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Hepatoma-derived growth factor functions as an unfavorable prognostic marker of human gliomas

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Abstract. Hepatoma-derived growth factor (HDGF) regulates various cellular processes involved in the onset and development of tumors. To evaluate the role of HDGF in human gliomas, western blotting analysis, immunohistochemistry staining and reverse transcription-quantitative polymerase chain reaction were performed to detect HDGF protein and mRNA expression levels in glioma and intractable epileptic brain tissue. Various clinicopathological characteristics, including age, gender, World health Organization grade, HDGF expression level, Karnofsky performance Status (KPS) and Ki-67 index were obtained from medical records. The correlation between HDGF expression and these clinicopathological characteristics was statistically evaluated. Following this, multivariate liner regression was used to evaluate their effect on patient survival time. HDGF expression, at the protein and mRNA levels, was observed to be more upregulated in glioma tissues compared with intractable epileptic brain tissue without tumor. Furthermore, the level of HDGF expression was positively associated with the grade of malignancy [grades II~IV, Ki-67 index ≥20% or KPS <80 (P<0.05)] and poor prognosis in glioma patients. Notably, the univariate survival analysis identified a negative correlation between HDGF-expression and survival time (P<0.01) and multivariate liner regression demonstrated that HDGF expression is an independent prognostic factor for gliomas (P=0.01). Overall, HDGF upregulation may be a crucial step in the development and invasion of glioma. Further survival analysis highlighted its prognostic value for this malignancy, implying its potential as a promising therapeutic target for gliomas.

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Abbreviations: WHO, world health organization; KPS, Karnofsky performance score; qRT-PCR, Quantitative Real-Time PCR

Key words: hepatoma-derived growth factor, expression, gliomas, WHO grade, KPS, Ki-67 index, prognosis

Introduction

Glioma is considered as the largest group of primary brain malignant tumor in adults, which shows an aggressive nature and is very likely to spread to the surrounding brain tissues (1,2). Although considerable progress had been made in surgical and anticancer therapy, the prognosis of glioma is still unfavourable. Glioblastoma multiforme (GBM), the glioma histology type reported to be the most malignant, results in a life expectancy of 10-12 months after diagnosis and it ranks the third fatal malignant tumor following lung and pancreatic cancer (3,4). As the mechanism and prognostic factors of glioma is still unclear, there is no effective and specific treatments for glioma so far (5). Therefore, developing new diagnostic approaches will be helpful to the early diagnosis and treatment for gilomas (6).

HDGF is an acidic heparin-binding growth factor which was first purified from the medium of human hepatoma cell line Huh-7 (7). In recent years, A various biological roles of HDGF have been found, including the effect on promoting mitosis and vascular development (8). The results were similar to those found in other studies that HDGF played an important roles in promoting cancer cell proliferation, vascular formation, invasion, and metastasis in several malignant tumor, such as oral squamous cell carcinoma, esophageal cancer, colon carcinoma as well as lung and stomach cancer. (9-13) Moreover, pathological analysis indicated that the over expression of HDGF is significantly related to poor outcome of multiple cancer types, such as pancreatic cancer (14), hepatocellular carcinoma (15)

and gastric cancer (16). However, the role of HDGF in the prognosis of human gliomas is still unclear.

To address this problem, immunohistochemistry staining, western blotting analysis and RT-PCR were used to evaluate the expression of HDGF protein and mRNA respectively in 130 patients with primary gliomas. The correlation between HDGF expression and these clinicopathological characteristics were statistically evaluated. Then, multivariate liner regression was also used to evaluate their effect on patients survival time.

Materials and methods

Patients and tissue samples. The study had obtained the approval from the Ethics Committee of Tangdu Hospital, Fourth Military Medical University, Xi'an, China. According to the ethical standards, informed consents were sighed by all subjects and the samples were handled anonymous.

Fresh glioma samples were obtained from totally 130 patients who was diagnosed with glioma at the Department of Neurosurgery, Tangdu Hospital from June 2009 to June 2013. Radiotherapy or chemotherapy was not performed for the subjects before surgery. Intraoperative histological examination was performed to make a definite diagnosis of glioma. Patients received adjuvant treatment after surgery according to a uniform guideline depending on the stage of disease. Histopathologically classification of the glioma samples were performed depending on the WHO classification (17). 26, 32, 40 and 32 patients were classified as WHO grade I, II, III and IV respectively.

Specimens got from each patient were divided into two parts. One part was made into paraffin sections by fixing tissues in formalin and then imbedding them in paraffin. Another was stored at -80˚C immediately after surgery for posterior Western Blot and qRT-PCR. Fifteen patients with intractable epilepsy were involved in the study and the nonneoplastic brain tissues obtained from them were taken as control.

The database of electronic medical record system was used to collect clinical information and we set the date of surgery as the starting point for survival analyses. Patients died of other reasons not related to glioma served as censored data. Follow-up was terminated until June 18, 2016.

Quantitative Real-Time PCR. TRIzol reagent (Invitrogen, Carlsbad, US) was used to isolate total RNA of glioma specimen following the operating instruction. qRT-PCR was conducted in CFX96™ PCR System (Bio-rad). PCR was done with the following primers: HDGF forward primer 5'-TGC TCCTACCCACGCAGATT-3', reverse primer 5'-GGCCAA CCCAGAGTTGGAA-3'; β-actin: Sense, 5'-CTACAATGA GCTGCGTGTGGC-3'; antisense, 5'-CAGGTCCAGACG CAGGATGGC-3'. β-actin was used to normalize the targets as a standard.

Western blotting analyses. Samples were lysed by lysis buffer for 30 min and then centrifuged (12,000 rpm) for 20 min. Protein quantitation was performed by the procedure of BCA Protein Assay kit (Beyotime Inst, Biotech, China). Samples were separated with 12% SDS-PAGE and transferred to nitrocellulose (NC) membrane by electrophoresis. Then, 5% skim milk was used to block the membranes for 1 h, and incubated with the suitable primary rabbit anti-human HDGF antibody (Abcam, USA) overnight at 4˚C. After washed by TBST, the HRP adjointed secondary antibodies (Jackson, USA) was used to incubate membranes for 1 h. Then, membranes were washed and the blots were visualized using enhanced chemiluminescence reagents (Millipore, USA). Bands were digitally scanned and analyzed using Image J software and the intensity signal was recorded for further statistical analysis.

Table I. Association of HDGF mRNA expression with various.

Clinicopathological features	No. of cases	HDGF mean (SD)	P-value
Tissue type			
Control	15	0.051 (0.079)	<0.05
Glioma	130	2.437 (0.190)	
WHO grade			
I	26	0.793 (0.009)	<0.05
II	32	1.635 (0.217)	
III	35	3.178 (0.316)	
IV	37	3.893 (0.427)	
Ki-67 index			
<20%	64	1.736 (0.109)	<0.05
≥20%	66	3.987 (0.520)	
KPS			
≥80	66	1.523 (0.215)	<0.05
<80	64	3.197 (0.296)	

Immunohistochemistry analyses. The slices were deparaffinized by a group of xylene and then dexylene by a group of ethanol with graded concentrations. Then they were incubated in 0.01 M citrate buffer (pH=6.0) for antigen retrieval by heating the tissues slices in pressure cooker for 5 min. Once the slices cooled to room temperature, the activity of endogenous enzyme was blocked by soaking the slices in a humidified chamber contained with 3% hydrogen peroxide for 10 min. After a brief wash in distilled water, they were incubated with 10% donkey serum (Abcam) and then the primary antibody were prepared to appropriate concentration using PBS. Antibodies adopted in our study include: Primary rabbit anti-human HDGF antibody (Santa Cruz, USA) and anti-human Ki-67 antibody (Santa Cruz, USA). Slices were incubated in a humidified chamber at 4˚C overnight. Following that, slices were incubated with goat anti-rabbit immunoglobulin G antibody (Santa Cruz, USA) conjugated by horseradish peroxidase for 30 min. Diaminobenzidine (DAB) staining and hematoxylic counterstaining were performed to show the location of HDGF in the glioma specimen. Two experienced neuropathologists, blinded to clinical information, rated the percentage of positive nuclei staining of the stained slices. The level of HDGF expression was defined as follows: Negative staining was classified as Level 0. More than 60% of positive staining was considered as level 2 and the rest of slices were graded as level 1.

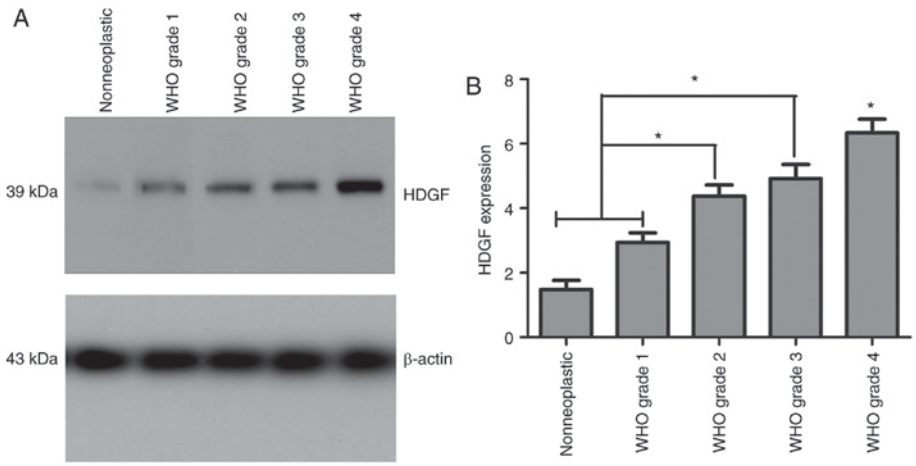

Figure 1. The expression of HDGF protein in normal brain tissues and each grade of glioma. (A) Compared with normal brain tissues, the HDGF protein expression was higher in glioma (WHO grades I-IV). (B) The level of HDGF protein in glioma and normal brain tissues. (ANOVA and Bonferroni's test, *P<0.01). No statistical difference was found between grade II and grade III (Bonferroni's test, P>0.05).

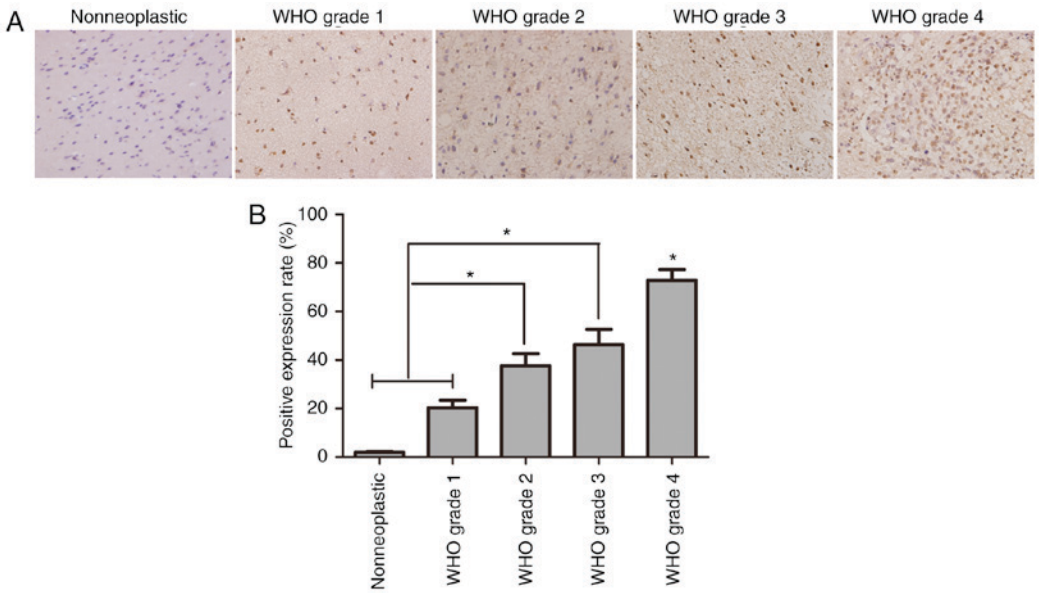

Figure 2. HDGF in representative specimens. (A) Nonneoplastic brain tissues of intractable epilepsy and WHO grade I-IV gliomas. (B) Overall positive rate of HDGF were obviously higher in WHO II-IV glioma than in WHO I glioma and nonneoplastic brain tissues (*P<0.05). But no statistical difference existed between grade II and grade III groups (P>0.05).

Statistical analyses. SPSS 13.0 software was applied to perform all statistical analyses. The relationship between HDGF levels and clinicopathologic data was analyzed by the χ^2 test. Data of western blotting analyses and qRT-PCR were dealt by using one-way classification of ANOVA followed by Bonferroni's test. The Kaplan-Meier method was used to generate survival curves and further analysis was performed using the log-rank test. Multivariable linear regression was adopted to analyze the effects of HDGF, age, gender, WHO grade and KPS on prognosis. A P-value of less than 0.05 was regarded as having statistical difference.

Results

Increased expression of HDGF mRNA in glioma tissues. The expression of HDGF mRNA was obviously increased in the glioma than in intractable epileptic brain (*P<0.05). Further statistical analysis was conducted to assess the relationship between HDGF mRNA expression and various clinical pathological features (Table I). Interestingly, HDGF mRNA expression was augmented as the WHO grades increased (*P<0.05) and was higher in subjects whose Ki-67 index ≥20% (*P<0.05) and KPS <80 (*P<0.05).

Increased expression of HDGF protein in glioma tissues. Western blotting indicated that the expressions of HDGF protein were obviously higher in both the high (WHO III-IV) and low (WHO I-II) grade glioma groups compared with normal brain tissue group (*P<0.01). Moreover, in the high-grade glioma group, the expression of HDGF protein expression was obviously higher compared with the low-grade glioma group (*P<0.01). But no statistical difference was

Table II. Association of HDGF protein expression with various clinicopathological features.

Clinicopathological features	No. of cases	HDGF expression (n)		P-value
		Level 1 and 0	Level 2	
WHO grade				
I	26	14	12	<0.05
II	32	10	22	
III	35	6	29	
IV	37	4	33	
Age				
<55	69	17	52	NS
≥55	61	15	46	
Gender				
Male	67	20	47	NS
Female	63	16	47	
Ki-67 index				
<20%	64	49	15	<0.05
≥20%	66	18	48	
KPS				
≥80	66	32	34	<0.05
<80	64	10	54	

Table III. Multivariate Cox regression analysis.

Parameter	Hazard ratio	95% confidence interval	P-value
Age	0.923	0.614-1.691	0.61
Gender	0.986	0.648-1.785	0.55
WHO grade	1.781	1.145-2.770	0.01
KPS score	1.952	1.251-3.048	0.006
Ki-67 index	2.671	1.827-4.727	<0.001
HDGF	4.028	2.542-6.380	<0.001

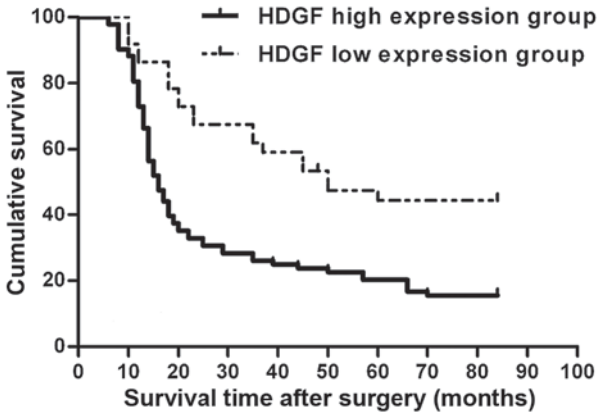

Figure 3. Kaplan-Meier survival curves for high and low HDGF expressive patients with glioma. As a result, patients expressing high HDGF had a significantly shorter survival time than patients expressing low HDGF (P<0.001).

observed between grade II and grade III group (P>0.05). (Fig. 1).

Positive rate of HDGF in glioma samples. The results of immunohistochemistry indicated a positive result of HDGF in glioma cells (Fig. 2A). The positive rate of HDGF in the control group and grade I-IV glioma groups was 1.96, 20.40, 37.64, 46.35 and 72.76%, respectively. These outcomes illustrated that the positive rate of HDGF was evidently higher in the WHO II-IV group than in WHO I and control groups (P<0.001). However, no statistical difference was observed between WHO II and III groups (P>0.05) (Fig. 2B).

Relationship between the HDGF expression and clinical pathologic parameters. The association of HDGF immunostaining with the clinical pathological parameters of glioma patients was summarized in Table II. As is shown in the table, the expression of HDGF was not markedly influenced by gender or age (P>0.05). In comparison, it was closely related to the WHO grade of gliomas and the KPS. The quantity of HDGF expression was significantly higher in glioma tissues with Ki-67 index ≥20%, KPS <80 and grades II ~IV than in those with Ki-67 index <20%, KPS ≥80 and grades I (Table II; *P<0.05).

Increase in HDGF protein expression indicates bad prognosis of patients with gliomas. The complete follow-up data obtained from 130 patients with gliomas and the results of HDGF expression level was used for survival analysis. 102 glioma patients (78.5%) died during follow-up (80 from the HDGF high expression group (level 2) and 22 from the HDGF low expression group (level 0 and 1)). Among the 102 dead patients, 6 died because of accidents or other diseases not directly related to gliomas (4 from HDGF high expression group (level 2) and 2 from the HDGF low expression group (level 0 and 1)). In the univariate survival analysis, the cumulative survival curve was plotted by using the Kaplan-Meier method and the difference in survival was determined by the log-rank method. The findings revealed that subjects with high level of HDGF had an obviously shorter survival time than patients with low HDGF expression level (P<0.001; Fig. 3). The average survival period of subjects with high and low HDGF expression were 16.6±2.0 and 49.8±1.5 months (log rank test: *P<0.01) respectively. Further more, the effect of age, gender, WHO grade, KPS and HDGF on prognosis was evaluated by multivariable linear regression. The results in Table III indicated that the WHO grade (HR=1.781, 95%CI: 1.145-2.770, P=0.01), KPS (HR=1.952, 95%CI: 1.251-3.048, P=0.006), Ki-67 (HR=2.671, 95%CI: 1.827-4.727, P<0.001) and HDGF expression (HR=4.028, 95%CI: 2.542-6.380, P<0.001) were significantly correlated with the prognosis of glioma patients, but no effect was found on age and gender.

Discussion

Despite huge progress in developing the diagnostic methods and strategies for therapy, such as radiation treatment and

chemotherapy, glioma is still one of the most lethal cancer in human (18,19). The average survival period of patients with glioma is less than 2 years and the 5-year survival rate is no more than 3%, which ranks the lowest among all cancers (20). Thus, it is urgent to develop novel diagnostic methods and effective treatment strategies. In recent 2 decades, extensive studies have identified HDGF as an important regulator that are critical to various biological processes, such as regeneration, growth, remodeling, mitosis promotion, vascular formation, transcriptional regulation, differentiation and apoptosis (21-26). The crucial role of HDGF overexpression on tumor progression and prognosis has been revealed in multiple cancer types, such as gastric cancer (16), hepatocellular carcinoma (15), pancreatic cancer (14), as well as lung and esophageal cancer (14,27). However, its role in human gliomas is still unknown.

In order to deal with the problem, 130 samples of human gliomas were collected to examine the HDGF expression and analyze the association between its expression and clinicopathological characteristics. Our data indicated that HDGF expression, at both protein and mRNA levels, was found to be more obviously up-regulated in glioma tissues than in intractable epileptic brain tissue without tumor. Moreover, high expression of HDGF was closely related to several clinicopathological parameters, including WHO grades II~IV, Ki-67 index ≥20% or KPS <80 ($^{*}P<0.05$). These outcomes may indicate an important role of HDGF in genesis or development of glioma.

Prior studies have mainly focused on the function of HDGF in other malignant tumors and accumulating evidence has revealed the effect of HDGF as a vital biomarker on cancer diagnosis and prognosis. Lots of studies have demonstrated that the over-expression of HDGF might play an important role in metastasis and eventually lead to poor results in various metastatic tumors. HDGF expression is significantly higher in breast cancer tissues and has a positive correlation with bad result severity, histology grades and tumor sizes. Thus, it is a strong predictor of the median survival time for breast cancer patients (28). Similar results were observed in several other types of cancer, including gastric cancer (14), lung cancer (26), pancreatic cancer (15) and esophageal carcinoma (14). For human glioma, current studies were mostly focused on the mechanism of carcinogenesis induced by HDGF. Hsu *et al* concluded that HDGF is a mitogenic growth factor in glioma progression (29). Zhang *et al* revealed that the knockdown of HDGF significantly inhibited tumorigenesis as well as colony formation, migration and invasion of U87 glioma cells (23). Song *et al*'s observed in their early studies that knocking out of HDGF obviously inhibited the formation, development and spread of glioma cell as well as restored the expression of E-cadherin and inhabited the biomarkers of mesenchymal cell such as β-catenin and N-cadherin and vimentin. They also found that HDGF probably participated in the activation of PI3K/Akt and TGF-β signaling pathways (30). In accord with these studies, our research also confirmed the carcinogenic role of HDGF as its expression, at both protein and mRNA levels, was up-regulated to a greater degree in glioma than in brain tissue without tumor. Moreover, the effect of HDGF expression on survival period of glioma patients was statistically analyzed. As a result, negative correlation was found between them. In addition, the results of multivariable linear regression suggested that WHO grade, KPS, Ki-67 and HDGF expression were closely related to glioma patients' prognosis. We have several innovations compared with these prior studies. These researches mostly based on glioma cell lines and animal as well as collected clinical features like age and gender. While we adopted glioma tissues of human brain in our study and more clinical data like Karnofsky performance Status (KPS) and Ki-67 index was collected in our research except for age and gender. So our research are more clinically relevant and tightly associated to human glioma.

Considering all of the results, animal experiments should be conducted by utilizing molecular biotechniques to evaluate the role of HDGF gene regulation on the development and invasion of glioma. Which may provide much more theoretical foundations for investigating prognostic and therapeutic potential of HDGF for glioma patients.

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