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Original article

Neuroglobin functions as a prognostic marker and promotes the tumor growth of glioma *via* suppressing apoptosis

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ABSTRACT

Neuroglobin (Ngb) has been reported to be upregulated by hypoxia and plays an anti-apoptotic function. Previous studies have reported that Ngb is expressed in human glioblastoma cells and up-regulated in hypoxic microregions of glioblastoma tumor xenografts. While, the clinical significance of Ngb and its function in human glioma keep unknown. Ngb expression was analyzed in 86 glioma tissues and 20 normal brain tissues. Results showed that Ngb was significantly overexpressed in glioma tissues compared to normal brain tissues. In addition, increased levels of Ngb also observed in glioma cell lines. Clinicopathological analysis verified that the positive expression of Ngb was associated with histological type and world health organization (WHO) grade of glioma. Moreover, Kaplan–Meier analysis found that Ngb overexpression led to a shorter survival. Multivariate Cox regression analysis demonstrated that Ngb expression was an independent prognostic marker. Further experiments illustrated that Ngb knockdown significantly inhibited proliferation and facilitated apoptosis in U251 cells. *In vivo* experiments further confirmed that Ngb silencing notably prohibited the tumor growth of glioma in nude mice. While, Ngb overexpression prominently promoted proliferation and suppressed apoptosis in U87 cells. Taken together, this work support the first evidence that Ngb can be potentially used as a promising biomarker and target for novel treatment of human glioma.

1. Introduction

Glioma represents one of the most common malignancies characterized by an adverse clinical outcome [1]. Radical surgery might be the only hope for curing glioma in the stage of precursor lesions [2]. However, even after surgical resection, radiotherapy and chemotherapy obtain little benefit [3]. Previous studies have identified that a few molecular markers were correlated with prognosis, but the mechanism involved in the pathogenesis of glioma is still unclear [4]. Therefore, there is urgent need to recognize oncogenesis-associated biomarker, which is helpful for developing novel treatment in view of glioma.

Neuroglobin (Ngb), firstly reported by Burmester T. et al. in 2000, may increase the availability of oxygen to brain tissue [5]. Increasing evidences prove that Ngb plays an antiapoptotic and neuroprotective role by facilitating O2 diffusion tomitochondria, scavenging oxygen free radicals and defending oxidative stress [6]. Recently, several studies have reported the expression of Ngb and its role in human cancers, especially in nervous system neoplasms. Ngb and cytoglobin protect human neuroblastoma cells against oxidative stress-induced cell death and prevent H2O2-induced apoptosis [7,8]. Emara M et al. report that the expressions of Ngb mRNA and protein are observed in human glioblastoma cells, and its expression is up-regulated by hypoxia *in vitro* and *in vivo*, providing a novel insight into how cancer cells adapt to and survive in hypoxic microenvironments [9,10]. Ngb is overexpressed in the non-small cell lung cancer (NSCLC) specimens compared to normal tissues, and upregulated by hypoxia [11]. Furthermore, Ngb exerts as a pro-survival player in estrogen receptor (ER) α-positive cancer cells including MCF-7, HepG2, SK-N-BE, and HeLa cells [12].

Abbreviations: Ngb, neuroglobin; WHO, world health organization; NSCLC, non-small cell lung cancer; ER, estrogen receptor; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; NHA, normal human astrocyte; DMEM, Dulbecco minimum essential medium; FCS, fetal calf serum; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction; TNFα, tumor necrosis factor α.

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ERβ-dependent Ngb up-regulation impairs 17β-estradiol-induced apoptosis in DLD-1 colon cancer cells upon oxidative stress injury [13]. Ngb overexpression reduces the sensitivity of MCF-7 breast cancer cells to paclitaxel [14]. However, Ngb functions as a tumor suppressor in hepatocellular carcinoma (HCC) by inhibiting proliferation, cell-cycle progression and colony formation *via* oncogenic Raf/mitogen-activated protein kinase (MAPK)/Erk signaling [15]. Recently, Qin H. et al. report that Ngb is expressed in a rat astrocytoma cell line (C6), human astrocytoma cell line (U251), and human astrocytoma tissues [16]. But, the clinical significant of Ngb and its role in human glioma remain poorly investigated.

This study showed that Ngb overexpression predicted a shorter survival. We also illustrated that Ngb promoted glioma cell growth and restrained apoptosis *in vitro* and *in vivo*. In conclusion, this work supported the first evidence that Ngb was a significant biomarker, and recognized as a potential therapeutic target for glioma.

2. Materials and methods

2.1. Patients

86 samples of glioma tissues were obtained from the Department of Pathology, Xi'an Central Hospital. And normal brain tissues were obtained from 20 cerebral hemorrhage patients, who underwent the resection of nonfunctional areas. Tissue specimens were conserved in liquid nitrogen for qRT-PCR or 10% formalin for IHC until use. All samples were used after obtaining informed consent. All clinicopathological information of patients were represented in Table 1. The Ethics Committee of Xi'an Jiaotong University approved all protocols according to the Declaration of Helsinki.

2.2. Cell culture and transfection

Human glioma cell lines (U87, T98, A172 and U251) and normal human astrocyte (NHA) cells (American Type Culture Collection, Manassas, VA, USA) were cultivated in Dulbecco minimum essential medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) tinting with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Small hairpin RNA (shRNA) targeting Ngb and non-targeting (NT) shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). shRNA vectors were transferred into cells using lipofectamine 2000 (Thermo Fisher Scientific) on the basis of the manufacturer's recommendation. Retroviral vector pMMP-Ngb was generated by inserting the cDNA into pMMP. Retrovirus packaging and transduction were described previously [17].

2.3. Immunohistochemistry (IHC)

The formalin-fixed and paraffin-embedded tumor tissues were sliced into 4 μm sections, and were deparaffinized in xylene and then rehydrated in graded alcohol. Antigen retrieval was accomplished by incubating the slides in Tris-EDTA Buffer for 3 min in a pressure cooker. Then, the slides were incubated in 0.3% H_2O_2 for 30 min to suppress endogenous peroxidase activity and then washed in PBS. Next, the slides were blocked with 10% skim milk in PBS for 30 min. Ngb (Abcam, Cambridge, MA, USA) antibody was used as a primary antibody at a 1:300 dilution in PBS overnight at 4 °C. The following day, after several washes with PBS, the slides were incubated with peroxidase conjugated secondary antibody (ZSGB BIO, Beijing China) for 90 min, and a peroxidase-labeled polymer, DAB solution was used for signal development for 5 min. The sections were counterstained with hematoxylin followed by dehydrating and mounting. Staining intensity was scored manually by two independent experienced pathologists as no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was drawn using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacture's procedure. The first strand cDNA was compounded using a Tianscript RT kit (Tiangen biotech, Beijing, China). Expressions of mRNA were detected using ABI 7300 system (Applied Biosystems, Foster City, CA, USA). GAPDH was employed to be the control. The primers used for target genes were purchased from Sangon Biotech (Shanghai, China).

2.5. Proliferation assay

Cells after transfection were gathered for MTT assay following the manufacturer's recommendation. In brief, cells were grown in 96-well plates containing 100 μl DMEM per well. After transfection for 24, 48, 72 and 96 h, respectively, 10 μl of MTT was added into each well, then the medium was undocked after incubation for 4 h and subsequently added with 150 μl DMSO per well. The results represent as the average of three independent replicates. As for colony formation assay, 2000 glioma cells were seeded on 6-well plates. 14–21 days after cell seeding, cell colonies with crystal violet staining were counted.

2.6. Flow cytometer detection for cell apoptosis

Glioma cells transduced with different vectors were subjected to flow cytometry. Apoptosis of glioma cells were evaluated with an Annexin-V/PI kit (BD Pharmingen, San Diego, CA, USA). In

Table 1
Correlation between the clinicopathologic characteristics and expression of Ngb protein in glioma.

Characteristics		n	Ngb expression		*P*
			Positive	Negative	
Age (y)	≥50	45	27	18	0.297
	<50	41	29	12	
Sex	Male	56	37	19	0.800
	Female	30	19	11	
Histological type	Astrocytic tumors	61	45	16	0.009*
	Oligodendrogial + Oligoastrocytic tumors	25	11	14	
WHO grade	I + II	27	12	15	0.007*
	III + IV	59	44	15	

Ngb, neuroglobin; WHO, world health organization.
* Statistically significant.