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ORIGINAL ARTICLE

Nectin-like Molecule 1 Inhibits the Migration and Invasion of U251 Glioma Cells by Regulating the Expression of An Extracellular Matrix Protein Osteopontin[△]

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Key words: nectin-like molecule 1; glioma cell line; extracellular matrix protein; osteopontin

Objective To investigate the molecular mechanism of nectin-like molecule 1 (NECL1) inhibiting the migration and invasion of U251 glioma cells.

Methods We infected U251 glioma cells with adeno-nectin-like molecule 1 (Ad-NECL1) or empty adenovirus (Ad). Transwell and wound healing assays were performed to observe the migration of U251 cells incubated with the cell supernatant from Ad-NECL1 or Ad infected U251 cells. DNA microarray was applied to screen the gene expression profile after the restoration of NECL1 in U251 glioma cell lines. The differential expression of osteopontin (OPN), a gene related to migration and invasion, was further analyzed with semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and immunohistochemistry.

Results The restoration of NECL1 inhibited migration of U251 cells significantly ($P < 0.05$). Altogether 195 genes were found differentially expressed by microarray, in which 175 were up-regulated and 20 down-regulated, including 9 extracellular matrix proteins involved in the migration of cells. Both mRNA and protein expressions of OPN, the most markedly reduced extracellular matrix protein, were found decreased in U251 cells after restoration of NECL1. Immunohistochemical assay also detected an increase of OPN in glioma tissues, related with the progressing of malignant grade.

Conclusion A link might exist between NECL1 and the extracellular matrix protein OPN in inhibiting the migration and invasion of U251 glioma cells.

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NECTIN-like molecule 1 (NECL1) is a member of the immunoglobulin superfamily, specifically expressed in nervous system.^{1,2} Members of this superfamily have the same conservative structure: a N-terminus consisting of three extracellular Ig-like domains, a single transmembrane domain, and a short C-terminus intracellular region. Extracellular domains of NECL1 could form homo- or heterodimer by itself or with other members of the family.^{1,3} The short C-terminus contains two relatively conservative motifs – FERM and PDZ. FERM motif is the binding motif of protein 4.1, while the PDZ binding motif can interact with other proteins containing PDZ domain.^{2,4} Expression of NECL1 focuses in neuronal cell bodies in a variety of brain regions, including the cerebellum, cerebral cortex, and hippocampus.² It is reported that NECL1 is lost in 12 different glioma cell lines, and that the loss of NECL1 in human glioma cell lines and tumor tissues could be epigenetically caused by histone deacetylation.^{5,6} NECL1 could inhibit the proliferation of T98G cells by inducing cell apoptosis,⁷ and inhibit migration and invasion of U251 glioma cells.⁸ Those effects of NECL1 on tumor cells could explain its limiting the tumor growth in xenograft model.⁶ However, the molecular mechanism of this inhibiting effect on tumorigenesis remains unclear.

In this research, we studied the migration of U251 glioma cells, analyzed their differential gene expression profile after NECL1 restoration, and probed into the relation between NECL1 and extracellular matrix proteins in the migration and invasion of U251 cells.

MATERIALS AND METHODS

Cell line and culture conditions

Human glioma cell line U251 was purchased from Cell Center of Peking Union Medical College. The cells were cultured in Minimum Essential Medium Eagle's with Earle's Balanced Salts (MEM/EBSS) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂.

Recombinant adenovirus package and infection

Empty adenovirus (Ad) and adeno-NECL1 (Ad-NECL1) (obtained from National Human Genome Center, Beijing, China) at a concentration of 10⁸ pfu/mL were used to infect U251 cells incubated in serum-free MEM/EBSS. Cells were cultured after adenovirus infection and shaken gently every 15 minutes. The cells were then maintained in complete medium after 2-hour incubation. Fluorescence was observable 24 hours after that.

Wound healing assay

The cell supernatant was collected after infection with Ad-NECL1 or Ad,⁸ in which U251 cells were cultured. At the same time, a scratch was drawn in the center of the well. The distance between the cells bordering the scratch was measured every 12 hours within 36 hours.

Transwell assay

Transwell, with or without 300 µg/mL matrigel pre-coating at 37°C for 3 hours, was incubated in serum-free MEM/EBSS for 30 minutes. U251 cells were harvested by centrifugation (2 000 ×g) for 5 minutes, then resuspended in serum-free medium at a density of 10⁶ cells/mL. Altogether 100 µL of the cell suspension was put onto the upper layer of transwell, while the same kind of cell supernatant as used in wound healing assay was collected and put onto the lower layer of transwell. After 24 hours, the cells were fixed by formalin and stained by 4',6-diamidino-2-phenylindole (DAPI).

Microarray screening

Total cellular RNA was extracted from U251 cells infected with Ad-NECL1 or Ad, using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Microarray analysis was performed by Phalanx Biotech Group, Taiwan, China. A fluorescence value higher than 100 and a coefficient of variation (CV) lower than 0.2 (CV=SD/mean) were considered positive hybridization signal. While an R value over 2 or under 0.5 ($|\log_2 R| \geq 1$) was considered to indicate differential expression.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was the same as that used in the microarray analysis. Oligo (dT) primers (Beijing TransGen Biotech, China) were used for reverse transcription. Detailed methods have been described in the manufacturer's instructions. RT-PCR exponential phase was determined on 25-35 cycles to allow semi-quantitative comparisons among cDNAs developed from identical reactions, performed in triplicate independently.

Protein extraction and Western blot

U251 cells infected with Ad-NECL1 or Ad were extracted with cold lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid pH 8.0, 1% NP-40, 10% glycerol, 25 mmol/L NaF, 1 mol/L NaVO₃, 100 µg/mL dithiothreitol, 100 µg/mL phenylmethanesulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin). After being lysed on ice for 30 minutes, the lysate and supernatant were centrifuged at 12 000 ×g for 20 minutes, and the supernatant was collected for Western blot.

Protein concentration was determined using the Bradford method. We separated the lysate with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred the gel onto nitrocellulose membrane. The protein was probed with rabbit anti-Human osteopontin (OPN) (1:100, Beijing Aviva Systems Biology, China) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000, Zhong Shan Gold Bridge Biotechnology, Beijing, China), followed by visualization with ECL.

Immunohistochemical analysis of primary glioma tissues

Human normal brain and glioma tissues were obtained from the Department of Neurosurgery of Beijing Tiantan Hospital, classified into normal, Grade II, Grade III, and Grade IV according to the third edition of the histological typing of tumors of the central nervous system (WHO, 2000). Written informed consents were obtained from all patients involved before the surgery. We performed immunohistochemical assay as previously described⁶ to examine the expression pattern of OPN in those tissues. Briefly, the 15 μ m formalin-fixed, frozen tissue sections were incubated with anti-Human OPN antibody (1 : 100, Aviva) at 4°C for 16 hours, followed by incubation in HRP-labeled secondary antibodies for 2 hours at room temperature. 3,3'-diaminobenzidine (DAB) was used as the chromogen.

Statistical analysis

Numerical data obtained in each experiment were analyzed with Student's *t* test. A *P* value lower than 0.05 was considered statistically significant.

RESULTS

Wound healing and transwell assays

Since many secreted proteins such as the extracellular matrix proteins have been reported to be involved in migration and invasion of tumor, cultured supernatant from the U251/Ad-NECL1 and U251/Ad cells was used for wound healing and transwell assays to further verify that secreted proteins are involved in NECL1's inhibiting the migration and invasion of tumor cells. The healing speed of the scratch in U251 cells cultured with supernatant from U251/Ad was higher than that in U251 cells cultured with supernatant from U251/Ad-NECL1 ($P < 0.05$) (Fig. 1A). Transwell assay also revealed that U251 cells cultured in supernatant from U251/Ad migrated faster than U251 cells cultured in supernatant from U251/Ad-NECL1 ($P < 0.0001$) (Fig. 1B). Those findings implied that some secreted proteins in the culture supernatant did participate in NECL1's inhibiting effect on the migration and invasion of U251 cells.

DNA microarray results

To further explore how NECL1 exerts its function, microarray was used to screen the genes whose expression changed after the restoration of NECL1 in U251 glioma cells. A total of 195 differentially expressed genes were identified, in which 175 were down-regulated, and 20 up-regulated. These genes were cluster analyzed according to their different functions such as involved in signaling pathway and translation, or encoding extracellular matrix proteins (Fig. 2). Among them, there are 9 extracellular matrix proteins down-regulated.

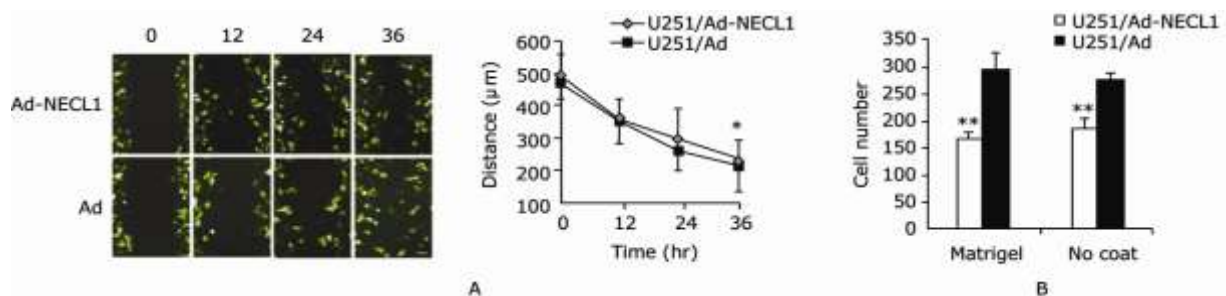


Figure 1. U251 cell migration in wound healing and transwell assays after incubated with the cell supernatant from U251 infected with adeno-neclin-like molecule 1 (Ad-NECL1) or empty adenovirus (Ad).

A. Wound healing assay.

Left: images of the same location taken at 0, 12, 24, and 36 hours after the scratch was made. Right: the distance between the scratch borders was measured at the fixed position on the photos taken at four different locations at each time point. Values on the curves are average distance.

B. Transwell assay.

Matrigel: chamber coated with matrigel (300 μ g/mL); No coat: chamber not coated with matrigel.

Cell number is counted in six parallel visions of each sample.

* $P < 0.05$, ** $P < 0.0001$ compared with the results of U251 cultured in the cell supernatant from U251 infected with Ad-NECL1.

Semi-quantitative RT-PCR and Western blot results

Based on the microarray data, secreted phosphoprotein 1 OPN was selected from the 9 down-regulated extracellular matrix proteins for further verification because of its marked reduction (the ratio of fluorescence value Ad-NECL1/Ad=0.191). Semi-quantitative RT-PCR results confirmed that the transcript of Opn gene was decreased in the U251 glioma cells infected with Ad-NECL1 (Fig. 3A). The expression of OPN protein was also decreased in supernatant and cell lysate from Ad-NECL1-infected U251 cells (Fig. 3B).

Immunohistochemical assay results

OPN expression was found increased along with the increasing malignant grade, demonstrated by the intensifying positive signal in the primary glioma tissues compared with the normal brain tissues (Fig. 4).

DISCUSSION

In conclusion, we confirmed that NECL1 could affect the migration and invasion characterization of glioma cell

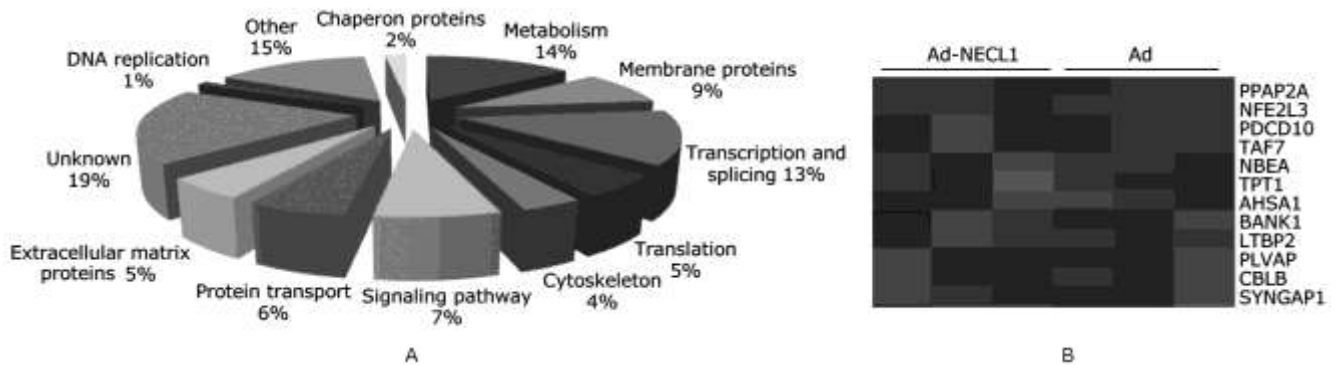


Figure 2. Gene expression profile after the restoration of NECL1 in U251 glioma cell lines detected by microarray.

- A. Microarray screening results. A fluorescence value higher than 100 and a coefficient of variation (CV) lower than 0.2 ($CV = SD/mean$) are positive hybridization signal. An R value over 2 or under 0.5 ($|\log_2 R| \geq 1$) indicates differential expression.
- B. Cluster analysis identifies 195 differentially expressed genes, in which 175 are down-regulated and 20 up-regulated, including 9 down-regulated extracellular matrix proteins.

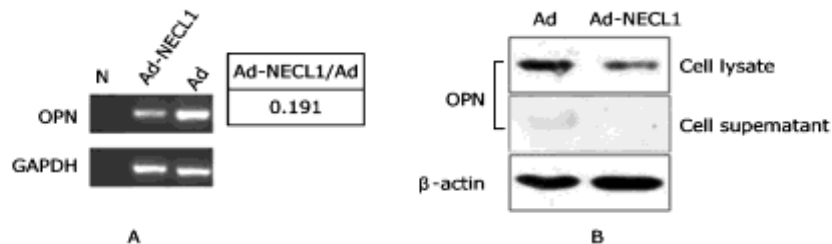


Figure 3. Osteopontin (OPN) expression detected by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot.

- A. Semi-quantitative RT-PCR reveals decreased transcript of Opn gene in the U251 glioma cells infected with Ad-NECL1; N: Negative control; Ad-NECL1/Ad: the ratio of fluorescence values.
- B. Western blot detects OPN reduction in supernatant and cell lysate from the U251 cells infected with Ad-NECL1. β -actin is used as control.

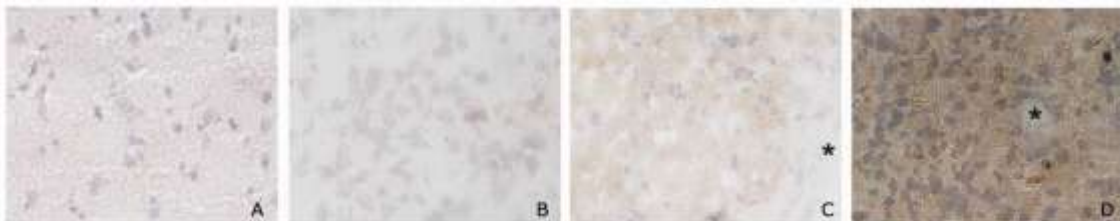


Figure 4. OPN expression in human normal brain and glioma tissues. $\times 200$

The tissues are classified into normal, Grade II, Grade III, and Grade IV based on the histological typing of tumors of the central nervous system (WHO, 2000).

A. normal; B. Grade II: astrocytoma; C. Grade III: anaplastic astrocytoma; D. Grade IV: neuroblastoma.

Asterisks represent blood vessels.

line. A link could be established between NECL1 and OPN based on the following evidence. There were obviously some secreted proteins in the supernatants participating in the process of NECL1 inhibiting the migration and invasion of U251 cells. In the microarray analysis and following assays, we observed that OPN expression was up-regulated in glioma cell line and glioma tissues, and restoration of NECL1 was related to the decreased expression of this secreted protein.

Expressed in a variety of tissues,⁹ osteopontin is a secreted phosphoprotein that has been reported playing an important role in tumor metastasis,¹⁰⁻¹² thus can be used as a biomarker for advanced disease and potential therapeutic target in the regulation of cancer metastasis.¹³ Exploration into the molecular mechanism of OPN in tumorigenesis implied that its N-terminal can interact with integrin, and C-terminal can interact with CD44 to promote the migration of tumor cells.¹⁴ OPN has also been found expressed at a high level in glioma.¹⁵ The transcriptional regulation of OPN in the U-251MG and U-87MG human malignant astrocytoma cell lines reveals that a proximal promoter element (-24 to -94 relative to the transcription initiation site) is essential for maintaining a high level of OPN expression in the tumor cells, and that transcription factors Sp1, the glucocorticoid receptor, and the E-box-binding factors, Myc and OCT-1, participate in forming DNA-protein complexes.¹⁴

It could be interesting to find out how NECL1-related signaling pathway is involved in OPN expression in glioma, also to use OPN as a biomarker and a potential therapeutic target in glioma.

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