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Expression of *HPA* in astrocytomas and its effect on invasiveness of tumor cells.

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Abstract

Objective: To observe the expression of heparanase (*HPA*) in human astrocytomas tissue and its effect on invasiveness of tumor cells.

Methods: Immunohistochemical Elivision™plus two step method and Realtime-PCR method were used to detect the expression of *HPA* protein and mRNA in 75 cases of astrocytoma tissues and 40 cases of normal brain tissues. Silencing *HPA* expression in astrocytoma U87 cells by siRNA interference technique, and adopting Realtime PCR, Western blot, Transwell chamber experiment, the expression and cell invasiveness change of tumor cells *HPA*, vascular endothelial growth factor (*VEGF*), matrix metalloproteinase -9 (MMP.9) were detected.

Results: The expression of *HPA* protein was not found in normal brain tissues, *HPA* positive expression rate in astrocytoma tissues was 78.67% (59/75), and the positive expression rate of *HPA* in astrocytoma tissues was significantly higher than that in normal brain tissues, and its expression level of mRNA was significantly higher than that in normal brain tissues, the difference of which was statistically significant (χ^2=67.8, P<0.05). The positive expression rate of *HPA* in grade II to IV group of astrocytoma increased gradually, the difference of which was statistically significant (χ^2=131.5, P<0.05); the positive expression rate of *HPA* in metastasis group was significantly higher than that in non-metastasis group (χ^2=89.3, P<0.05). SiRNA can effectively inhibit the expression of *HPA* in astrocytoma U87 cells, and down regulate the expression of *VEGF*, *MMP-9*, at the same time the number of transmembrane cells decreased significantly, and the difference was statistically significant (P<0.05).

Conclusion: *HPA* is highly expressed in astrocytomas tissues, and with the increase of malignancy of tumor, the expression increases gradually. The cell invasiveness decreased significantly after siRNA silenced *HPA*, and *HPA* might be a potential target for the treatment of astrocytomas.

Keywords: Astrocytoma, Heparanase, Invasion.

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Introduction

According to World Health Organization (WHO), grade III and IV gliomas are primary brain tumors with the highest incidence in adults [1]. Among these high-grade gliomas, the grade IV astrocytic tumor known as glioblastoma multiforme (GBM) is the most common, aggressive, and deadly tumor. The therapeutic strategy for astrocytic tumor involves surgery, irradiation, and chemotherapy [2], and this treatment regime has resulted in an increase in median progression-free and overall survival [3,4]. Astrocytoma is a primary brain tumor which is the most common in adults, and its incidence is increasing year by year, accounting for about 70% of primary malignant brain tumors [5]. Despite the rapid development of neuroimaging greatly helps the diagnosis and treatment of astrocytoma, and treatment technology of astrocytoma has made great progress, its clinical effect is not ideal, the main reason for which is most of astrocytomas show invasive growth, and it is difficult to cure completely by operation [6]. Heparanase (*HPA*) is a degrading enzyme in extracellular matrix found in recent years, it can damage the structure of natural barrier formed by extracellular matrix and basement membrane of blood vessels, playing an important role in the process of tumor cell invasion and metastasis. Heparanase is the only mammalian enzyme able to cleave heparan sulfate internally, generating short fragments (of 10 to 20 residues) endowed with biological activity. Elevated heparanase expression by tumor cells correlates with increased tumor

angiogenesis, tumor invasiveness and metastasis [7-10]. A large number of studies have confirmed that *HPA* is highly expressed in a variety of malignant tumors [11]. However, few studies was reported on the effect of the expression of *HPA* in astrocytomas on tumor cells invasion. Tumor tissue samples come from the Second Hospital of Hebei Medical University and the Third Hospital of Shijiazhuang. From January 2004 to December 2014, a total of 75 patients with primary astrocytomas and complete data were collected. In this study, we aimed to explore the effect of the expression of *HPA* in astrocytomas on tumor cells invasion.

Experimental Methods and Reagents

Immunohistochemistry

Three sections were taken from each paraffin tissue, and were stained with immunohistochemical Elivision™plus two step method. The known positive slices were used as controls, and PBS staining as negative control. The used primary antibody was mouse anti human *HPA* monoclonal antibody (SantaCruz Co., USA), ElivisionTMPlus immunohistochemistry kit and DAB coloring kit were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. Results judgment: *HPA* positive sites were in cytoplasm, presenting brownish yellow granules, otherwise it's negative if they didn't appear. At high magnification, 5 high-power visual fields at the angle and in the center of each slice were taken and the percentage of positive cells in 100 cancer cells was calculated to obtain the mean value. Then, according to the staining intensity to record scores: 0-3 points were colorless, pale yellow, yellow, brown yellow; according to the percentage of the positive cells to record scores: 0 was negative, 1, 2, 3 and 4 points were respectively positive cells accounting for <11%, 11%~50%, 51%~75% and >75%, and the calculation method was as follows: the staining intensity * positive cells percentage; among them: >3 points was (+), 6-9 points was (+ +), 10-12 points was (+ + +), and (+) or above (+) was seen as positive expression. The preserved paraffin tissue sections of all cases were diagnosed and reviewed by two senior pathologists according to 'tumor of the nervous system' of WHO in 2010.

Cell culture medium transfection

In 37°C 5% CO incubation box, DMEM medium containing 10% fetal bovine serum was used for routine culture of U87 cells, cells in logarithmic phase were seeded in culture dishes, the cell density was adjusted to 1*10/ml, and when the cells fuse to 30%~50%, they were divided into 3 groups: 1) The blank control group; 2) The negative control group: transfection of empty plasmid; 3) Transfection group: transfection of *HPA*-siRNA.

Realtime PCR

Trizol was adopted for extraction of tissue or cell total RNA, application of sample was conducted for reverse transcription reaction according to the kit instructions, and fluorescent quantitative PCR instrument of ABI7300 type was used for amplification. It was composed by Shanghai Shenggong Biological Engineering Technology & Services Co. Ltd. The analysis software coming with instrument was adopted to obtain CT value amplified by various samples and genes with B-actin as the reference gene and relative value of target gene expression RQ=2 0, (Table 1).

Table 1. *Realtime PCR primer sequence.*

Gene	Primer sequence/5'3'
HPA	
Upstream	GAATGGACGGACTGCTAC
Downstream	CCAAAGAATACTrGCCTCA
VEGF	
Upstream	CGGCGAAGAGAAGAGACACATTG
Downstream	CGGGAAGGGAAGGGAAGGAC
MMP-9	
Upstream	GCAGAGGACCTGTACCGC
Downstream	AGGTTTGGAATGTGCCCAGGT
β-actin	
Upstream	CTACAATGAGCTGCGTTGTGGC
Downstream	CAGGTCCAGACGCAGGATGGC

Western blot

Collection, lysis of cells, extraction of total protein, 4°C, 12000 rpm centrifugation for 30 min, and obtain of supernatant were conducted. Half dry method electrophoresis was used for transferring to the PVDF membrane with 10% skimmed milk powder for 2 h close. Sheep anti mouse IgG labeled with specific primary antibody and horseradish peroxidase were added successively, and chemiluminescence method was adopted for coloration and fixation. The protein band IOD value was measured by UVP software, and the ratio between the target protein and the internal reference B-actin was calculated.

Transwell chamber experiment

Matrigel was diluted by pre cold and serum-free DMEM culture medium, transfected cells were made into cell suspension and adjusted to the cell density of 3*10/mL, and 100 μL cell suspension was put into upper chamber of Transwe11 chamber, and 600 L DMEM medium containing 10% BSA was added into lower chamber. 18 h Incubation was conducted at 37°C and 5% CO with culture medium abandoned, PBS for washing and fixation for 15 min by formaldehyde at room temperature. The upper surface cells were dabbed gently with cotton bud, stained with hematoxylin, and drought overnight at room temperature. The microporous membrane was placed on the slide, and the number of cell transmembrane was counted under microscope. The mean value was obtained after repeating 3 times.

Statistical analysis

SPSS21.0 statistical software was used, measurement data were expressed by $\bar{x} \pm s$ with t test and enumeration data were compared by χ^2 test with $P<0.05$ seen as statistically significant difference.

Ethical considerations

The study was carried out in compliance with the Declaration of Helsinki of the World Medical Association, and according to a protocol approved by Second Hospital of Hebei Medical University and the Third Hospital of Shijiazhuang, the approval number is 2004014. The objectives of the study were explained to the study participants and verbal consent was obtained before interviewing each participant.

Result

In the selected 75 cases, 45 cases were male, and 30 female; age ranged from 12 to 78 years old with the average age (59 ± 25); pathological grading: 25 cases of grade II, 29 cases of grade III, and 21 cases of grade IV. The depth of invasion: 44 cases were involved in serosa, and 31 cases were not. In addition, 40 cases of normal brain tissues of patients with craniocerebral decompression after craniocerebral injury were selected randomly as control, including 28 males and 12 females; the age ranged from 21 to 65 years old, and the mean age was (41 ± 12) years old.

Expression of HPA in astrocytomas and normal brain tissues

Immunohistochemistry results showed that: *HPA* positive staining were localized in the nucleus and cytoplasm, presenting brownish yellow granules, and in 75 cases of astrocytoma tissues and 40 cases of normal brain tissues, *HPA* protein expression rate was 78.67% (59/75), 0 (0/40), the positive expression of *HPA* protein in astrocytoma tissues was significantly higher than that in normal brain tissues, the difference of which was statistically significant ($P<0.05$) (Table 2).

Table 2. *Positive expression rates of HPA protein in two groups (%).*

Classification	Positive expression rate of *HPA*
Normal brain tissues (n=40)	0
Astrocytoma (n=75)	59 (78.67)*
Note: compared with normal brain tissues, *P<0.05	

Realtime PCR results showed that the expression level of *HPA*mRNA in astrocytoma tissues was significantly higher than that in normal brain tissues, the difference of which was statistically significant ($P<0.05$) (Table 3).

Table 3. *HPA expression situation in astrocytomas tissues with different pathological grades (x ± s).*

Classification	Expression level of *HPA*mRNA
Normal brain tissues (n=40)	0.00 ± 0.00
Astrocytoma (n=75)	1.92 ± 0.32*
Note: compared with normal brain tissues, *P<0.05	

The relationship between HPA and histopathological grading of astrocytoma

The positive expression rate of *HPA* in grade IV astrocytoma tissue was significantly higher than that of grade III astrocytomas ($P<0.05$), while the latter was significantly higher than that of grade II astrocytoma tissue, suggesting that positive expression rate of *HPA* showed a linear upward trend with astrocytoma differentiation decreasing (Table 4).

Table 4. *HPA expression situation in non-metastatic and metastatic astrocytomas tissues.*

Histological types	Expression situation of *HPA*		Positive rate (%)
	-	+	
Grade II (n=25)	10	15	60.00
Grade III (n=29)	6	23	79.31
Grade IV (n=21)	2	19	90.48
Note: compared with grade II, *P<0.05			

The positive expression rate of *HPA* in metastatic astrocytomas tissues was significantly higher than that in non-metastatic astrocytoma tissues ($P<0.05$), indicating that the positive expression rate of *HPA* is related to whether astrocytoma has metastasis (Table 5).

Table 5. *The relationship between HPA and metastasis.*

Metastasis or not	Expression situation of *HPA*		Positive rate (%)
	-	+	
No (n=31)	12	19	61.29
Yes (n=44)	2	42	95.15
Note: compared with non-metastasis, *P<0.05			

The influence of *HPA*siRNA on the expression of *HPA*mRNA in U87 cells and protein Realtime-PCR and Western-blot results showed that after *HPA*siRNA transfected for 48 h, *HPA*mRNA in U87 cells and protein expression were significantly decreased, and compared with blank control group and negative control group, the difference was statistically significant ($P<0.05$), while the comparison of blank control group and negative control group had no significant difference ($P>0.05$), which indicated that *HPA*siRNA could effectively silent the expression of *HPA* (Table 6).

Table 6. *The influence of HPAsiRNA on the expression of HPAmRNA in U87 cells and protein (x ± s).*

Group	Expression level of *HPA* mRNA	Protein expression level of *HPA*
Transfection group	0.18 ± 0.03	0.14 ± 0.02
Negative control group	0.67 ± 0.11*	0.60 ± 0.10*
Blank control group	0.65 ± 0.10*	0.55 ± 0.09*
Note: compared with the transfection group, *P<0.05		

The influence of *HPA*siRNA on the expression of *VEGF*mRNA in U87 cells and protein Realtime-PCR and Western blot results showed that after *HPA*siRNA transfected for 48 h, *VEGF*mRNA in U87 cells and protein expression were significantly decreased, and compared with blank control group and negative control group, the difference was statistically significant (P<0.05), while the comparison of blank control group and negative control group had no statistically significant difference (P>0.05), which indicated that *HPA*siRNA could obviously inhibit the expression of *VEGF* (Table 7).

Table 7. *The influence of HPAsiRNA on the expression of VEGFmRNA in U87 cells and protein ($\bar{x}$ ± s).*

Group	Expression level of *VEGF*mRNA	Protein expression level of *VEGF*
Transfection group	0.21 ± 0.03	0.17 ± 0.02
Negative control group	0.89 ± 0.14*	0.79 ± 0.12*
Blank control group	0.90 ± 0.15*	0.77 ± 0.11*
Note: compared with the transfection group, P<0.05		

The influence of *HPA*siRNA on the expression of *MMP-9*mRNA in U87 cells and protein Realtime-PCR and Western blot results showed that after *HPA*siRNA transfected for 48 h, *MMP-9*mRNA in U87 cells and protein expression were significantly decreased, and compared with blank control group and negative control group, the difference was statistically significant (P<0.05), while the comparison of blank control group and negative control group had no statistically significant difference (P>0.05), which indicated that *HPA*siRNA could obviously inhibit the expression of *MMP-9* (Table 8).

Table 8. *The influence of HPAsiRNA on the expression of MMPOmRNA in U87 cells and protein (x ± s).*

Group	Expression level of MMPO mRNA	Protein expression level of *MMP-9*
Transfection group	0.15 ± 0.02	0.11 ± 0.02
Negative control group	0.76 ± 0.12*	0.68 ± 0.11*
Blank control group	0.74 ± 0.11*	0.64 ± 0.10*
Note: compared with the transfection group, *P<0.05		

The influence of *HPA*siRNA on the invasiveness of U87 cells The results of Tran.swell chamber experiment showed that after *HPA*siRNA transfected for 48h, the number of transmembrane cells in the transfection group, the negative control group, and the blank control group were respectively (21 ± 3), (51 ± 7), (53 ± 9), and compared with negative control group and blank control group, the number of transmembrane cells in the transfection group were significantly decreased, the difference of which was statistically significant (P<0.05), while the comparison of blank control group and negative control group had no statistically significant difference (P>0.05), which indicated that *HPA*siRNA could obviously reduce the invasiveness of U87 cells.

Discussion

Astrocytoma arises from the embryonic ectoderm, and is the most common tumor in brain tissue. Its incidence is increasing year by year, and according to statistics, astrocytoma accounts for 70% of primary malignant brain tumors [1], which is the first in brain tumor, and is a kind of disease with serious harm to human life and health, in addition, the prognosis is often poor [12]. The biological characteristics of astrocytomas have specialty, which are characterized by invasive growth, regardless of whether the degree of differentiation is high or low. Although the rapid development of neuroimaging greatly helps the diagnosis and treatment of astrocytoma, and the progress and diversification of medical treatment methods improves the survival rate of the patients with astrocytoma, the prognosis of patients with astrocytomas is still not ideal.

The main reason for the high mortality rate of patients with astrocytoma is the invasive growth and metastasis of tumor. Invasive growth of malignant tumors must degrade various components of the extracellular matrix (ECM) and heparan sulfate proteoglycan (HSPG) before passing through the barrier composed by the extracellular matrix (ECM) and the vascular basement membrane (BM). *HPA* is a ECM degrading enzyme found in recent years, which is obtained from clones of human placenta tissue and platelet. Study from Hulett et al. [13] considered that *HPA* was a membrane protein that could degrade HSPG in BM and ECM, and regulate the transcription of HSPG; and *HPA* in a variety of metastatic tumor cell lines appeared the phenomena of high expression, and in cell lines with transfected *HPA*cDNA, it also showed the characteristics of high metastasis. The current study found that *HPA* in breast cancer, ovarian cancer, thyroid cancer and other primary tumors tissues appeared abnormal high expression, which played an important role in continuous pathological state of malignant tumors [14-22]. And the increased expression was related to reduced postoperative survival period of tumor patients, metastasis and increased microvessel density.

There were 59 cases of *HPA* positive expression in 75 cases of astrocytoma specimens collected in this study, the positive rate of which was 78.67%, while the positive expression was not found in normal brain tissues, and with the pathological grade of astrocytoma increased, its expression also increased, suggesting that *HPA* is involved in the occurrence and development of astrocytoma. In addition, this study used

siRNA interference technology to successfully induce the *HPA* gene silence, and the results showed that invasiveness of astrocytoma U87 cells was decreased significantly. At the same time, the expression of *VEGF* and *MMP-9* was down regulated obviously, which further confirmed that high expression of *HPA* was closely related to tumor invasion and metastasis, and its expression level could be used as a reliable index to evaluate tumor recurrence and metastasis. To sum up, high expression of *HPA* plays an important role in the occurrence, development, invasion and metastasis of astrocytomas. Through gene targeting therapy, the down regulated expression of *HPA* may inhibit the invasion and metastasis of tumor. *HPA* can be used as a marker for judging the biological behavior of astrocytomas and a potential target for the treatment of this tumor [23-27].

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