Expression pattern of osteopontin splice variants and its functions on cell apoptosis and invasion in glioma cells

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Osteopontin (OPN) is widely overexpressed in various cancers, including gliomas, and plays an important role in tumorigenesis. However, the expression pattern and functions of OPN splice variants expressed in gliomas remain unclear. The aims of our current study were to examine the expression pattern and functions of OPN splice variants in gliomas. In present study, the mRNA levels of OPN splice variants are markedly increased in gliomas tissues, and all OPN splice variants were also found in U251 and U87 cells. Furthermore, knock-down and regain of function experiments were designed to explore the functions of OPN splice variants in U251 and U87 cells. Lentiviral vectors of OPN small interference RNA (siRNA) targeting all three endogenous mRNAs of OPN and OPN splice variants synonymous mutant that were not silenced by OPN siRNA were constructed. Our results showed that all OPN splice variants synonymous mutant-protected glioma cells from apoptosis induced by OPN siRNA through alteration of the levels of Bcl-2 family proteins and OPN-b Mu elicited a significant effect. Both OPN-a Mu and -c Mu promoted glioma cell invasion through alteration of the levels of uPA, MMP-2, and MMP-9 expressions and the activities of MMP-2 and MMP-9 via activation PI-3K/AKT/NF-κB signaling pathway. Moreover, OPN-c Mu showed the strongest effect on glioma cell invasion, while OPN-b Mu showed no effect on the invasion of U251 and U87 cells. Thus, different splice variants of OPN have divergent functions in regulating apoptosis and invasion of glioma cells, which broadens their importance in glioma biotherapy.

Keywords: apoptosis, glioma, invasion, Osteopontin, siRNA, splice variant, synonymous mutant.
to exist between normal and transformed rat bone cells. A recent study showed that all three messages, including OPN-a, OPN-b, and OPN-c, were present in breast cancer tissues and had different roles in human breast cancer progression, and increased OPN-c levels were also detected in metastatic hepatocellular carcinoma samples, a highly invasive cell line (MHCC-97). Three types of splice variants of OPN in glioma cells have been isolated and sequenced by Saitoh et al. However, to date, there have been no reports which have analyzed the expression pattern and functions of OPN splice variants in glioma cells.

In this study, we examined, for the first time, the expression profile of three OPN splice variants in glioma tissues and glioma cell lines by real-time reverse transcription polymerase chain reaction (real-time PCR) and reverse transcription–polymerase chain reaction (reverse-transcription–PCR), and our data showed that multiple splice variants of OPN are significantly expressed in glioma tissues and U251 and U87 cells. To further investigate the biological function of OPN splice variants in the progression of gliomas, knockdown and gain of function experiments were employed, and differences in the biological roles of OPN splice variants were found.

### Materials and Methods

#### Materials

Our study was approved by the Medical Review Board of Nanjing Medical University. Human glioma tissue samples were obtained from Department of Neurosurgery of the first affiliated hospital of Nanjing Medical University after informed consent from adult patients diagnosed with glioma; 15 WHO-II, 15 WHO-III, and 15 WHO-IV glioma tissues were resected during surgery and immediately frozen in liquid nitrogen for subsequent total RNA extraction. Twelve normal brain tissues were obtained during surgery of severe traumatic brain injury after informed consent from the patients who needed post-trauma surgery. Human glioma cell lines, such as U251, U87, SHG44, and TJ905, were purchased from the Chinese Academy of Sciences Cell Bank. All glioma cell lines were cultured in 37°C with 10% fetal bovine serum (FBS), and routinely passaged at 2–3-day intervals. In the present study, integrin αvβ3 antibody was purchased from Chemicon International and applied at a concentration of 20 μg/mL. LY294002 was from Sigma and used at a concentration of 10 μM. Experiments were divided into 6 groups as blank control group, negative control (pGC-FU-GFP) group, OPN small interference RNA (siRNA) group, OPN siRNA + OPN splice variant-a synonymous mutant group (OPN-a Mu group), OPN siRNA + OPN splice variant-b synonymous mutant group (OPN-b Mu group), and OPN siRNA + OPN splice variant-c synonymous mutant group (OPN-c Mu group).

#### Lentivirus Vector Construction and Infection

Expression vectors mediated by lentivirus for human OPN siRNA, OPN splice variants synonymous mutants, and negative control vectors were constructed via technical support from Shanghai GeneChem. The siRNA sequences targeting OPN were designed to silence three different endogenous OPN splice variants using an Internet application system (Invitrogen). Double-stranded oligonucleotide-encoding shRNA sequence, its mismatch mutant, and the siRNA of 3 OPN splice variants synonymous mutants tagged with green fluorescent protein (GFP) were annealed and inserted into pGC-LV expression vector (Invitrogen). Then, the siRNA and OPN splice variants Mu expression vectors (pGC-LV) and packaging vectors (pHelper 1.0 and pHelper 2.0; Invitrogen) were cotransfected into 293FT cells with Lipofectamine 2000 (Invitrogen). The culture supernatants were collected, concentrated, and stored in a refrigerator maintained at −70°C. Enhanced GFP (eGFP) was expressed in all lentiviral vectors for titering and measuring their infection efficiency in infected cells. The lentiviral vectors were infected into U251 and U87 cells with a multiplicity of infection (MOI) from 5 to 20 in the presence of polybrene (10 μg/mL).

#### RNA Isolation, Reverse-Transcription PCR, and Real-Time PCR

RNA was extracted from tissues and glioma cell lines (U251, U87, SHG44, and TJ905) using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Expression of OPN splice variants in glioma tissues were analyzed by real-time PCR on the ABI 7300 HT Sequence Detection system (Applied Biosystem) using the SYBR® PrimeScript™ RT-PCR kit (TaKaRa), the OPN-a, -b, and -c were specifically amplified with the primer pairs: 5’-ATC TGC TAG CCC CAC AGA AT-3’ (forward) and 5’-CAT CAG ACT GTG GAT AAT CAT C-3’ (reverse) for OPN-a, 5’-ATC TGC TAG CCC CAG AGA C-3’ (forward) and 5’-AAA ATC AGT GAC CAG TTC ATC AG-3’ (reverse) for OPN-b, 5’-TGA GAA AAA GCA GAA TGC TG-3’ (forward) and 5’-GTC AAT GGA GTC CTG GCT GT-3’ (reverse) for OPN-c. Three isoforms of OPN mRNA in glioma cell lines were also confirmed by reverse-transcription PCR using the primer 5’-CTG TGC CAT ACC AGT TAA-3’ (forward) and 5’-GAT GTC AGG TCT GCC AAA-3’ (reverse) designed by Primer Premier 5.0 which can amplify the region comprising exon 4 and exon 5 of OPN cDNA. The amplification of β-actin with primers 5’-AAG ACC TGT ACG CCA ACA AGG T-3’ (forward) and 5’-AGA AGC ATT TGC GGT GGA CGA T-3’ (reverse) was taken as an internal control for both reverse-transcription PCR and real-time PCR.

#### ELISA Assays

To analyze the secretion of total OPN protein from each group, OPN in culture supernatant was measured with
the commercial ELISA kit according to the manufacturer’s instructions (Quantikine assay, R&D Systems). Briefly, cells were plated at 5 x 10^4 cells per well in 6-well culture plates in 2 mL medium with 10% FCS following infection for 24 hours. After 72 hours, culture supernatants were collected and subjected to ELISA analysis. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad, Richmond).

**Apoptosis Detection Assay**

U251 and U87 cells infected with lentiviral vector were trypsinized, washed with PBS (4°C), and suspended in 500 μL binding buffer (4°C) containing 5 μL of Annexin V-PE and 5 μL of 7-AAD (eBioscience), then incubated for 15 minutes in the dark at room temperature according to the manufacturer’s instructions. Then, the cells were analyzed by FACScan (Becton Dickinson). Annexin V-PE^- and 7-AAD^- cells were used as controls. Annexin V-PE^+ and 7-AAD^- cells were designated as early apoptotic, and Annexin V-PE^+ and 7-AAD^+ cells displayed late apoptotic and necrotic cells. Tests were repeated in triplicate.

**In Vitro Invasion Assay**

Invasion assays were done using Transwell membranes coated with Matrigel (BD Biosciences). The infected cells (5 x 10^4) were plated in the upper chamber and incubated at 37°C in 5% CO2 humidified air for 72 hours. The lower compartment was filled with 20% FBS as a chemoattractant. After incubating 24 hours, cells remaining in the upper chamber were carefully removed with cotton swabs, while invaded cells were fixed with 3% paraformaldehyde, stained with crystal violet, counted and photographed with a microscope in 5 independent 10 x fields for each well. Three separate experiments were performed.

**Subcellular Fractionation and Western Blot Analysis**

To investigate the increase of cytochrome C (Cyto.c) in the cytosol, cytosol and mitochondria fractionations were performed by centrifugation technique as described in Zhao et al. To evaluate the translocation of NF-κB p65 to nucleus, nuclear-protein extractions were made using Nuclear and Cytoplasmic Extraction Reagents (KeyGEN Biotechnology) according to the manufacturer’s protocol.

For the analysis of the levels of protein expression, the cells were lysed in RIPA buffer (15 mM EGTA, 15 mM MgCl2, 137 mM NaCl, 0.1% Triton X-100, 25 mM MOPS, 0.1 mM sodium orthovanadate, 100 μM phenylmethylsulfonylfluoride, and 20 μM leupeptin, adjusted to pH 7.2). Proteins at equal amounts were separated by 12% SDS–PAGE and transferred onto polyvinylidene difluoride membranes, then probed with individual antibodies. Antigen–antibody reaction was detected and visualized with enhanced chemiluminescence (Amersham Life Science). GAPDH or Tubulin was reprobed as an internal control on the same membrane. The antibodies used were anti-OPN, anti-Bcl-2, anti-Bcl-xl, anti-Bax, anti-Cyto.c, anti-Proapase-3, anti-iPA, anti-MMP-2, anti-MMP-9, anti-Akt, anti-p-Akt, anti-NF-κB p65 (Santa Cruz Biotechnology). In addition, the OPN antibody used in our study was a rabbit polyclonal antibody raised against amino acids 1-314 representing full-length OPN of human origin and could recognize three isoforms of OPN splice variants Mu in our study.

**Gelatin Zymography**

Gelatin zymography was performed with a Gelatin Zymography kit (YU-68001; Yagai). Briefly, 96 hours after infection, equal amounts of supernatant from the treatment groups were electrophoresed on 5–10% SDS–PAGE containing gelatin (1 mg/mL). Then the gel was incubated in the washing buffer (50 mM Tris–HCl, pH 7.5, 2.5% Triton X-100) for 1 hour, and incubated in a reaction buffer (50 mM Tris–HCl, pH 7.5, containing 5 mM CaCl2, 150 mM NaCl, 0.02% NaN3) for 36 hours at 37°C according to the manufacturer’s instructions. Then enzyme activity was visualized by staining with 0.05% Coomassie Blue.

**Statistical Analysis**

All statistical analysis was performed using SPSS Graduate Pack 11.0 statistical software (SPSS). Statistical significance (P < .05) was determined by descriptive statistics, including mean and SE along with one-way ANOVAs.

**Results**

**Expression of OPN Splice Variants in Human Glioma Tissues and Cell Lines**

To investigate the expression of OPN splice variants mRNA in glioma tissues, real-time PCR was carried out using specific primers for human OPN splice variants; β-actin was taken as an internal control. As shown in Fig. 1A, the average expression of OPN splice variants mRNA including OPN-a, -b, and -c was significantly higher in high-grade gliomas (Grade III–IV) than in low-grade gliomas (Grade I–II). To confirm the expression pattern of OPN splice variants in human glioma cell lines, reverse-transcription PCR was carried out. The three bands of OPN splice variants were detectable in U251 and U87 cells, but scanty bands were seen in SHG44 and TJ905 cells (Fig. 1B). On the basis of these results, we used U251 and U87 cells as models for investigation of OPN splice variants biological functions in our knock-down and regain of function experiments.

**Glioma Cells Stably Infected with OPN SiRNA and Splice Variants Synonymous Mutant Mediated by Lentiviral Vector**

As shown in Fig. 2, the synonymous mutational bases of 3 splice variants Mu are located in the sequence targeted by
Fig. 1. OPN splice variants mRNA expression levels in human normal adult brains and glioma tissues. (A) Levels of OPN splice variants expression in glioma tissues and normal brain tissues. The average expression OPN splice variants mRNA including OPN-a, -b, and -c was significantly higher in high-grade tumors (Grade III–IV) than low-grade tumors (Grade I–II) and normal brain tissues. $P < 0.01$. (B) Expression of OPN splice variants in glioma cell lines, TJ905, SHG44, U87, and U251. Three mRNAs of OPN splice variants were detectable in U251 and U87 cells, but scanty bands were seen in SHG44 and TJ905.

Fig. 2. Constructions for synonymous mutants of OPN splice variants. The synonymous mutational bases of three splice variants Mu are located in the sequence targeted by OPN siRNA. Therefore, OPN siRNA, which silenced endogenous splice variant of OPN, cannot degrade all three synonymous mutants of OPN splice variant in the respective group.
OPN siRNA. Therefore, OPN siRNA, which silenced endogenous splice variants of OPN, cannot degrade all 3 OPN splice variants synonymous mutant. Coinfection of U251 and U87 cells with OPN splice variants synonymous mutant and OPN siRNA mediated by lentivirus can express stable OPN splice variants synonymous mutant in the respective group. After infected with an MOI of 5–20 for 96 hours both in U251 and U87 cells, cells were collected and examined for OPN splice variants mRNA expression in each group. As shown in Fig. 3, the three messages of OPN splice variants mRNA in OPN siRNA group were nearly downregulated by 90% compared with the cells infected with the negative control vector or the uninfected control cells, and OPN splice variants synonymous mutant were stably expressed in the respective group analyzed by real-time PCR and Western blot analysis. Furthermore, the secretion of OPN could be efficiently blocked by OPN siRNA and high concentration of OPN could be detected in culture supernatants of OPN-a and -c Mu group, while not in OPN-b Mu group by ELISA assay targeting all splice variants of OPN.
Effects of OPN Splice Variants on Apoptosis of Glioma Cells

To study the biological function of OPN splice variants on the apoptosis of glioma cells, U251 and U87 cells from each group were infected with lentiviral vector for 96 hours, and then the apoptosis of U251 and U87 cells was measured by flow cytometry. As shown in Fig. 4, statistically significantly more apoptotic cells were found in OPN siRNA-treated cells of U251 and U87 compared with negative controls. Furthermore, the pro-apoptotic effects induced by OPN siRNA could be reversed by infection with all 3 OPN splice variants synonymous mutant, and OPN-b Mu elicited the strongest effect.

Effects of OPN Splice Variants on Cell Apoptosis-Related Proteins in Human Glioma Cell Lines

The alterations of Bcl-2 family proteins, which regulate the release of Cyto.c through the outer mitochondrial membrane, the assembly of the apoptosome and caspase activation sequentially, play a very important role in cellular apoptosis. To further explore the molecular mechanism of OPN splice variants on apoptosis of glioma cells, Western blot analysis was performed. Our data showed that OPN siRNA resulted in release of Cyto.c to cytosol from mitochondria, increase of Bax expression, an obvious decrease of Bcl-xL, Bcl-2, and cleavage of procaspase-3 (Fig. 5), OPN splice variants synonymous mutant could reverse the change of apoptosis-related proteins. These experiments demonstrated that OPN siRNA resulted in apoptosis of U251 and U87 cells, and all 3 OPN splice variants synonymous mutant can reverse the apoptosis induced by OPN siRNA.

Influence of OPN Splice Variants on the Invasion of Glioma Cells In Vitro

To measure effects of OPN splice variants on the invasive ability of U251 and U87 cells, the in vitro Matrigel invasion assay was employed. We employed OPN siRNA to silence all 3 endogenous OPN mRNA of U251 and U87 cells, and infected OPN splice variants synonymous mutant, respectively. In this study, the total viable cell numbers were obtained via subtracting the numbers of apoptotic cells induced by OPN siRNA from that of total cells when analyzing the reduced cell invasion. And the percentages of invading cells adjusted to the total viable cells were evaluated statistically. Cells infected with lentivirus-mediated OPN siRNA showed an average slightly more than 50% reduction of invasive ability of glioma cells compared with cells infected with negative control vector. OPN-a and -c Mu could reverse this reduction and promote the invasion significantly in

![Fig. 4. Effects of OPN splice variants on cell apoptosis in human glioma cell lines. (A) At 96 hours post-infection, U251 and U87 cells were harvested, double stained for Annexin V-PE and 7-AAD, and analyzed by flow cytometry. Early apoptotic cells are Annexin V-PE⁺/7-AAD⁻, late apoptotic and necrotic cells are Annexin V-PE⁺/7-AAD⁺ and healthy cells are Annexin V-PE⁻/7-AAD⁻. (B) Columns, mean percentage of apoptosis from triplicate-independent experiments, each in triplicate; bars, SE. Data were adjusted to blank control. Statistically significant differences were observed. *P < .01 compared with blank control; **P < .01 compared with OPN siRNA group.](https://academic.oup.com/neuro-oncology/article-abstract/12/8/765/1074124)
both U251 and U87 cells and OPN-c Mu showed a stronger effect, while OPN-b Mu did not (Fig. 6).

**OPN-a and -c Mu Increase the Expression of UPA, MMP-2, and MMP-9 and the Activities MMP-2 and MMP-9 via PI-3K/AKT/NF-κB Pathway by Ligation of αvβ3 Integrin**

The PI-3K/AKT/NF-κB signaling pathway is of great importance in regulating cellular invasion and expression of some invasion-related proteins, such as uPA, MMP-2, and MMP-9. To elucidate the molecular mechanisms of OPN splice variants on the invasion of U251 and U87 cells, the expression levels of uPA, MMP-2, and MMP-9 in each group were analyzed by Western blotting, and MMP-2 and MMP-9 activities were evaluated by gelatin zymography. Our data showed that uPA, MMP-2, and MMP-9 expression and activities of MMP-2 and MMP-9 were suppressed by OPN siRNA and increased by OPN-a and -c Mu (Fig. 7), while OPN-c Mu showed a stronger effect. In addition, we found that activation of AKT and translocation of NF-κB p65 to nucleus could be impaired by OPN siRNA and promoted by OPN-a and -c Mu. Moreover, uPA, MMP-2, and MMP-9 expression, activities of MMP-2 and MMP-9, AKT activation and translocation of NF-κB p65 to nucleus induced by OPN-a and -c Mu can be inhibited by anti-αvβ3 antibody and PI-3K inhibitor LY294002.

LY294002 and anti-αvβ3 antibody effectively blocks the increased cellular invasion induced by infection with OPN-a and -c Mu lentivirus (Fig. 8). These results indicated that OPN-a and -c Mu may promote invasion of U251 and U87 cells significantly by PI-3K/AKT/NF-κB pathway via ligation of αvβ3 integrin.

**Discussion**

High levels of OPN expression correlate with cell adhesion, migration, invasion, and cell proliferation of various cancers and have been recognized as a key prognostic marker. OPN has been identified as the most upregulated gene in ENU (N-ethyl-N-nitrosourea)-induced glioma in rat brains\(^1\) and the expression level of OPN may correlate with human astrocytomas grade.\(^12\) A recent study also shows that OPN knockdown exerts anti-tumor activity in a human glioblastoma model.\(^2\) In contrast, OPN secreted by activated helper T lymphocytes is essential for the induction of cellular immune responses and acts as a macrophage chemoattractant and thus contributes to the antitumor host defense.\(^13,14\) Thus, the functions of OPN secreted by different cells may be contradictory. Osteopontin is a complex molecule with diverse alternative splice forms that may be one mechanism of the different functions of OPN derived from various cellular sources. Recent reports that focused on the bio-functional characters of OPN splice variants have found that the
soluble form (OPN-c) supports invasiveness and the aggregated form (OPN-a) promotes adhesion in breast cancers. Furthermore, OPN-c has been shown as a diagnostic and prognostic marker of breast cancer. In addition, overexpression of OPN-c with physiological levels of MMP-9 enhanced cellular invasion and released a fragment of OPN that was essential to cellular invasion and appeared to correlate with metastatic potential for hepatocellular carcinoma. In this study, we examined the expression pattern in human glioma tissues and cell lines, and designed knockdown and regain of function experiments to investigate the functions of the 3 splice variants of OPN. To the best of our knowledge, this is the first study to demonstrate the expression pattern and functions of OPN splice variants in glioma cells. We found that all OPN splice variants were significantly higher in high-grade tumors (Grade III–IV) than in low-grade tumors (Grade I–II) and normal brain tissues. The three mRNAs of OPN splice variants were also detected in U251 and U87 cells relative to SHG44 and TJ905 cells. Thus, U251 and U87 cells were used to explore the biological functions of OPN splice variants. And our data demonstrated that OPN-b Mu effectively induced the inhibition of apoptosis in glioma cells. However, OPN-b Mu had no effect on the invasion of glioma cells, whereas OPN-c Mu elicited the strongest effect on the invasion of glioma cells distinctively. These data show that OPN splice variants have divergent functions in the progression of gliomas.

Apoptosis plays a critical role in tissue homeostasis. Tumor cells are often deficient in apoptotic regulation, which leads to the progression of transformed cells. Osteopontin has been shown to exert strong effects on preventing programmed cell death in response to diverse stimuli in various cells, protect monocyte from apoptosis, and induce endothelial cell survival. It has been reported that OPN-elicited anti-apoptosis is mainly attributed to the engagement of CD44V isoforms, and OPN decreases Cyto.c oxidase activity in RAW 264.7 murine macrophages. In addition, a previous study has indicated that no significant increase of the OPN-b expression level was observed in the breast tumor tissues when compared with normal tissues, and the function of OPN-b is still unclear. However, in the present study, silencing of OPN induced apoptosis significantly, and the death of glioma cells induced by OPN siRNA was reversed by infection of OPN splice variants.
variants synonymous mutant. Moreover, we found that OPN-b was significantly expressed in glioma tissues relative to normal brain samples and elicited the strongest inhibition of the apoptosis of U251 and U87 cells induced by OPN siRNA. To our knowledge, our data demonstrates that OPN-b plays an important role in cell apoptosis for the first time.

The mitochondrial stage of apoptosis, which is upstream of caspase activation, is mediated by alteration of the Bcl-2 family of proteins. Bcl-2 family proteins regulate the release of Cyto.c through the outer mitochondrial membrane. Then, the release of Cyto.c activates the assembly of the apoptosome that leads to caspase activation. Our results showed that OPN siRNA induced obvious upregulation of Bax expression, downregulation of Bcl-2, Bcl-xl, increased Cyto.c release from mitochondria to cytosol significantly, and decrease of pro-caspase-3. Meanwhile, the processes could be blocked by all three OPN splice variants Mu. It suggests that OPN splice variants regulate apoptosis via abnormal Bcl-2 family protein expression.

A multitude of studies has shown that increased OPN expression is associated with invasion or metastasis in cancers. Higher levels of OPN expression correlate with increased tumor grade and elevated migratory capacity of tumor cells. Cells expressing high levels of OPN showed higher levels of cell invasiveness than control vector transfectants. In this study, downregulation of all messages of OPN suppressed the invasion of glioma cells significantly, and infection of OPN-a and -c Mu could promote U251 and U87 cells invasion when compared with the OPN siRNA group. It should be pointed out that OPN-b Mu did not display any effect on the cellular invasion, while OPN-c Mu exerted a stronger role on glioma cells invasion when compared with OPN-a Mu. These findings indicate that the functions of OPN splice variants on the invasion of glioma cells are in great diversity.

Previous studies have shown that OPN may promote cellular invasion through interaction with avb3 integrins. OPN-avb3 interaction induced the phosphorylation 

Fig. 7. OPN-a and -c increased the expression of uPA, MMP-2, and MMP-9 and the activities MMP-2 and MMP-9 via PI-3K/AKT/NF-κB pathway by ligation of avb3 integrin. Cytoplasmic and nuclear-protein extractions of U251 and U87 cells infected with lentivirus vector for 96 hours in each group were made using Nuclear and Cytoplasmic Extraction Reagents. OPN siRNA suppressed uPA, MMP-2, and MMP-9 expression, the activities of MMP-2 and MMP-9 (A), Akt phosphorylation (B), and the translocation of NF-κB p65 to nucleus (C) (P < .05 compared with blank control). While OPN-a and -c Mu reversed and enhanced the processes and OPN-c Mu showed a significant effect (P < .05 compared with OPN siRNA group), which could be inhibited by a supplement with a PI-3K inhibitor LY294002 and anti-avb3 antibody (P < .01 compared with OPN-a and -c Mu groups, respectively). Quantification of the bands was calculated by densitometric analysis. Data were adjusted to blank control and fold changes were indicated.

Yan et al.: Expression and function of OPN splice variants in glioma cells

NEURO-ONCOLOGY • AUGUST 2010 773
Akt in the survival pathway of PI-3K/AKT. Then, phosphorylated Akt may activate IκBα kinase (IKK) activity that induces phosphorylation and degradation of IκBα and release of NF-κB p65 to nucleus sequentially. The translocation of NF-κB p65 to nucleus may increase transcriptional activities of uPA.24,25 To explore the possible role of avβ3/PI-3K/AKT/NF-κB signaling pathway and several invasion-related proteins in OPN-a and -c Mu–induced cellular invasion, PI-3 kinase inhibitor LY294002, anti-avβ3 antibody, Western blot, and gelatin zymography were employed. A significantly decreased Akt phosphorylation, translocation of NF-κB p65 to nucleus, uPA, MMP-2, and MMP-9 expression and activities were found. Our results also demonstrated that OPN-a and -c Mu could stimulate Akt phosphorylation, translocation of NF-κB p65 to nucleus, and the expression and activities of uPA, MMP-2, and MMP-9. Moreover, the above processes induced by OPN-a and -c Mu can be blocked by a supplement with LY294002 and anti-avβ3 antibody. Meanwhile, cellular invasion induced by OPN-a and -c Mu can also be suppressed by addition of LY294002 and anti-avβ3 antibody. By inference, OPN-a and -c Mu increased the expression and activities of uPA, MMP-2, and MMP-9 and promoted the invasion of glioma cells by PI-3K/AKT/NF-κB pathway via ligation of avβ3 integrin.

Why does the idea that alternative splice variants of OPN have different roles in regulating apoptosis and invasion attract our attention? It is known that OPN-a is a full length of OPN, whereas OPN-b lacks exon 5 and OPN-c lacks exon 4. To date, the role of exon 4 or 5 remains unclear. Here, we hypothesize that loss of exons 4 and 5 may result in alteration of molecular structure that may affect the affinity of OPN splice variants to its receptors. Furthermore, in our study, the secretion of OPN was suppressed by OPN siRNA. Meanwhile, high concentration of OPN could be detected in culture supernatants of OPN-a and -c Mu groups, but not in the OPN-b Mu group by ELISA assay targeting all splice variants of OPN. Thus, we presume that the alteration of molecular structure resulted by loss of exon 4 or 5 may also play an important role in the secretable ability of OPN splice variants. Therefore, OPN-b may be less secretable and OPN-c may be easily secreted to culture supernatants due to the alteration of molecular structure induced by the absence of exon 5 or 4. Therefore, OPN-b aggregates in cytoplasma and shows a significant antiapoptotic effect. OPN-c in the supernatants is more available for ligation of avβ3 integrin and shows a
significant effect on cellular invasion. However, the function of exons 4 and 5 in the diversity of OPN splice variants will be under investigation in our further studies.

In conclusion, we have shown, for the first time, the expression pattern and functions of OPN splice variants in glioma cells. All 3 OPN splice variants show an inhibition of apoptosis induced by OPN siRNA in U251 and U87 cells in vitro and OPN-b elicited the strongest effect. Furthermore, both OPN-a and -c Mu, but not OPN-b Mu, improve glioma cell invasion significantly. In view of our evidence that splice variants of OPN have different roles in determining the fate of malignant glioma cells, we recommend that the difference among OPN splice variants could be a novel anticancer direction when developing new anticancer therapeutic approaches targeted to OPN splice variants in malignant gliomas.

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References