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Negative correlation of Nogo-A with the malignancy of oligodendroglial tumor

Nan-Xiang XIONG, Hong-Yang ZHAO, Fang-Cheng ZHANG, Zhu-Qiang HE
Department of Neurosurgery, Xiehe Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

Abstract: Objective Nogo-A is an axon regeneration inhibitor, and its function in central nervous system (CNS) is still unknown. The present study is to explore the relationship between the expression of Nogo-A and the malignancy of oligodendroglial tumors in patients. **Methods** Tumor tissue samples with different malignancy grade were obtained from the hospitals. The samples used for detection had been diagnosed as oligodendroglial tumors (oligodendroglioma or anaplastic oligodendroglioma). The expression of Nogo-A was detected by immunohistochemistry and western-blot analysis. The correlation test between the Nogo-A expression and the morphological changes (the percentages of atypical cells and mitotic cells in the tumors) related to the malignancy of tumor tissues was performed. **Results** There was significant negative correlation between the Nogo-A expression and the morphological change of tumor tissues according to immunohistochemistry. Western-blot analysis also indicated that the gray value of Nogo-A protein band in the oligodendroglioma group was significantly higher than that in the anaplastic oligodendroglioma group. **Conclusion** Nogo-A expression was negatively correlated with the malignancy grade of oligodendroglial tumors.

Keywords: Nogo-A; oligodendroglial tumors; immunohistochemistry

1 Introduction

Nogo, a mammalian reticulon family gene, could encode three splice isoforms, Nogo-A, Nogo-B and Nogo-C, resulting from both alternative promoter usage and alternative splicing[1]. Nogo-A is known as an axon outgrowth inhibitor in central nervous system (CNS)[2], while Nogo-B is found associated with the apoptosis of tumor cells[3,4]. Nogo-A have a same apoptosis-inducing domain[6] to Nogo-B, but no experimental result so far to indicate that Nogo-A also has the apoptosis-inducing function. Liao H *et al*.[9] reported that the substratum adherence and migration of human U87MG glioma cells in the culture were significantly attenuated by the extracellular domains of Nogo-A, indicating that Nogo-A can modulate glioma growth and migration. However, Teng FY *et al*.[14] hypothesized that Nogo isoforms might have the antitumorigenic or tumor-suppressing activity.

It is known that Nogo-A mainly localizes in oligodendrocytes[5], but the expression pattern of Nogo-A in oligodendroglial tumors is still unknown. In this study, we attempted to exam the expression of Nogo-A in oligodendroglial tumors. The relationship between the expression of Nogo-A and the malignancy of oligodendroglial tumor was also investigated.

2 Materials and methods

2.1 Tumor samples Twenty-six samples, which had been histologically diagnosed as oligodendroglial tumors, were collected from Tongji Hospital and Xiehe Hospital, Huazhong Science and Technology University, China. All cases were divided into oligodendrogliomas (n = 16) and anaplastic oligodendrogliomas (n = 10) according to their atypia, mitosis, vascular angiogenesis, and necrosis. The donor patients included 13 male and 10 female aged from 21 to 47 (mean 35.5 years old). In this study, we obtained informed consent from all the patients.

2.2 Immunohistochemistry Immunohistochemistry was done as previously described[15]. Briefly, samples were cut

Corresponding author: Hong-Yang ZHAO
Tel: 86-27-85726833
E-mail: hyzhao750@sina.com
Article ID: 1673-7067(2007)01-0041-05
CLC number: R739.4
Document code: A
Received date: 2006-09-25

into sections at 30–50 μm on a vibrating microtome. Before staining, free-floating sections were incubated sequentially in 0.3% Triton X-100 (in PBS) and 10% goat serum (in PBS) for 30 min each. Incubate the sections with primary antibodies (anti-Nogo-A polyclonal antibody, 1:200; Santa Cruz, USA) at 4 °C for 48 h followed by at 37 °C for 2 h, and then wash the specimens with PBS at room temperature. Diluted biotinylated anti-rabbit IgG was used as the secondary antibody (1:400; Zhongshan Company, Beijing, China). The antigen-antibody reaction sites were visualized by staining the sections in 0.033% 3,3'-diaminobenzidine (DAB kit, Zhongshan Company) and 0.014% H_2O_2 (in 0.05 mol/L Tris-HCl buffer) for 7 min. Stop the reaction in PBS. Then the sections were mounted, air-dried, dehydrated, restained by hematoxylin, and coverslipped.

Photos were taken with light microscope (Nikon YS100, Japan). At least 20 pictures (× 200) were selected from each sample. The optical density (OD) value of Nogo-A-immunopositive cells and the percentages of atypical cells and mitotic cells in the same field were analyzed by HMIAS-2000 image analysis software (Qianping Imaging Ltd., Wuhan, China).

2.3 Western-blot analysis Western-blot was done according to Ghorbel *et al*. described[15]. Briefly, tumor tissues were homogenized in the solution consisted of 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 0.5 mmol/L phenylmethylsulphonyl fluoride, 0.5 mmol/L dithiothreitol, and 25% (v/v) glycerol. The homogenate was frozen on dry ice for 5 min, thawed in ice water for 15 min, and centrifuged at 14 000 *g* for 15 min. The supernatant containing total protein extract was stored at –70 °C. Protein concentration was determined by the Bradford method.

Equal amounts of protein (30 μg) were separated on a 16% (w/v) SDS-polyacrylamide gel, and then electrotransferred onto the Immobilon-P membrane. Membranes were blocked in 5% (w/v) bovine serum albumin (BSA) in TBS-T [20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, 0.1% (w/v) Tween 20], and then incubated with affinity-purified primary antibody (goat polyclonal Nogo-A antibody, 1:400; Santa Cruz, USA) in 5% (w/v) BSA in TBS-T overnight at 4 °C. Rabbit anti-actin antibody was used as the internal control. Following washes with TBS-T (four times with 10 min each), membranes were incubated with anti-goat and anti-rabbit secondary antibodies IgG (1:2000; Zhongshan Company, Beijing, China) for 2 h at room temperature. The bound antibody was visualized with the ECL chemiluminescence system (Amersham Biosciences, USA). Membranes were opposed to X-ray films and developed. The films were scanned and subjected to the densitometric analysis by using ImageMaster quantification software (Amersham Biosciences, USA). Data were shown as mean ± SD.

2.4 Statistical analysis The correlation between the Nogo-A expression and the morphological changes of tumor cells was accomplished by the nonparametric Spearman's rank correlation test. Comparison of the Nogo-A expression between the oligodendroglioma group and the anaplastic oligodendroglioma group was accomplished by one-way ANOVA analysis. $P < 0.05$ was considered significant.

3 Results

3.1 Immunohistochemistry Nogo-A-immunopositive materials were localized in the cytoplasm of oligodendroglial tumor cells, which had a round shape with hematoxylin positive nuclear. As can be seen, the nuclear atypia and mitosis were less in the low grade tumor cells (oligodendroglioma, Fig. 1A) than those in the anaplastic tumor cells (anaplastic oligodendroglioma, Fig. 1B).

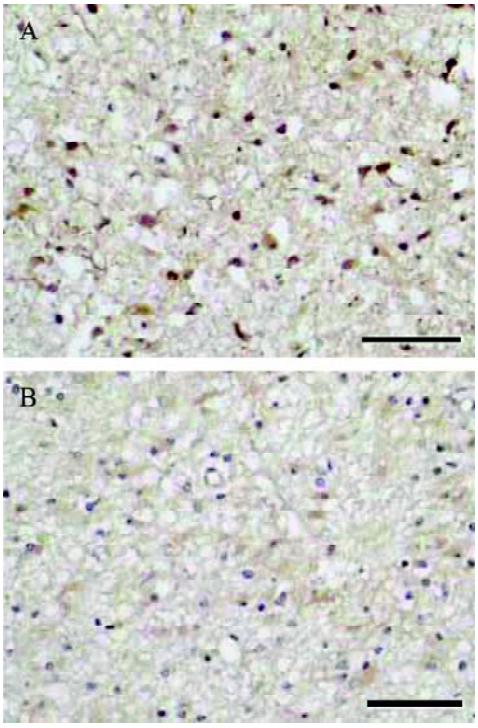

Fig. 1 Immunohistochemistry results of oligodendroglial tumors. The immunopositive materials were mainly localized in cytoplasm, nuclei were stained by hematoxylin. A: oligodendroglioma, B: anaplastic oligodendroglioma. Scale bar, 40 μm.

By statistics, we found a significant negative correlation between the OD of Nogo-A-positive cells and the percentages of atypical cells ($P = 0.0294$, $r = –0.387$) and mitotic cells ($P = 0.0175$, $r = –0.486$) (Fig. 2), indicating that the Nogo-A expression was negatively correlated with the malignancy of oligodendroglial tumor.

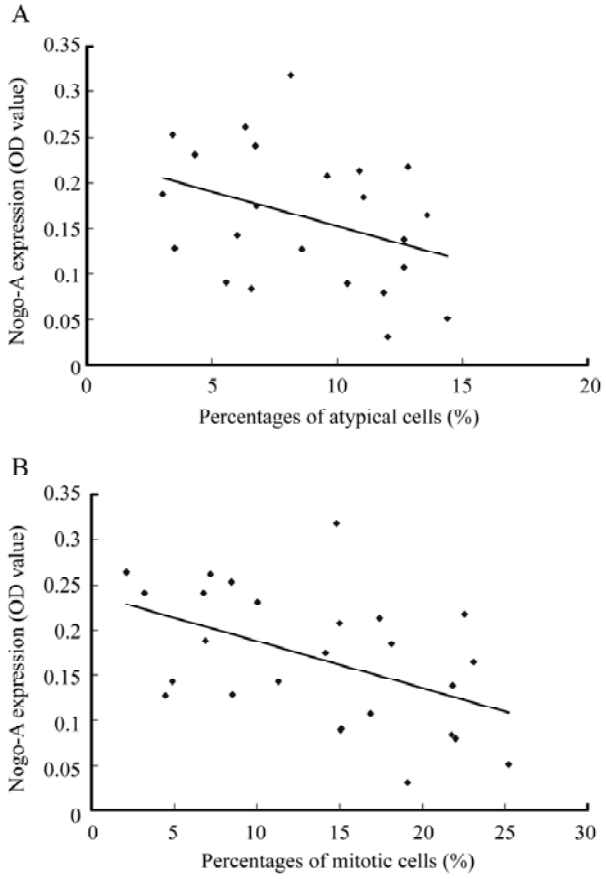

Fig. 2 Correlation between the Nogo-A expression and the percentage of atypical cells (A) or mitotic cells (B) in the oligodendroglial tumor.

3.2 Western-blot analysis The result of western-blot assay was shown in Fig. 3. The densitometric analysis showed that the relative gray scale value of Nogo-A to actin in the oligodendroglioma group (3.48±0.85) was significantly higher than that in the anaplastic oligodendroglioma group (1.81±0.59) ($P = 0.0328$).

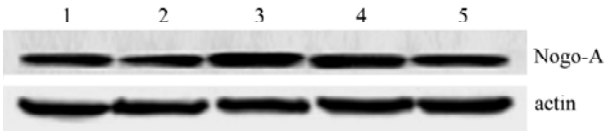

Fig. 3 Western-blot results of Nogo-A in oligodendroglial tumors. Lane 1, 2: anaplastic oligodendrogliomas; lane 3, 4, 5: oligodendrogliomas.

4 Discussion

Li Q *et al*. first reported that Nogo might be related to any kind of cancer, and showed that the ectopic expression of Nogo-B led to apoptosis in some kinds of cancer cells[3]. Tambe Y *et al*.[7] also showed that the product of *drs* gene can interact with Nogo-B, and the coexpression of drs and Nogo-B can increase the efficiency of apoptosis. Previous experiments demonstrate that overexpressed Nogo-B protein induces apoptosis in various cancer cells. It is known that Nogo-A and Nogo-B share much the same molecular structure—the N-terminus and a conserved 66-amiod acid domain (Nogo-66), and the protein sequence of Nogo-A covers all that of Nogo-B. Therefore we wondered whether Nogo-A also has the same tumor suppressive activity as Nogo-B's.

According to our results of immunohistochemistry experiments, there were significant negative correlations between the Nogo-A level and the extent of atypia and mitosis in the oligodendroglial tumor. Western-blot analysis also indicated that the gray scale value of Nogo-A band was negatively correlated with the malignancy grade of oligodendroglial tumors. The preliminary results suggested that Nogo-A may have a tumor-inhibiting activity.

Amberger VR *et al*. have examined a great number of human brain tumor cell lines and cultured specimens from human brain tumors for their ability to spread and migrate on CNS myelin-coated culture dishes[8]. Although primary and low-grade glioma cultures were sensitive to inhibitory proteins, high-grade glioblastomas were able to spread and migrate. For glioma, therefore, the ability of resistance to the inhibition of CNS myelin appears to be correlated to its malignancy grade.

We postulate that Nogo-A may suppress tumor in two ways according recent relevant researches.

First, Nogo-A might execute the growth-inhibitory function by inhibiting the adhesion or migration of tumor cells. Liao H *et al*.[9] showed that the substratum adherence and migration of human U87MG glioma cells in culture were significantly attenuated by the extracellular domain of Nogo-A (Nogo-66) and the myelin-associated glycoprotein (MAG). U87MG cells contained large amounts of endogenous Nogo-66 receptors (NgRs). Nogo-66 and MAG may modulate glioma growth and migration by activating NgR, since treating U87MG cells with phosphatidylinositol-specific phospholipase C (PI-PLC), or NgR antibodies, resulted in

an increase in their ability to adhere to or migrate through Nogo-66- and MAG-coated substrates. Oertle T *et al*. demonstrated the presence of three different active sites with distinct growth inhibitory activities: 1, an N-terminal region involved in the inhibition of fibroblast spreading; 2, a stretch encoded by the Nogo-A-specific exon that restricts neurite outgrowth and cell spreading and induces growth cone collapse; and 3, a C-terminal region (Nogo-66) with growth cone collapsing function[10]. The N-terminal end of Nogo-A (amino acids 59–172) and the Nogo-A-specific domain (amino acids 544–725) appear to contain regions with the general growth inhibitory activity such as fibroblast spreading. Since the N-terminal region and Nogo-A-specific domain can inhibit fibroblast spreading, they would also exert an inhibitory effect on CNS tumor growth, invasion, and mignation.

Second, recent research results led researchers to speculate that Nogo-A might enhance the susceptibility of cancer cells to apoptosis. Li Q *et al*. have found that Nogo-B could induce intensive apoptosis in various cancer cell lines such as SaOS-2, HT-1080, MeWo, and CGL4[3]. Although the mechanism of Nogo-induced apoptosis is not yet clarified, it has been reported that Nogo associates with the anti-apoptotic proteins Bcl-2 and Bcl-X_L: changing their localization to the endoplasmic reticulum (ER), and reducing their anti-apoptotic activity in the cells treated with tunicamycin and staurosporine[11]. Recent results identified Nogo-B as a new physiological substrate of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2)[12]. These studies suggest that overexpressed Nogo protein may induce apoptosis by disturbing the anti-apoptotic mechanism of Bcl-2 family proteins in the ER.

Furthermore, Qi B *et al*. have isolated the Nogo-interacting protein (Nogo-IP) gene using yeast two-hybrid method[13]. Nogo-IP is a member of the reticulon protein family and forms a complex with Nogo protein in the ER. In preliminary experiments, Nogo-IP showed the anti-apoptotic activity against certain apoptosis inducers. Therefore, it is possible that the apoptotic activity of endogenous Nogo is suppressed by binding with some inhibitory proteins such as Nogo-IP, and the ectopic overexpression of Nogo may disrupt this control balance. Further studies are needed to isolate and characterize the genes of Nogo-IP.

Another important subject requiring attention is the relationship between Nogo proteins and ER stress-response-related proteins, especially the interaction of Nogo with inositol-requiring kinase 1 (IRE1), or caspase-12. The interactions of Nogo-A with pro- or anti-apoptotic genes could potentially regulate or impact a tumor's malignancy by influencing cancer cell apoptosis. This line of study may lead to clarification of the mechanism of stress-induced apoptosis in the ER.

It is not known whether Nogo-A could interact with oncogenic or anti-oncogenic proteins. It is not clear either if the mRNA and protein expression of Nogo-A are subjected to any physiological regulation. The two issues remain to be investigated and resolved[14].

Acknowledgements: This work was supported by grants from the National Nature Science Foundation of China (No. 30471775) and the Project of Science and Technology of Hubei Province (No. 2005AA301C15).

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Nogo-A 蛋白的表达与少突胶质细胞肿瘤恶性程度呈负相关

熊南翔，赵洪洋，张方成，何主强
华中科技大学同济医学院附属协和医院神经外科，武汉 430022

摘要：目的 探讨Nogo-A蛋白在少突胶质细胞肿瘤中的表达水平与该肿瘤恶性程度之间的关系。**方法** 选取来自临床上经病理检查确诊为少突胶质细胞肿瘤的组织标本，其中少突胶质细胞瘤16例，间变型少突胶质细胞瘤10例。免疫组织化学染色检测Nogo-A的表达，并与同视野下瘤细胞的核分裂与核异型性比例作相关分析；另外，把病理标本按照分级进行蛋白印迹试验。**结果** 免疫组织化学结果显示，Nogo-A免疫阳性染色的光密度值与肿瘤细胞核分裂、核异型比例呈明显的负相关，对蛋白印迹试验结果进行光密度分析也发现少突胶质细胞瘤中的Nogo-A蛋白条带灰度值明显高于间变型少突胶质细胞瘤中的灰度值。**结论** Nogo-A蛋白在少突胶质细胞肿瘤中的表达水平与该肿瘤恶性程度呈负相关。

关键词：Nogo-A；少突胶质细胞肿瘤；免疫组织化学