%), air-dried and mounted in neutral resins. PBS was used as a substitute for the primary antibody to serve as a negative control.

Evaluation of immunolabeling. Two of the authors initially examined the sections simultaneously using a dual-headed light microscope. The evaluation of HSPgp96 positive cells was performed on high-power fields (\times 400) using a standard light microscope.

Cell with distinctive brown particles was considered positive. Those samples whose positive cells account for 25%-49% of the total cells were counted as (+), 50% -74% as (++) , 75% -99% as (+++). 100% as (++++), those less than 24% were defined as negative. No specific immunoreactivity was detected in the tissue sections of negative control group.

Statistical analysis. All data were dealt statistically with SPSS13.0 statistical software package. The overall differences among the three groups (the osteoblastic group, the chondroblastic group and the control group) and the differences between each two groups were analyzed respectively using Kruskal-Wallis method. The relationship of HSPgp96 immunoreactivity with Price degree, clinical stage and histological subtypes were analyzed respectively using Spearman Rank Relation test. $P < 0.05$ was considered as statistically significance.

Results

Immunopositivity of HSPgp96 in osteosarcoma and normal tissues adjacent to sarcoma. The results of immunohistochemistry of HSPgp96 were summarized in Table 1. HSPgp96

Table 1. Relationship between clinical pathology and immunoreactivity of HSPgp96 expression in osteosarcoma.

Pathologic types	n	HSPgp96				
		- (%)	+ (%)	++ (%)	+++ (%)	++++ (%)
Normal tissues adjacent to sarcoma	44	35(79.5)	9 (21.5)	0 (0)	0 (0)	0 (0)
Osteosarcoma tissues*	44	0 (0)	2 (4.5)	4 (9.1)	8 (18.2)	30 (68.2)
Subtypes						
Osteoblastic type**	24	0 (0)	1 (4.2)	2 (8.3)	5 (20.8)	16 (66.7)
Chondroblastic type**	20	0 (0)	1 (5.0)	2 (10.0)	3 (15.0)	14 (70.0)
Price grade						
Grade I	11	0 (0)	1 (9.1)	2 (18.2)	2 (18.2)	6 (54.5)
Grade II	18	0 (0)	1 (5.6)	1 (5.6)	3 (16.7)	13 (72.1)
Grade III	15	0 (0)	0 (0)	1 (6.7)	3 (20.0)	11 (73.3)
Enneking stages						
I A	12	0 (0)	1 (8.3)	1 (8.3)	2 (16.7)	8 (66.7)
II A	18	0 (0)	0 (0)	1 (5.6)	3 (16.7)	14 (77.7)
II B	10	0 (0)	1 (10.0)	2 (20.0)	2 (20.0)	5 (50.0)
III	4	0 (0)	0 (0)	0 (0)	1 (25.0)	3 (75.0)

* $P < 0.01$, vs. normal tissues adjacent to sarcoma.

** $P < 0.01$, vs. normal tissues adjacent to sarcoma.

* $P < 0.05$, vs. Price grade I group.

** $P < 0.05$, vs. Price grade I and II groups.

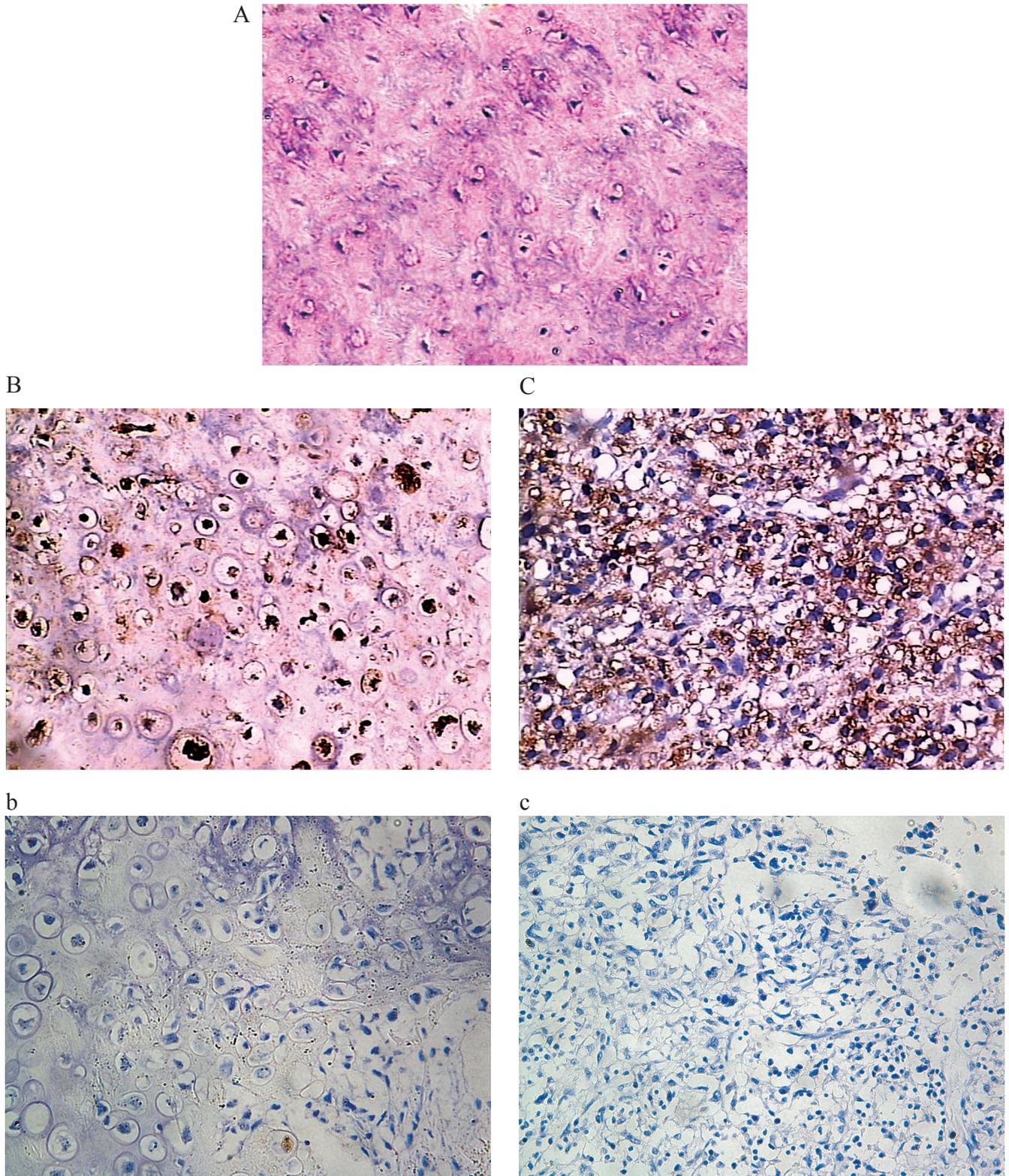


Figure 1. Immunohistochemistry for HSPgp96 in osteosarcoma cells and normal tissues adjacent to sarcoma (A); (B) distinctive cell nucleus and cytoplasm immunoreactivity was detected for HSPgp96 in chondroblastic osteosarcoma, negative control shows in (b); (C) distinctive cell cytoplasm immunoreactivity was detected for HSPgp96 in osteoblastic osteosarcoma, negative control shows in (c), (arrows). Scale bars: 20 μm.

immunopositivity was detected in all of the 44 osteosarcoma cases, and in the positive cell, brown and tan particles of different size were observed, illustrated in Figure 1. In contrary, HSPgp96 expressed mildly and stained lightly in adjacent normal tissues, the positive samples account for 21.5% (9/44). Thus, there was significantly difference between the osteosarcoma group and the control group ($P < 0.01$).

Immunolocalization of HSPgp96 two subtypes of osteosarcoma tissues. There are two expression patterns of HSPgp96. In the osteoblastic osteosarcoma, HSPgp96 was detected mainly in cytoplasm, illustrated in Figure 1(C). Meanwhile, in the chondroblastic osteosarcoma, HSPgp96 was localized extensively in nucleus though it can also be found in cytoplasm lightly, illustrated in Figure 1(B).

Relationship of HSPgp96 immunoreactivity with Price degree, clinical stage and histological subtypes. The immunoreactivity of HSPgp96 in osteosarcoma was significantly higher than in the tissues adjacent to sarcoma ($P < 0.01$). Price Grade II and Grade III groups showed distinctly higher immunopositive values than Grade I groups ($P < 0.05$). There were no significantly differences of HSPgp96 immunoreactivity among the Enneking IA, IIA, IIB and III stage group ($P > 0.05$). These result suggest that there exists a significant correlation between immunopositivity of HSPgp96 and progression of osteosarcoma ($P < 0.05$, $r = 0.92$), but not clinical stage ($P > 0.05$). As well, the immunopositive difference between the two distinctive subtypes of osteosarcoma had no statistical significance ($P > 0.05$). Results are summarized in Table 1.

Discussion

Heat shock protein (HSP) gp96 that is a member of the HSP90 family has been described as a classical ER-resident protein, but later studies show its surface expression on plasma membrane of some tumor cells [12, 13]. It is still unknown that the mechanism by which gp96 is directed and retained on the plasma membrane. Another study showed that gp96 exerts also protective function during cellular stress and plays an important role in the maintenance of protein homeostasis. The expression and function of HSPgp96 in osteosarcoma have not been reported up to now.

In this study, HSPgp96 immunopositivity was detected in 9 of 44 adjacent normal tissues, it was expressed mildly, stained lightly in these positive cells, which suggests that HSPgp96 was of low and weak expression in normal tissues. In contrast, HSPgp96 immunopositivity was detected in all osteosarcoma tissues, significantly higher than the control group. This is coincident with the viewpoint that HSPgp96 which serves as molecular chaperone is related with antigen-presenting of tumor cell, and its expression enhances with the increased immunogenicity [6, 12].

In normal cells, the expression of heat shock protein 90 (HSP90) is regulated by cell cycle, but in tumor cells the continuous high-expression of HSP90 does not require stress conditions. The mutational or abnormal proteins can

directly stimulate the synthesis of HSP90 [14]. As HSPgp96 is a member of HSP90 family, we speculate that the high-expression of HSPgp96 in osteosarcoma cells is stimulated by the mutational or abnormal proteins as well. At the same time, HSPgp96 also known as glucose-regulated protein 94 (grp94), is easily induced by lack of glucose. Along with the tumor volume increasing, ischemia and hypoxia aggravated, consumption and shortage of glucose accumulating, HSPgp96 expressed more visibly. HSPgp96 was positive expressed in the osteosarcoma cells on account of compensation for the disruption of gene expression or various harmful stresses. Both constituted and induced types of HSPgp96 were transported from cytoplasm to nucleus (especially transported in the nucleolus) under the stress state, so as to rectify the disorder of information transfer.

Now we reported a novel finding that there exist two distinct expression patterns during the two subtypes of osteosarcoma studied in our research. In the osteoblastic osteosarcoma, HSPgp96 was detected mainly in cytoplasm, illustrated in Figure 1(C). Meanwhile, in the chondroblastic osteosarcoma, HSPgp96 was localized extensively in nucleus though it can also be found in cytoplasm lightly, illustrated in Figure 1(B). The mechanism that HSPgp96 expresses mainly in cytoplasm or nucleus discriminately in the osteoblastic and chondroblastic osteosarcoma needs further research.

Besides, we observe that the tumor cells with high pathology grade and poorly differentiated account for a larger proportion of positive cells. It shows that HSPgp96 expression rise with decreasing of the cell differentiation level, which is coincident with the report of Wang XP [10]. It means that the increased expression of HSPgp96 in human osteosarcoma tissues can preliminary reflect the proliferation of the tumor cells, plays important roles in the promotion of the pathogenesis and development in human osteosarcoma, may be used as the important indicators of the clinical degree of malignant tumors and of estimating the prognosis. It is reported that HSP expression significantly increased in the oncogene transformed cells and human malignant cell line, and HSP in human malignant tumor tissues also expressed higher than normal tissues or the tissues adjacent to tumor, which suggest that HSP is closely related to the process of the cell transformation and exacerbation [15, 16]. Subtypes of osteosarcoma, including osteoblastic type, chondroblastic type, fibroblastic type and mixed pattern. In this study, 24 cases of osteoblastic osteosarcoma and 20 cases of chondroblastic osteosarcoma were obtained. HSPgp96 expressed extensively in the two subtypes, between which there was no significant difference of HSPgp96 immunoreactivity ($P > 0.05$). It suggests that the expression of HSPgp96 has nothing to do with the pathological subtype of tumor.

HSPgp96 in the tumor immunity has functions of promoting innate immunity and enhancing acquired immunity. The HSPgp96 molecules that were purified from tumor tissues may present peptides associated with them to major histocompatibility complex (MHC, or HLA in human) class I molecules for recognition by cytotoxic T lymphocytes (CTL) and thereby

elicit protective and therapeutic cellular immune responses [17–19]. Parmiani et al. found that the tumor vaccine made up of the HSPgp96 purified from the colorectal cancer patient can improved the T cell-mediated immunity reaction by 50% [20]. HSPgp96 which is the effective activating factor in the innate immunity can activate the IL-12 and dendritic cell (DC) [21, 22], and could made the DC matured by means of activating the production of pro-inflammatory factor and of transmitting signal through Toll-like receptor-2 and receptor-4. Injecting the HSPgp96 vaccine can elicit hosts protective immunity response [23].

A series of HSP (HSP70, HAP90, gp96 .etc) were distilled and purified by Srivastava from animal tumors (lung cancer and colon cancer), and they can induce protective immune response in mouse anti-tumor immunity so that the tumor is not easy to form. However, HSP, distilled from normal tissues, can't induce the protective immune response [24]. In our research, HSPgp96 expressed in all osteosarcoma tissues, while in little of the normal tissues. HSPgp96 maybe have important functions in the immunity response, that is to say, enhanced expression of HSPgp96 maybe contributes to the body's own protective mechanisms. Recently, phase II clinical trial approved that treating by tumor vaccine dealt with HSPgp96, the patients could survive longer [25]. In this study, we observed that HSPgp96 expressed highly in osteosarcoma tissues. It could be suggested a new treatment approach by obtaining quantities of the HSPgp96 from osteosarcoma tissues for constructing the vaccine and then inject directly to the patients to treat osteosarcoma in future.

In conclusion, HSPgp96 is highly expressed in osteosarcoma and has different immunolocalization during two subtypes of osteosarcoma. The immunopositivity is significantly higher in tumors with lower differentiation. The research implies that HSPgp96 may play a contributive role on the pathogenesis and development in human osteosarcoma, and there is hope in its application in determining the malignancy of cancer and utilization as a target for tumor immunity.

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