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ORIGINAL ARTICLE

# Nm23-H1 inhibits proliferation of glioma cells via regulation of PKC signaling pathway

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## Summary

***Purpose:*** *To explore the influence of nm23-H1 on the proliferation and apoptosis of glioma cells and the mechanism therein.*

***Methods:*** *The changes in the messenger ribonucleic acid (mRNA) expression of nm23-H1 were detected via quantitative real-time-polymerase chain reaction (qRT-PCR), and the relative protein expression level of nm23-H1 was determined using immunohistochemistry. The glioma H4 cells were transfected exogenously with nm23-H1 gene (nm23-H1 group) or empty vector (Vector group), and the biological influences of the expression level of nm23-H1 on H4 cells were then assessed through in vitro functional experiments. Besides, the cells transfected with nm23-H1 were incubated with the protein kinase C (PKC) pathway inhibitor Calphostin C, and functional experiments were performed to observe the changes in the proliferation and apoptosis of cells after incubation.*

***Results:*** *According to the immunohistochemistry and qRT-PCR results, the protein and mRNA expression levels of nm23-H1 declined notably in glioma tissues ($p<0.01$). The cells with up-regulated nm23-H1 expression had substantially weakened proliferation and migration abilities, but exhibited dramatically enhanced apoptosis ($p<0.01$). The PKC pathway inhibitor considerably potentiated the effects of nm23-H1 protein on the proliferation and apoptosis of H4 cells ($p<0.05$), and the protein expression level of nm23-H1 rose in the cells treated with the PKC inhibitor ($p<0.01$).*

***Conclusions:*** *Compared with that in normal brain tissues, nm23-H1 is lowly expressed in glioma tissues and affects the expression of PKC to influence the biological behaviors of H4 cells.*

***Key words:*** *nm-23-H1, glioma, protein kinase C*

## Introduction

Glioma is the most common malignant tumor in the central nervous system and one of the malignancies with a high mortality rate [1], and its stage affects the prognosis of patients [2-4]. The prognosis is still very poor in the glioma patients who have received treatments such as resection, radiotherapy and chemotherapy [5,6]. As molecular targeted therapy develops, there has been growing evidence that molecular heterogeneity is a key barrier to improving the clinical outcomes of glioma [7]. Therefore, it is necessary to elucidate the molecular mechanisms of the proliferation and apoptosis of glioma cells to find efficacious therapeutic targets.

Nm23 gene, firstly discovered in 1988, has 8 subtypes, of which nm23-H1 is a relatively impor-

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tant one and has been confirmed to participate in the metastasis of cancer cells in multiple tumors. Current studies have proven that nm23-H1 is negatively correlated with tumor invasion and metastasis. Nm23-H1 may function to repress the invasion of tumor cells. Compared with that in distant tumor metastases, nm23-H1 has a higher expression level in the primary melanoma and breast cancer tissues [8,9]. Although the direct role of this gene in metastases has not yet been determined via assays, reducing its expression level may benefit metastasis and diffusion.

Protein kinase C (PKC) is a phospholipid-dependent serine/threonine kinase family that can be transported from cell membranes to nuclei via the ligand-receptor interaction to regulate the growth and differentiation of cells. The excessive activation and/or expression of PKC is associated with the abnormal proliferation of malignant glioma cells indeed [2, 10-12]. In fact, the proliferation of astrocytes is closely related to the expression of PKC. Fetal astrocytes with active mitosis are characterized by a high expression level of PKC [13,14], whereas PKC is almost not expressed in less proliferative gliocytes [15]. The relationship between the PKC signaling pathway and the mechanism of action of nm23-H1 gene in glioma cells remains elusive, so the present study preliminarily explored this relationship.

## Methods

*Experimental materials*

This study was approved by the Ethics Committee of Jiaozhou People's Hospital of Qingdao. Signed informed consents were obtained from all participants before the study entry. A total of 56 pairs of glioblastoma multiforme (GBM) tissues and normal brain tissues were collected from the patients undergoing surgical resection in our hospital from 2015 to 2016. Upon removal, the tissues were preserved in liquid nitrogen for later use. The PKC pathway inhibitor Calphostin C was purchased from Sigma (St. Louis, MO, USA), Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA), RNAiso Plus from TaKaRa (Dalian, China) and primers from Biosune (Shanghai, China).

*Experimental grouping*

The GBM tissues and normal control tissues were assigned into GBM group and Non-tumor group. The GBM H4 cells were transfected with nm23-H1 (nm23-H1 group) to raise its expression therein or with empty vector (Vector group). Additionally, the cells treated with the PKC pathway inhibitor Calphostin C were assigned into Untreated group and Treated group.

*Experimental methods*

*Cell transfection*

H4 cells were first inoculated into a 24-well plate, and when the confluence reached 70%, the cells were transfected with the transfection reagents targeting nm23-H1 and vector. As specified by the manufacturer, the cells were transfected using Lipofectamine 2000 at a concentration of 10 nmol/L.

*Quantitative real-time-polymerase chain reaction (qRT-PCR)*

Total ribonucleic acids (RNAs) were extracted from glioma cell line or tissues using RNAiso Plus, and reversely transcribed into complementary deoxyribonucleic acids (cDNAs) via PrimeScript™ reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, 2 μL of cDNA template was amplified in the reaction mixture system of SYBR Green (20 μL) through the following procedures: reaction at 95°C for 30 s, and 40 cycles of reaction at 95°C for 5 s, 55°C for 30 s and 72°C for 30 s. Unless otherwise stated, the relative messenger RNA (mRNA) expression level of nm23-H1 was calculated based on $2^{-\Delta\Delta Ct}$, with the relative expression level of β-actin as a reference. The primer sequences are listed as follows: nm23-H1-F: 5'-GCGTACCTTCATTGCGATCAAAC-3', nm23-H1-R: 5'-ATCCAGTTCTGAGCACAGCTCGTG-3'. β-actin-F: 5'-TGCGTGACATTAAGGAGAA-3', β-actin-R: 5'-AAGGAAGGCTGGAAGAGT-3'.

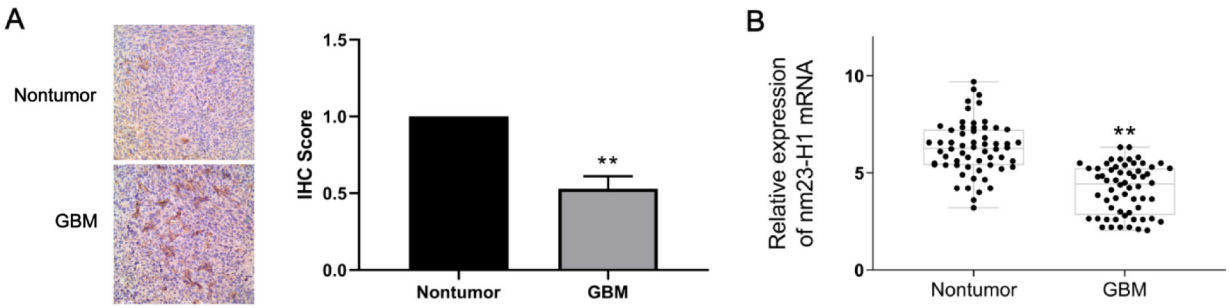


**Figure 1.** Changes in nm23-H1 expression in glioma tissues. **A:** Protein expression of nm23-1 protein detected via immunohistochemistry: The protein expression of nm23-H1 declined substantially in GBM group in comparison with that in Non-tumor group ($p<0.01$) (magnification: 400×). **B:** MRNA expression of nm23-H1 in glioma tissues: The mRNA expression level of nm23-H1 in GBM group was considerably lower than that in Non-tumor group ($p<0.01$) (**$p<0.01$ *vs.* Non-tumor group).

*Western blotting*

The protein extraction reagent was placed on ice in advance and dissolved, and the cell lysis mixture was then prepared. Subsequently, the mixture was ultrasonicated for 3 times. Afterwards, the absorbance of the protein sample at 540 nm was measured by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) and converted into protein concentration. The protein sample was then isolated via electrophoresis, transferred onto a membrane for 1 h and incubated with the primary antibody overnight and with the secondary antibody for 1 h, followed by image development.

*Methyl thiazolyl tetrazolium (MTT) cell proliferation assay*

Following successful transfection, the cells seeded into 96-well plates in nm23-H1 group and Vector group were incubated with 100 μL of 10% MTT and Dulbecco's Modified Eagle's Medium (DMEM)) (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 0, 24 , 48, 72 and 96 h after culture. Then, the absorbance at 490 nm was determined.

*Wound-healing assay*

The cells in the 6-well plates grew to full confluence, and the single-layer cells were scraped using a 20 μL pipette tip to simulate wounds and added with serum-free medium. Images were acquired at 0 and 24 h after scratching, before which the floating cells were washed clean using phosphate buffered saline (PBS). Finally, the scratch zones were quantified using ImageJ software (NIH, Bethesda, MD, USA).

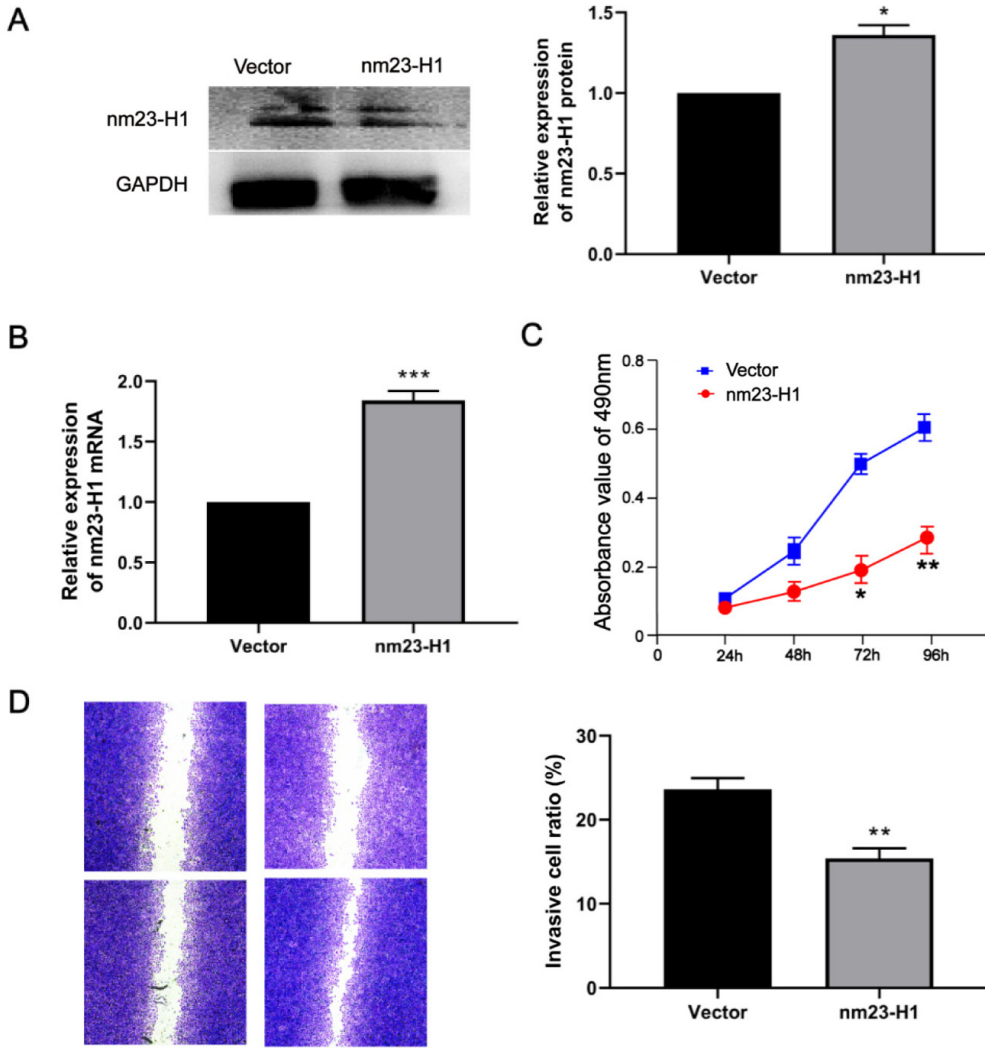


**Figure 2.** Influence of nm23-H1 expression on the proliferation and migration of H4 cells. **A:** Changes in nm23-H1 protein expression in the transfected cells: Compared with that in Vector group, the protein expression of nm23-H1 rose substantially in nm23-H1 group ($p<0.05$). **B:** Changes in nm23-H1 mRNA expression detected via qRT-PCR in the H4 cells in nm23-H1 and Vector groups: The mRNA expression level of nm23-H1 in cells in nm23-H1 group was markedly higher than that in Vector group ($p<0.001$). **C:** Changes in the proliferation of the transfected cells examined by MTT assay: The absorbance of cells at 490 nm in nm23-H1 group was notably lower than that in Vector group. **D:** Changes in the migration of the transfected cells detected via wound-healing assay: In comparison with that in Vector group, the healing area of cells was decreased in nm23-H1 group ($p<0.05$) (**$p<0.01$ *vs.* Vector group, ***$p<0.001$ *vs.* Vector group). (magnification: 20×).

*Statistics*

Unpaired *t*-test was adopted to evaluate the differences in the experimental data among groups, and SPSS 22.0 (IBM, Armonk, NY, USA) was used for all statistical analyses. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P<0.05 showed statistical significance.

## Results

*Nm23-H1 expression in glioma tissues*

First, the changes in the protein and mRNA expressions of nm23-H1 in the glioma tissues were determined via immunohistochemistry and qRT-PCR, respectively. Immunohistochemistry showed that nm23-H1 protein was located in the cytoplasm and that GBM group exhibited a considerably lighter staining intensity and a remarkably lower expression level of nm23-H1 than Non-tumor group (p<0.01). Besides, the qRT-PCR results revealed that the mean mRNA expression level of nm23-H1 in GBM group was prominently lower than that in Non-tumor group (p<0.01) (Figure 1).

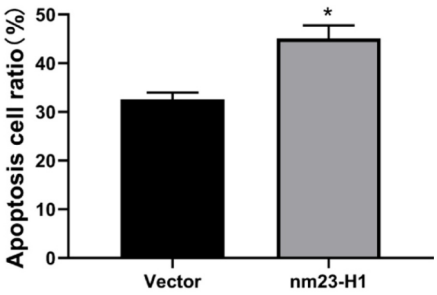


**Figure 3.** Proportion of apoptotic cells determined via flow cytometry after transfection: Compared with Vector group, nm23-H1 group exhibited a considerably increased percentage of apoptotic cells (*p<0.05 *vs.* Vector group).

*Influence of nm23-H1 expression level on the proliferation and migration of H4 cells*

To investigate the role of nm23-H1 in the progression of glioma, an *in vitro* functional assay was performed to assess the influence of the nm23-H1-induced exogenous overexpression of nm23-H1 on the phenotypic changes of glioma cells. Moreover, the transfection efficiency of nm23-H1 was evaluated at mRNA and protein levels. According to the assay results, the expression of nm23-H1 rose notably in H4 cells due to the transfection with nm23-H1 (p<0.05), and the absorbance at 490 nm was dramatically decreased in the H4 cells with overexpressed nm23-H1 at 72 h after inoculation. The wound healing assay showed that the migration distance was markedly longer after overexpression of nm23-H1 than that in Vector group (p<0.05). Therefore, it can be inferred from these data that nm23-H1 plays an active role in promoting the growth of glioma (Figure 2).

*High expression of nm23-H1 promoted H4 cell apoptosis*

After transfection, the cell pellets were harvested, and the percentage of apoptotic cells was measured via flow cytometry. It was discovered that the up-regulation of nm23-H1 expression in glioma cells induced cell apoptosis (p<0.05), corroborating that nm23-H1 protein inhibits the proliferation and migration of glioma H4 cells and promotes their apoptosis (Figure 3).

*Impact of PKC pathway on the proliferation and apoptosis of H4 cells*

The excessive activation and/or expression of PKC is associated with the abnormal proliferation and apoptosis of malignant glioma cells, so the cells in nm23-H1 group were treated with 1.0 μM Calphostin C to explore the influence of the PKC path-

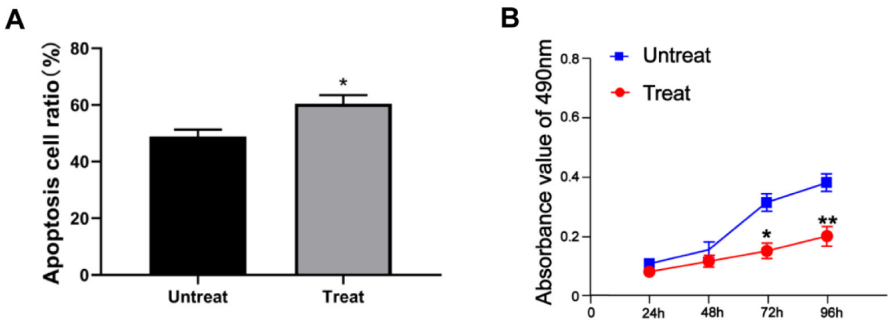


**Figure 4.** Influence of PKC pathway on the apoptosis and proliferation of H4 cells. **A:** Changes in the proliferation of cells treated with Calphostin C in nm23-H1 group: The treated cells had a dramatically lower absorbance at 490 nm than the untreated cells (p<0.05). **B:** Changes in the apoptosis of cells treated with Calphostin C in nm23-H1 group detected via flow cytometry: The proportion of apoptotic cells in Treated group was substantially higher than that in Untreated group (p<0.05) (*p<0.05 *vs.* Untreated group, **p<0.01 *vs.* Untreated group).

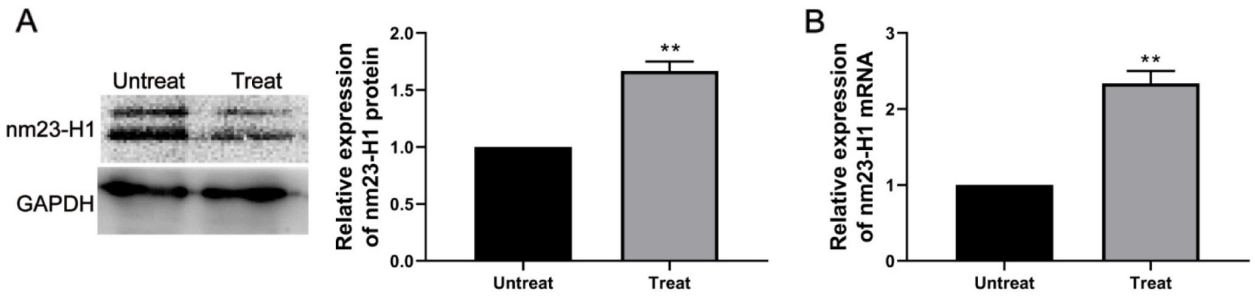


**Figure 5.** Influence of PKC inhibitor on the changes in the protein and mRNA expressions of nm23-H1 in H4 cells. **A:** Changes in protein expression of nm23-H1 detected by Western blotting after treatment with the inhibitor: Treated cells had a notably higher protein expression level of nm23-H1 than untreated cells ($p<0.01$). **B:** Changes in mRNA expression of nm23-H1 detected via qRT-PCR after treatment with the inhibitor: The relative mRNA expression level of nm23-H1 in Treated group was markedly higher than that in Untreated group (*$p<0.05$ *vs.* Untreated group, **$p<0.01$ *vs.* Untreated group).

way on the proliferation and apoptosis of glioma cells. The results of the MTT assay and apoptosis assay revealed that the proliferation ability of cells was notably weakened and their apoptosis was induced after addition of the PKC pathway inhibitor ($p<0.05$). The above results suggest that the PKC inhibitor enhances the effects of nm23-H1 on the proliferation and apoptosis of H4 cells (Figure 4).

*Nm23-H1 protein affected the behaviors of H4 cells through negative feedback regulation of the PKC pathway*

According to the findings, the inhibitor of the PKC pathway enhanced the effects of nm23-H1 protein on the proliferation and apoptosis of glioma cells, and the protein and mRNA expression levels of nm23-H1 in the cells incubated with the PKC pathway inhibitor were considerably higher than those in Untreated group ($p<0.01$). These results imply that the inactivated PKC expression induces protein expression of nm23-H1, and the increase in its protein expression represses the proliferation of H4 cells and promotes their apoptosis. It can be seen that nm23-H1 protein negatively regulates the PKC pathway to affect the behaviors of H4 cells (Figure 5).

## Discussion

Cancer metastasis is not only a basic aspect of tumor progression, but also the leading factor for the related deaths. The insidious infiltration of cerebral tumor cells into the peripheral normal brain cells makes it impossible to radically remove tumors under a microscope, so it is also the leading cause of treatment failure. Although nm23 gene was initially recognized as a metastasis-inhibiting gene, the clinical correlation between the role of nm23 as a metastasis-inhibiting factor and the prognosis indicators of human cancers remains unknown. Nm23 gene encodes nucleoside diphosphate kinase that inhibits metastasis in some tumors, including hepatocellular carcinoma and ovarian cancer [16,17]. However, such a metastasis-inhibiting effect has not been detected in other tumors such as neuroblastoma and pancreatic cancer [18-20]. The nm23 family is a multi-functional protein with enzymatic and DNA-binding functions [21]. Nm23 consists of 5 isotypes, of which nm23-H1 and nm23-H2 have their metastasis-inhibiting function extensively researched. These two gene isotypes are located on chromosome 17q21, and other isotypes on chromosome 16 [22,23]. Biological factors such as cell movement and migration and changes in adhesion molecules and proteolytic enzymes are associated with metastatic and locally invasive cells. Nm23 has been confirmed to be important in controlling metastasis, but there have been few data on the role of this gene family in modulating the invasion of cerebral tumors.

Therefore, this study aimed to explore the expression changes of nm23-H1 in human glioma and its biological influence on glioma cells. First, immunohistochemistry and qRT-PCR were performed to determine the changes in the protein and mRNA expressions of nm23-H1 in glioma tissues. It was found that the transcription and translation levels of nm23-H1 were considerably higher than those in normal brain tissues, indicating that nm23-H1 protein may be a tumor suppressor in glioma. Then, the results of the *in vitro* functional assays revealed that the increase in the protein expression of nm23-H1 decreased the proliferation and migration rates of glioma cells and induced their apoptosis. The above results fully confirm that nm23-H1 also plays a role as a tumor suppressor in glioma, so it may become a potential target protein for the treatment of glioma.

It has been corroborated that excessive activation and/or expression of PKC is truly associated with the abnormal proliferation of malignant glioma cells. The proliferation of astrocytes is closely related to the expression of PKC. PKC is highly expressed in fetal astrocytes with active mitosis [13,14], whereas the non-tumor mature astrocytes with a lower proliferation rate hardly express PKC [15,24]. Furthermore, the cells transfected with nm23-H1 were incubated with the inhibitor of PKC to explore the function of PKC in glioma cells and its molecular mechanism in this study. According to the results, the H4 cells incubated with the inhibitor had poorer proliferation and migration abilities than the non-incubated cells, and the apoptosis showed the opposite condition. Therefore, the PKC inhibitor can potentiate the effects of nm23-H1 protein on the proliferation and apoptosis of H4 cells. Besides, the protein and mRNA expression levels of nm23-H1 rose considerably in the cells incubated with the inhibitor. The reason is that the inactivated PKC expression induces the protein expression of nm23-H1, and the increase in its protein expression represses the proliferation of H4 cells and facilitates their apoptosis. It can be seen that nm23-H1 protein affects the behaviors of H4 cells through negative feedback regulation of the PKC pathway.

## Conclusions

In conclusion, nm23-H1 protein has a considerably low expression in glioma tissues and inhibits the activation of PKC to repress the proliferation and migration of glioma cells and induce their apoptosis.

## Conflict of interests

The authors declare no conflict of interests.

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