Expression of Heat Shock Proteins in Brain Tumors

Beyin Tümörlerinde Isı Şok Proteinlerinin Ekspresyonu

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ABSTRACT

AIM: Heat shock proteins (HSP) are an evolutionary conserved family of proteins that serve as molecular chaperones, preventing the formation of nonspecific protein aggregates and assisting proteins in the acquisition of their native structures. Furthermore, HSPs have anti-apoptotic properties and have been found to be elevated in many human cancers; their overexpression has been associated with poor survival and response to therapy. In the present study we assessed the HSP expression in brain tumors.

MATERIAL and METHODS: Simultaneous detection of HSP27, HSP40, HSP60, HSP70, HSP90a, total Akt and phospho- Akt in 19 brain tumor specimens was performed using the multiplex bead array assay.

RESULTS: There was expression of HSP27 (pSer^{82}), HSP27 (pSer^{15}), HSP40, HSP60, HSP70, HSP90a, total-Akt and phospho- Akt in both gliomas and meningiomas. Significantly higher levels of HSP70 and a trend towards higher levels of HSP40 were found in meningiomas compared to gliomas. There was a significant correlation between HSP27 (pSer^{82}) and HSP27 (pSer^{15}) expression and between HSP90a and both total-AKT and phospho- AKT. A significant correlation between HSP27 and total-AKT was observed.

CONCLUSION: Since HSPss are an attractive target for anticancer therapy, further studies are needed in order to better assess their relationship with tumor aggressiveness and patient prognosis.

KEYWORDS: Heat shock proteins, Brain tumor, Glioma, Meningioma

ÖZ

AMAÇ: Isı şok proteinleri (HSP) moleküler bakıcılar görevi görüp non-spesifik protein agregatlarının oluşmasını önleyen ve proteinlere doğal yapılarının kazanılmasında yardımcı olan, evrimsel olarak korunmuş bir protein ailesidir. Ayrıca, HSP'lerin anti-apoptotik özellikleri vardır ve birçok insan kanserinde arttıkları bulunmuştur; aşırı ekspresyonları zayıf sağkalım ve tedavi cevabıyla ilişkilendirilmiştir. Mevcut çalışmada, beyin tümörlerinde HSP ifadesini değerlendirdik.

YÖNTEM ve GEREÇLER: Multipleks boncuk dizisi testini kullanarak 19 beyin tümörü numunesinde HSP27, HSP40, HSP60, HSP70, HSP90a, total Akt ve fosfo- Akt'yi eş zamanlı olarak saptadık.

BULGULAR: Hem gliomalar hem de menenjiyomlarda HSP27 (pSer^{82}), HSP27 (pSer^{15}), HSP40, HSP60, HSP70, HSP90a, total-Akt ve fosfo-Akt ifadesi yoktu. Menenjiyomlarda gliomalara göre önemli ölçüde daha yüksek HSP70 seviyeleri ve daha yüksek HSP40 seviyeleri eğilimi bulunmaktaydı. HSP27 (pSer^{82}) ve HSP27 (pSer^{15}) ifadesi ile HSP90a ve hem total-AKT hem fosfo-AKT arasında önemli bir korelasyon vardı. HSP27 ile total-AKT arasında önemli bir korelasyon gözlendi.

SONUÇ: HSP, antikanser tedavi için çekici bir hedef olduğundan tümör agresifliği ve hasta prognozuyla ilişkisini daha iyi değerlendirmek için ek çalışmalar gerektirir.

ANAHTAR SÖZCÜKLER: Isı şok proteinleri, Beyin tümörü, Glioma, Menenjiyom

INTRODUCTION

Heat shock proteins (HSP) are evolutionary conserved family of proteins that serve as molecular chaperones, preventing the formation of nonspecific protein aggregates and assisting proteins in the acquisition of their native structures (2, 5, 6, 21). Furthermore, HSPs have anti-apoptotic properties and have been found elevated in many human cancers; their overexpression has been associated with poor survival and response to therapy (5, 6, 14). HSPs have therapeutic implications since they can be targeted by drugs or may provide a possible target for immunotherapy (5, 20). Regarding brain tumors, HSP expression in selected cell lines under heat shock or normal environments has been studied (13). Using mainly immunohistochemistry, high HSP expression has been found in brain tumors (2, 3, 13). Nevertheless, the majority of studies have only investigated selected HSP. Herewith, we report on the simultaneous detection of HSP27, HSP40, HSP60, HSP70, HSP90a, total Akt and phospho Akt in 19 brain tumor specimens by using multiplex bead array assay. This

is the first study in which such a large number of HSPs have been investigated in human brain tumors.

MATERIAL and METHODS

We studied 19 patients (10 males, 9 females, mean age 55.3, range 34-78) with brain tumors that were treated surgically in our institute. Nine patients suffered from glioblastoma, 1 from anaplastic oligoastrocytoma, 6 from typical meningiomas, 1 from atypical meningioma, 1 from acoustic neuroma and 1 from a metastatic brain tumor. We used tissue from surgically excised brain tumor specimens. All patients gave their informed consent to participate in the study.

Sample Preparation

We used frozen tissues from brain tumor specimens. For the tissue extracts, we followed the protocol suggested in the kit insert. In brief, we placed ~0.5 cm^3 piece of tissue in a mortar and added a sufficient volume of liquid nitrogen to cover the tissue, allowing the liquid nitrogen to evaporate (the tissue should be thoroughly frozen). Then we grinded the frozen tissue to a powder with a pestle and added 1 ml of HSP/ Chaperone Lysis Buffer supplement with protease inhibitor cocktail (Sigma, Catalog # P8340) at a concentration of 0.5 μL/ mL, phosphatase inhibitor cocktail (Sigma, Catalog # P2850) at a concentration of 10μL/mL, and PMSF (Sigma Catalog # P7626) at a concentration of 10μL/mL. We then continued to homogenize the tissue with the pestle until the tissue suspension became homogeneous and passed through a syringe 21g 5 times in order to ensure full lysis of the cells. The extracts were transferred to a polypropylene tube vortex and incubated on ice for 30 min. Then, they were centrifuged at 16,000 x g for 20 minutes in a 4°C refrigerated microfuge. The supernatant was transferred to a labeled polypropylene tube. The supernatant collected was the tissue extract, ready for analysis. The resulting pellet was discarded. Alternatively, the tissue extracts can be frozen at -70°C and assayed at a later date.

Multibead Analysis Procedure

We quantified 8 different molecules (Total Akt, Akt phospho – ser473, HSP60, HSP70, HSP90a, HSP40, HSP27 phospho – ser82, HSP27 phospho – ser15) per sample following the suggested procedure of Multibead HSP/Chaperons 8-plex kit (Catalog # ADI – 980-002, Enzolifescienses). In brief, we used 50μl of cell extracts diluted with Assay Buffer (1:4) for each sample. Each one of the analyte-specific antibodies was captured in beads with the same size (5.4 μm) and different intensity in the red fluorescence channel (FL4). The analysis of the results was performed with a dedicated analysis Software (MultiBead Analysis Software, Enzolifescienses). The sensitivity of each analyte, based on the lower used standard, was the following: Akt (0.2 ng/mL), Hsp27 phospho - Ser82 (5.2 U/mL), Hsp27 phospho - Ser15 (3.4 U/mL), Hsp40 (0.2 ng/ mL), Hsp60 (3.4 ng/mL), Hsp70 (0.2 ng/mL), Hsp90a (0.3 ng/ mL), Akt phospho - Ser473 (2.0 U/ml) (8,34). For the analysis we used the FACS – Calibour flow cytometer equipped with 2 lasers (488nm, 635nm) and 6 parameters (Fsc, Ssc, FL1 – FL4).

Statistical Analysis

Correlation among total Akt, Akt phospho, HSP60, HSP70, HSP90a, HSP40, HSP27 phospho–ser82 and HSP27 phospho–ser15 indices of tumor tissue were analyzed statistically using Spearman analysis. The differences in HSP expression between high-grade gliomas and meningiomas were estimated using two-tailed paired t-test. A 2-sided *P*-value < 0.05 was considered statistically significant.

RESULTS

Table I and II summarizes the patients' data. There was a significant positive correlation between HSP27 (pSer82) and HSP27 (pSer15) expression (p=0.001). There was also a significant correlation between HSP90a and both total Akt and Akt (phospho) (p<0.0001 and p=0.0057 respectively). A significant positive correlation between HSP27 (pSer82), HSP27 (pSer15) and total Akt (p=0.014 and p=0.02 respectively) was also observed. HSP70 and HSP90 showed a strong parallel expression (p<0.0001). When comparing high-grade gliomas with meningiomas, no significant difference was found for Akt (total) (p=0.3), HSP27 (pSer82) (p=0.9), HSP27 (pSer15) (p=0.4), HSP60 (p=0.9), HSP90a (p=0.3) and Akt (phospho) (p=0.17). We found significantly higher levels of HSP70 expression in meningiomas compared to gliomas (p=0.04) and a trend towards higher levels of HSP40 expression in the meningiomas group compared to gliomas (p=0.076). No significant correlation was found between Ki-67 index and HSP expression in gliomas or meningiomas.

DISCUSSION

The present study demonstrated substantial expression of HSP in brain tumors. We found expression of HSP27 (pSer82), HSP27 (pSer15), HSP40, HSP60, HSP70, HSP90a in both gliomas and meningiomas. Akt (total) and Akt (phospho) were also detected. Significantly higher levels of HSP70 and a trend towards higher levels of HSP40 were found in meningiomas compared to gliomas.

HSP expression is induced by stress such as irradiation, heat, anticancer agents and oxidating stress (11). Mammalian HSPs have been classified according to their size into six families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (15 to 30 kDa) including HSP27 (17). HSP27 can form oligomers up to 1000 kDa. HSP27 is an ATP-independent chaperone that protects the cells from protein aggregation. HSP27 can be phosphorylated at three serine residues (Ser15, Ser78 and Ser82), and its dephosphorylation enhances oligomerization. HSP27 inhibits apoptosis by regulating upstream signaling pathways (27). Rane et al. showed that Akt phosphorylated HSP27 on Ser82 and this phosphorylation of HSP27 resulted in its dissociation from Akt. Additionally, the interaction between HSP27 and Akt was necessary for activation of Akt in intact neutrophils. Thus, HSP27 regulates apoptosis through control of Akt activity (25). Moreover, HSP27 facilitate the activation of the ubiquitin-proteasome pathway resulting in an increase in NF-κB activity that has an anti-apoptotic effect (24). HSP27 accumulation reduces the apoptotic process

induced by alkylating agents in human colorectal cancer cells (13). Khalid et al. reported that HSP27 expression in gliomas correlated with histological grades of astrocytoma and with the Ki-67 index. However, HSP27 expression was only analyzed immunohistochemically (17). In our study we used the multiplex bead array assay, which allows quantification of multiple proteins simultaneously, in contrast to ELISA or

Western blot that require a similar amount of sample but where only one protein can be analyzed (8,22,34). No significant correlation between Ki-67 index and HSP expression in gliomas or meningiomas was found in the present study, possibly due to the limited number of cases. In another study, a comparative analysis of HSP27, alphaBC, HSP72, HSP73, and HSP90 expression in 24 paired samples of first resections

Table I: Patients' Data

Age/ Sex	Diagnosis	Ki-67	AKT (Total) ng/ml	Hsp27 (pSer82) u/ml	Hsp27 (pSer15) u/ml	Hsp40 ng/ml	Hsp60 ng/ml	Hsp70 ng/ml	Hsp90 alpha ng/ml	Akt phospho u/ml
55/F	GBM	5	0.88	14.95	6.62	1.85	5.23	2.88	0.86	2.19
48/F	GBM	30	1.78	14.38	6.08	2.36	87.22	4.57	5.89	12.97
68/M	GBM	30	0.78	1.74	4.92	1.1	1.1	1.77	1.17	0
34/M	AOA	15	2.06	5	6.49	2.99	160.21	4.67	7.7	10.24
59/M	GBM	20	3.03	17.86	7.17	5.69	70.15	9.71	16.9	29.89
57/F	GBM	60	1.69	54,96	9.1	2.94	108.4	7.3	1.58	12.97
58/M	GBM	60	0.98	8.47	5.87	1.52	17.17	1.95	1.3	4.85
54/M	GBM	35	0.71	8.47	5.93	1.16	0	1.23	1.18	0
57/M	GBM	70	0.8	9.5	5.87	1.61	1.32	1.06	1.13	0
62/F	GBM	n/a	2.13	113.46	15.02	5.77	449.99	35.88	81	7.53
50/F	Meningioma Meningothelial	0,5	3.09	17.86	6.3	9.14	421.55	158.4	63.43	27.01
52/M	Meningioma Psammomatous	4	6.25	33.05	7.68	20.49	134.69	118.03	44.14	155.51
51/F	Meningioma Meningothelial	5	1.99	87.78	5.93	19.47	97.24	276.07	66.96	29.89
51/F	Meningioma Meningothelial	1.5	0.89	9.5	6.3	1.88	1.75	1.77	1.43	15.73
78/F	Meningioma Atypical	25	1.1	6.46	6.11	2.16	1.96	3.46	1.99	0
50/M	Neurinoma	1.29	1.29	6.46	5.36	1.7	17.17	4.06	2.5	12.97
51/M	Meningioma Meningothelial	0,5	0.87	14.95	6.82	1.46	5.6	0.97	1.28	2.19
48/F	Meningioma Transitional	0,5	0.86	13.26	6.39	1.29	5.73	2.32	1.67	0
68/M	Metastasis	N/A	1.56	12.16	6.49	3.01	139.65	8.72	15.98	0

AOA: *Anaplastic oligoastrocytoma,* ***F:*** *Female,* ***GBM:*** *Glioblastoma,* ***M:*** *Male,* ***N/A:*** *Non-available.*

Table II: Average of Analytes Based on Different Diagnosis

Diagnosis	AKT (Total) ng/ml Average	Hsp27 (pSer82) u/ml Average	Hsp27 (pSer15) u/ml Average	Hsp40 ng/ml Average	Hsp60 ng/ml Average	Hsp70 ng/ml Average	Hsp90 alpha ng/ml Average	Akt phospho u/ml Average
GBM	1.42	27.09	7.40	2.67	82.29	7.37	12.33	7.82
Meningioma	2.04	23.66	6.36	7.20	85.71	70.63	22.92	30.41

GBM: *Glioblastoma.*

and recurrences of glioblastomas revealed no impact of HSP expression on response to adjuvant radiochemotherapy and no modulation of HSP expression by radiochemotherapy (15). Recently, Shen et al. studied low-grade gliomas and reported that the HSP27 expression was downregulated compared to autologous para-cancerous brain tissues (29). We investigated site-specific phosphorylation of HSP27 at Ser82 and Ser15 in high-grade gliomas for the first time in the present study. There was a parallel high expression of HSP27 at Ser82 and Ser15 in these tumors. We also found a substantial expression of several HSPs in medulloblastoma with the more important HSP27 and HSP70 in a previous study (2). Regarding meningiomas, an immunohistochemical study revealed low HSP27 cytoplasmic immunoreactivity in 26 of 64 meningiomas (3). Using Western blotting, Yokoyama et al. detected HSP27 in every meningioma tissue homogenate of the cases studied (33). Similarly, we found substantial HSP27 expression at Ser82 and Ser15 in all meningioma cases. Thus, HSP27 is highly phosphorylated at both Ser82 and Ser15 in brain tumors.

HSP70 and HSP90 have been considered anti-apoptotic proteins similar to HSP27. Both HSP70 and HSP90 function as ATP-dependent molecular chaperones (30). In the present study, we found a strong linear correlation between HSP70 and HSP90a. HSP70 acts to suppress signals leading to Bax activation and thereby prevents the release of pro-apoptotic proteins from mitochondria (30). HSP70 and HSP90 can also suppress apoptosis by directly associating with Apaf-1 and blocking the assembly of a functional apoptosome (4). Furthermore, JNK activation is believed to be an essential mediator of stress-induced apoptosis. Binding of HSP70 to JNK prevents its phosphorylation (20). HSP90 associates with a number of signaling proteins. Client proteins of HSP90 such as AKT, p53, MEK, STAT3, and Bcr-Abl are vital in tumor progression and HSP90 is required for the stability and function of these proteins (18). Hsp90 can bind to Akt *in vivo* and contributes to the functional stabilization of PI3K/Akt signaling and cell survival. The blockade of the binding to HSP90 inactivates Akt and increases the sensitivity of the cells to apoptosis-inducing stimuli (26). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 and by phosphorylation within the carboxy terminus at Ser473 (1). Hsp90 inhibition dramatically depleted intracellular PDK1, suggesting that loss of PDK1 may be the primary reason for Akt deactivation (31). In the present study we confirm the parallel expression of Hsp90a and both total Akt and (phospho) Akt. We chose to study HSP90a because only the induced HSP90α and not the constitutive HSP90β is upregulated in glioma (23). HSP90a has therapeutic implications since both Hsp90a gene silencing and the protein inhibitor approaches resulted in a dramatic reduction in cell viability (7).

The role of HSP60 in cancer has not been fully elucidated yet. Xanthoudakis et al reported that HSP60 has a pro-apoptotic role by facilitating the activation of pro-caspase-3 by different upstream caspases, including caspase-6 (32). Cytosolic HSP60

prevents translocation of the pro-apoptotic protein Bax into mitochondria and thus promotes cell survival (28). Ghosh et al. reported that HSP60 is up-regulated in human cancers including some cases of glioblastoma and orchestrates a cytoprotective pathway that involves stabilization of survivin levels and restraint of p53 function (12). To date, no other study has evaluated HSP60 expression in brain tumors. Herewith, we found expression of HSP60 in nearly all tumors studied, both in high-grade gliomas and meningiomas. Furthermore, we found expression of HSP40, also known as chaperone DnaJ, in all brain tumors. A trend towards higher expression in meningiomas was also detected. HSP40 has not been previously studied in brain tumors. HSP40 regulates HSP70 function. Both HSP90 and the HSP40/HSP70 system act to enhance the Akt pathway, which is important for cell survival (9,26). The present study showed a parallel high expression of HSP40/HSP70/HSP90 in brain tumors.

In conclusion, our results demonstrated substantial HSP production in the tumor specimens examined, which we believe is the first time in which many different HSPs have been reported in brain tumors. Based on this finding and given that HSPs are an attractive strategy for anticancer therapy, further studies involving larger number of patients are obviously needed to clarify their relationship with tumor aggressiveness and prognosis.

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