

Neuritin expression and its relation with proliferation, apoptosis, and angiogenesis in human astrocytoma

Lei Zhang · Yonggeng Zhao · Cheng-guo Wang ·
Zhou Fei · Yuan Wang · Lexiang Li ·
Liang Li · Hai-ning Zhen

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Abstract Neuritin, a new member of the neurotrophic factor family, plays an important role in promoting neuronal survival, differentiation, function, and repair. However, whether neuritin is expressed in human astrocytoma and involved in their proliferation, apoptosis, and angiogenesis remains unclear. The expression of neuritin messenger RNA, protein and the relationship with proliferation, apoptosis, and angiogenesis were examined in human astrocytoma samples and three glioma cell lines by immunohistochemistry, Western blot, and quantitative real-time RT-PCR and so on. And neuritin immunoreactivity score (IRS), proliferative index (PI), apoptotic index (AI), overall daily growth (ODG), and microvessel density (MVD) in brain astrocytoma were measured. The results showed that neuritin was overexpressed in human astrocytoma samples, and the overexpression correlated positively with the malignancy of astrocytomas as reflected by changes in proliferation, apoptosis, and angiogenesis markers. In our study, we found neuritin is overexpressed

in astrocytoma, which may be an important factor in tumorigenesis and progression of astrocytoma, and can be used as a target for biological therapy.

Keywords Neuritin · Astrocytoma · Proliferation · Apoptosis · Angiogenesis

Introduction

To design effective therapeutic strategies to the common malignant primary brain tumors, especially infiltrating astrocytoma, it is essential to understand their molecular mechanisms and to find specific tumor markers for tumorigenesis, proliferation, and apoptosis. The neurotrophin proteins (NTs) family is an important factor in promoting angiogenesis, neuronal differentiation and function, and also cell proliferation and apoptosis [1, 2]. Up to now, six mammalian NTs have been discovered, i.e., nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, NT-4/5, NT-6, and neuritin [3]. Neuritin, first identified by Nedivi et al., is a novel member of the NTs family [4], it (also called candidate plasticity-related gene 15 [CPG 15]) is induced by neuronal activity in the hippocampal area of rat brain. Recently, neuritin was found to be expressed by non-nervous system tissues, such as liver, invasive breast carcinoma, and Kaposi's sarcoma and to play a crucial role in apoptosis, neuronal network reconstruction and maintenance, axonal regeneration, and possibly in tumorigenesis and nerve development [5–7]. However, whether neuritin is expressed in human astrocytoma and whether neuritin expression is correlated with proliferation, apoptosis, and angiogenesis in astrocytoma are not clear. Our study examined neuritin expression in brain astrocytoma and human glioma cell lines and

Lei Zhang, Yonggeng Zhao, and Cheng-guo Wang contributed equally to this work.

L. Zhang · Y. Zhao · Z. Fei (✉) · Y. Wang · L. Li · H. Zhen
Department of Neurosurgery, Xijing Hospital, Fourth Military
Medical University, 15 Changlexi Street, Xi'an,
Shaanxi Province 710033, China
e-mail: feizhou@fmmu.edu.cn

Y. Zhao · L. Li
Department of Emergency, The 456th Hospital of PLA, 25
Wuyingshan Street, Jinan, Shandong Province 250031, China

C. Wang
Department of General Surgery, Tangdu Hospital,
Fourth Military Medical University, 17 Changlexi Street,
Xi'an, Shaanxi Province 710033, China

investigated the relationship of neuritin expression to proliferation, apoptosis, and angiogenesis.

Materials and methods

Tissue samples

All tissue specimens were obtained according to institutional review board-approved procedures for consent. All the archival paraffin blocks and tissue specimens were provided by the specimen bank of the Institute of Neurosurgery of People's Liberation Army (Xi'an, China) and the 456th hospital of People's Liberation Army (Ji'nan, China). Human astrocytoma tissue sections and frozen samples were from 108 patients (61 women and 47 men; median age, 46 years; age range, 14–73 years; 17 of Grade I, 29 of Grade II, 24 of Grade III, and 38 of Grade IV, respectively) [8]. Normal human brain tissue specimens were obtained from 10 patients (median age, 45 years; age range, 20–65 years). All tumor tissues were obtained from the initial surgery.

Cell culture

Two human glioma cell lines (U251, U87) and one human normal astrocyte line (SVG p12) were purchased from American Type Culture Collection (Rockville, MD), and the human astrocytoma cell line (SHG44) was obtained from the First Affiliated Hospital of Suzhou University (Suzhou, China) in March 13, 2009. All cells were cultured at 37°C in a 5% CO₂ incubator (Life Technologies, Baltimore, MD).

Immunohistochemistry

The sections were treated with anti-neuritin, anti-proliferating cell nuclear antigen (PCNA), and anti-factor VIII-related antigen (FVIII-Rag) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). In the negative control sections, PBS with 1.0% BSA was used instead of the primary antibodies.

Western blot analysis

Protein concentration was determined using either the Bio-Rad protein assay kit (Hercules, CA) or micro-BCA protein assay (Pierce, Rockford, IL). And the following protocols of Western blot were described by Raggo et al. [7].

Quantitative real-time RT-PCR

The methods of quantitative real-time RT-PCR were described by Savino et al. [9]. The quantitative real-time

PCR primers were designed using ABI Primer Express software. The forward and reverse primer sequences were as follows: neuritin: sense primer, 5' -GUG CGA UGC AGU CUU UAA GTT- 3'; anti-sense primer, 5' -GGG CUU UUC AGA CUG UUU GTT- 3'; GAPDH: sense primer, 5' -GCA CCG TCA AGG CTG AGA AC- 3'; anti-sense primer, 5' -ATG GTG GTG AAG ACG CCA GT- 3'.

Fluorometric TdT-mediated dUTP nick end-labeling (TUNEL) analysis

TUNEL assays were performed using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI) essentially according to the manufacturer's instructions. Experiments and control experiments were performed twice on different days using the same protocol and times of exposure.

Evaluation of staining results

Neuritin-positive reactivity in the cytoplasm, PCNA-positive reactivity in the nucleus [10], and FVIII-Rag-positive reactivity in vascular endothelial cells were indicated by brown-yellow staining, while apoptosis was indicated by purple staining in the nucleus and in the apoptotic bodies. To measure the neuritin immunoreactivity score (IRS), proliferative index (PI), apoptotic index (AI), and overall daily growth (ODG) in astrocytomas [11], especially in the most strongly stained tumor area of each section, 1,000 cells were observed in 5–10 adjacent high-powered fields at ×400 magnification. Neuritin protein expression was assessed semiquantitatively by calculating neuritin IRS according to the method described by Friedrich et al. [12]. Because the staining of tumor cells is heterogeneous, SI was determined by the staining intensity of most cells. The percentages of PCNA-positive cells and apoptotic cells were regarded, respectively, as the PI and AI of tumor tissues. The ODG of astrocytomas was calculated using the equation: $ODG = (PI/2) - (AI/0.5)$, described by Brown et al. [11]. Microvessel density (MVD) of astrocytomas was measured using the method described by Leon et al. [13].

Statistical analysis

All statistical analyses were performed using the SPSS 12.0 software package (Chicago, IL). The neuritin IRS, PI, AI, ODG, and MVD for each group of specimens are all expressed as mean ± SD. Differences in neuritin IRS, PI, AI, ODG, and MVD in different pathologic grade groups were first compared using analysis of variance (ANOVA), and then the Student-Newman-Keuls test (SNK test, i.e., *q* test) was used for comparison of the differences between

two groups. Differences in PI, AI, ODG, and MVD between the neuritin-positive group and neuritin-negative group were compared using the Student *t*-test. Correlation coefficients of neuritin IRS with PI, AI, ODG, and MVD were determined using Pearson’s correlation analysis with a two-tailed test for significance. Statistical significance was defined as $P < 0.05$.

Results

Neuritin is expressed in astrocytoma and glioma cell lines

In our study, immunoreactive neuritin was overexpressed in the cytoplasm of the U87 and U251 cell lines, but not in the SHG cell line (Fig. 1a). Both neuritin mRNA and protein expression increased with astrocytoma grade (Fig. 1b, c). Neuritin immunoreactivity was obviously localized within the cytoplasm of cells (Fig. 2a).

Neuritin is associated with pathologic grade in astrocytomas

Low neuritin expression of neuritin was demonstrated in 3 samples of normal brain tissue. However, neuritin

overexpression was detected in 69.7% of 108 astrocytoma specimens with an IRS of 4.88 ± 5.07 (Fig. 2a). The neuritin IRS increased markedly from Grade I to Grade IV ($F = 6.141$, $P < 0.001$, Table 1). In particular, neuritin IRS was significantly higher in Grade III than Grade I astrocytomas ($P = 0.031$), in Grade IV than Grade II astrocytomas ($P = 0.013$), in Grade IV than Grade I astrocytomas ($P < 0.001$).

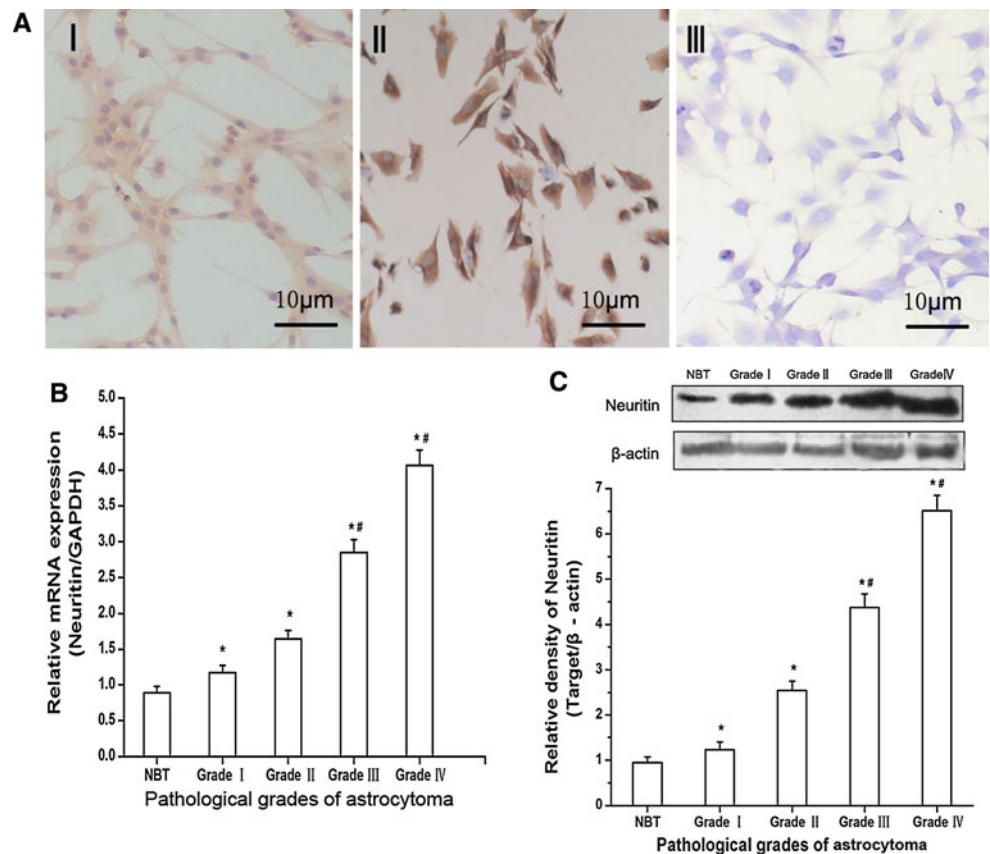
PI is positively related to pathologic grade and neuritin expression in astrocytomas

PCNA was expressed in all specimens, and the range of PI was 3.41–92.15% ($37.28 \pm 21.39\%$, Fig. 2b). Moreover, PI not only increased greatly with increase in pathologic grade ($F = 13.115$, $P < 0.001$), but was also significantly higher in neuritin-positive astrocytomas ($42.77 \pm 21.53\%$) than in neuritin-negative astrocytomas ($19.64 \pm 13.38\%$, $t = 4.224$, $P < 0.001$). Moreover, PI correlated positively with neuritin IRS ($r = 0.712$, $P < 0.001$, Table 1 and Fig. 3a).

ODG is positively related to pathologic grade and neuritin expression in astrocytomas

The range of ODG in brain astrocytomas was 0.84–47.63% ($17.15 \pm 12.13\%$). ODG increased significantly with

Fig. 1 Immunohistochemical detection of human neuritin proteins: (A-I) U87 is neuritin-positive human glioma cell line; (A-II), U251 also is neuritin-positive human astrocytoma cell line; (A-III) SHG44 is neuritin-negative human astrocytoma cell line; scale bar = 10 μm. **b** Quantitative RT-PCR was performed to determine neuritin mRNA levels in grade I to IV of astrocytomas (* $P < 0.05$, ** $P < 0.01$ vs normal brain tissues (NBT)). **c** Western blot was performed to determine neuritin protein levels in grade I–IV of astrocytomas. (* $P < 0.05$, ** $P < 0.01$ vs NBT)



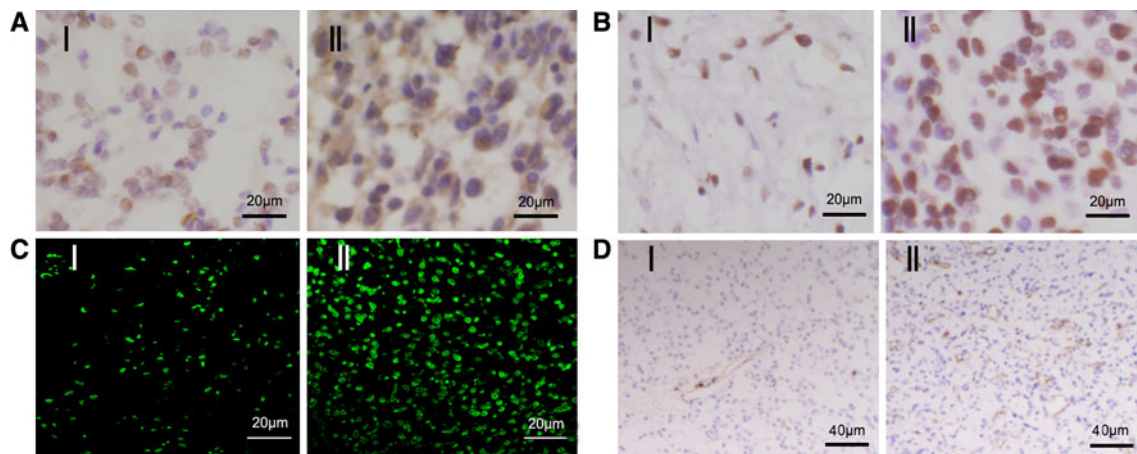


Fig. 2 Immunohistochemical analysis of neuritin expression, proliferation, apoptosis, and angiogenesis in brain astrocytomas. **a, b, d** Immunostaining of neuritin (A-III), proliferating cell nuclear antigen (PCNA) (B-III), and factor VIII-related antigen (FVIII-RAg) (D-III);

c Apoptosis detection by fluorometric TUNEL. A–D:I: Astrocytoma (WHO Grade II). A–D:II: Glioblastoma (WHO Grade IV). scale bar = 20 μ m

Table 1 Measurements of Neuritin IRS, PI, AI, ODG, and MVD in brain astrocytoma

| Glioma tissues | Neuritin IRS | PI% | AI% | ODG% | MVD |
|----------------|------------------------------|--------------------------------|------------------------------|--------------------------------|--------------------------------|
| Grade I | 1.35 \pm 1.79 | 13.61 \pm 17.58 | 0.91 \pm 1.20 | 5.47 \pm 7.07 | 32.31 \pm 41.72 |
| Grade II | 2.61 \pm 2.57 | 23.43 \pm 22.84 | 1.98 \pm 1.95 | 9.52 \pm 9.28 | 52.25 \pm 51.91 |
| Grade III | 4.63 \pm 3.09 | 33.52 \pm 22.01 | 2.70 \pm 1.78 | 14.25 \pm 9.37 | 76.93 \pm 50.43 |
| Grade IV | 6.74 \pm 3.37 | 46.36 \pm 23.05 | 3.80 \pm 1.92 | 19.68 \pm 9.79 | 90.62 \pm 45.05 |
| Total | 4.21 \pm 3.49 ^a | 31.63 \pm 24.65 ^a | 2.57 \pm 2.04 ^a | 13.26 \pm 10.40 ^a | 67.40 \pm 51.41 ^a |

^a $P < 0.001$ when compared among Grade I to IV brain astrocytomas (by ANOVA)

increase in pathologic grade ($F = 9.420$, $P < 0.001$). ODG was significantly higher in the neuritin-positive group ($20.17 \pm 13.24\%$) than in the neuritin-negative group ($7.56 \pm 6.33\%$, $t = 3.977$, $P < 0.001$) and positively correlated with neuritin IRS ($r = 0.716$, $P < 0.001$; Table 1 and Fig. 3b).

AI is negatively related to pathologic grade and neuritin expression in astrocytomas

In all tumor specimens, there were apoptotic astrocytoma cells and/or apoptotic bodies mainly in astrocytoma cells (Fig. 2c). The characteristic morphologic changes due to apoptosis included chromatin condensation, nuclear disintegration, and formation of crescentic caps of chromatin at the nuclear periphery. The range of AI in brain astrocytomas was 0.38–9.45% ($2.79 \pm 2.11\%$). AI increased as pathologic grade increased ($F = 3.987$, $P = 0.001$). Although not significantly different in the neuritin-positive group ($2.85 \pm 2.71\%$) and the neuritin-negative group ($2.14 \pm 1.48\%$, $t = 1.197$, $P = 0.242$), AI was inversely correlated with neuritin IRS ($r = -0.267$, $P = 0.0332$; Table 1 and Fig. 3c).

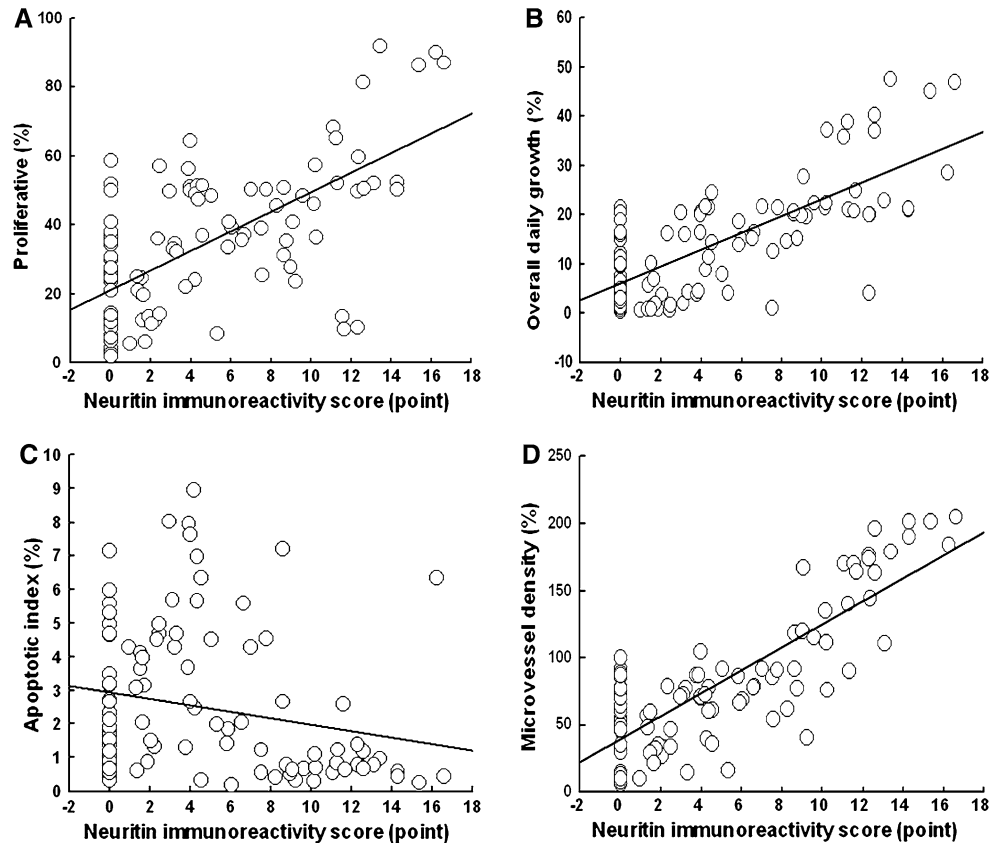
MVD has close relationship with pathologic grade and neuritin expression in astrocytomas

Microvessels were investigated in all tumor specimens (Fig. 2d). MVD of brain astrocytomas ranged from 15–218 (78.42 ± 49.88), which increased markedly with the increase in pathologic grade of brain astrocytomas ($F = 13.613$, $P < 0.001$). Although there was no significant difference in MVD in the neuritin-positive group (92.21 ± 53.25) and that in the neuritin-negative group (51.87 ± 27.41 , $t = 3.918$, $P < 0.001$), MVD was positively correlated with neuritin IRS ($r = 0.799$, $P < 0.001$, Table 1 and Fig. 3d).

Discussion

Astrocytoma is a common malignant primary brain tumor with a poor clinical prognosis in both adults and children. And tumorigenesis of astrocytoma involves multiple factors, multiple gene mutations, and many signal transduction pathways, which is the role especially played by NTs

Fig. 3 Scatterplots of correlation of neuritin immunoreactivity score (IRS) with proliferative index (PI), apoptotic index (AI), overall daily growth (ODG), and microvessel density (MVD) in brain astrocytomas. A trend line is provided in each plot, which represents a 'best fit' as determined by simple linear regression. With neuritin IRS increase **a** PI **c** ODG, and **d** MVD increased significantly, whereas **b** AI decreased markedly ($P < 0.05$ for all, Pearson correlation analysis)



[12]. However, the mechanisms are not fully understood at present.

Neuritin has been cloned and characterized as an important neurotrophin [4]. The expression of neuritin is closely associated with the growth of afferent nerves and the development of dendrites, axons, and synapses [9, 14]. Recently, neuritin expression was found in tissues besides nervous system and tumor tissues [5–7, 15]. In our study, the neuritin protein was highly expressed in astrocytomas. Furthermore, neuritin IRS increased with increase in astrocytoma pathologic grade, indicating that neuritin has an important role both in the promotion and in the progression of astrocytomas.

In the present study, we found that PI, ODG, and MVD in neuritin-positive specimens all significantly increased compared with neuritin-negative specimens [16], and neuritin IRS was positively correlated with PI, ODG, and MVD [12]. Although there was no significant difference between AI in neuritin-positive specimens and that in neuritin-negative specimens, there was a decreasing tendency of AI in neuritin-positive specimens. Moreover, neuritin IRS was inversely correlated with AI. The results indicate that neuritin may play a key role in tumorigenesis and progression of astrocytomas by its biologic activities.

To the best of our knowledge, only one report has shown that expression of neuritin is related to proliferation. In that

study, Kaposi's sarcoma cells contained genes for neuritin, which was essential for the transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus [5]. In our study, PI, ODG, and MVD all increased significantly in neuritin-positive specimens in contrast to neuritin-negative specimens, and neuritin IRS was positively correlated with PI, ODG, and MVD. Neuritin IRS was inversely related to AI possibly because neuritin inhibits caspase-3 activity, which thereby can inhibit apoptosis [5]. Our results illustrate that neuritin may play a key role in tumorigenesis and progression of astrocytoma through its biologic activities including anti-apoptosis, promotion mitosis, and angiogenesis. We propose that neuritin may be a valuable biomarker for the molecular diagnosis of astrocytomas and an ideal therapeutic target in the management of astrocytomas.

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