O-GlcNAc Regulated Proliferation and Deterioration of the Nasal Inverted Papilloma

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Research

Keywords: O-GLcNAc, Nasal Inverted Papilloma, ogt, oga

Posted Date: September 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-845783/v1

Abstract

Background: The nasal inverted papilloma occurs mostly in the epithelium of the nasal mucosa. Histopathological manifestations of the nasal inverted papilloma are benign but it has the characteristics of aggressive growth, strong local destruction, frequent recurrence, and malignant change. Nasal inverted papilloma is a tumor with malignant biological behavior. O-GLcNAc is a posttranslational modification that is ubiquitous in cells. This seemingly simple carbohydrate modification played a key role in cell physiology and disease progression.

Methods: In this study, immunohistochemical staining and western blot were used to determine the expression of O-GlcNAc in the nasal inverted papilloma; RT-qPCR was used to detect the expression of *ogt*. The expression levels of *ogt* and *oga* genes were detected by RT-qPCR in the SCC6 and CNE-E1 cells. An *ogt* and *oga* small-interference RNA fragment was transfected into cells to both reduce and increase the O-GlcNAc. The effect of O-GlcNAc on the proliferative ability of cells was detected by CCK8. The migration and invasion of cells was detected by wound healing assay and transwell invasion assay.

Results: The expression of O-GlcNAc and *ogt* mRNA levels in nasal inverted papilloma were higher than that in the control group. O-GlcNAc enhanced SCC6- and CNE-E1- cell proliferative, migratory, and invasive ability. This study found that changes in the glycosylation level of O-GLcNAc affected the proliferation, invasion, and migration of the NIP.

Background

The Nasal inverted papilloma (NIP) is one of the more common benign tumors of the nasal cavity and sinus[1, 2]. It originated from the schneiderian membrane, and its biological behavior is one of malignance. It has the characteristics of frequent recurrence and malignant transformation. Reports had shown that the recurrence rate of NIP is 28–74%, and the percentage of NIP deterioration to sinonasal squamous cell carcinoma (SNSCC) is 7–27%[3]. O-GlcNAc is a dynamic and reversible protein posttranslational modification that is widely present on protein serine or threonine residues. Unlike the classic glycosylation modification, it is distributed mainly in the cytoplasm and nucleus[4]. The addition and removal of O-GlcNAc modification groups are completed by O-GlcNAc transferase (OGT) and glycosidase (O-GlcNAcase, OGA), respectively. O-GlcNAc didn't only participat in the regulation of most normal physiological functions of the organism, but it also may play an important role in the pathogenesis of various diseases[5]. Some recent studies have shown that the abnormalities of O-GlcNAc are related to diabetes, immune diseases, and stroke[6, 7]. In the research of O-GlcNAc and tumors, although it has been found that many tumor-related proteins could be modified by O-GlcNAc (such as p53 and c-Myc), the exact relationship between O-GlcNAc and NIP or whether O-GlcNAc is involved in the occurrence and development of NIP have not been reported[8]. Therefore, in this article, we systematically studied the occurrence and development of O-GlcNAc in the NIP and explored the molecular mechanism of the O-GlcNAc regulation of NIP.

The high recurrence and malignancy rate of NIP have alerted clinicians to treat it as a precancerous lesion. Therefore, this study aimed to investigate the role of O-GlcNAc in the pathogenesis of different NIP. It is intended to provide a reference for early clinical diagnosis, surgical method selection, treatment of NIP recurrence, and malignant transformation.

Materials And Methods

Study population and clinical examinations

Thirty-five patients with NIP and 30 patients with SNSCC (sinonasal squamous cell carcinoma) who were admitted to the Second Affiliated Hospital of Dalian Medical University from June 2016 to March 2017 and 30 control patients (based on normal mucosa at the back of the inferior turbinate) were selected. The 20 cases of NIP, 20 cases of SNSCC, and 20 control cases were used to detect the O-GlcNAc protein expression with immunohistochemistry and western blotting. The 10 cases of NIP, 15 cases of SNSCC, and the 10 control cases were used to detect the *ogt* gene expressions with RT-qPCR.

Inclusion criteria were (1) NIP confirmed by pathology before or after surgery, excluding specimens containing a large amount of necrotic tissue or with a large number of infiltrated inflammatory cells, (2) patients in the SNSCC group needing to be diagnosed as NIP before malignant transformation, (3) patients generally in good condition undergoing routine examination and comprehensive evaluation before surgery and having no surgical contraindications, and (4) patients confirmed to have squamous cell carcinoma and not having undergone any physical, chemical, or immunological antitumor treatment before surgery. All patients were approved by the Ethics Committee of Dalian Medical University (2009 No. 006), giving informed consent.

Cell Culture

SCC6 and CNE-E1 cells (American Type Culture Collection) were grown in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented by 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C under 5% CO_2 in humidified air. The cells (10^6) were seeded onto 6-well plates. When the cells reached 80% confluence, they were washed three times with phosphate-buffered saline (PBS) and subsequently starved of serum for 3 h before progestogen treatment.

Immunohistochemistry

The tissue was treated by conventional methods and embedded in paraffin, serially sectioned, sliced to a thickness of 3 μm, and the attached sections and reagents required for the experiment were placed on a fully automated immunohistochemical staining instrument. O-GlcNAc antibody dilution was 1:100 (Abeam). Immunohistochemistry images were taken using a Nikon microscope and a CCD image sensor. All sections were photographed in the same light-intensity room using the same aperture, the same light source, and the same magnification (400×). Each slice was randomly photographed with five fields of

view. The image was analyzed by the US Image-Pro Plus 6.0 image analysis system. The immunohistochemical results of each group were analyzed. After standardization, the area-weighted average optical density (IOD/area) was used as the analysis standard.

RNA Isolation and RT-qPCR

The tissues were combined, and total RNA was isolated using Trizol (Takara Biotechnology). The cDNA was formulated from 100 ng of total RNA using the PrimeScript RT reagent Kit (Takara Biotechnology) and subjected to quantitative PCR (qPCR) using SYBR Green in an RT-qPCR thermal cycler. Relative quantification was used to analyze the RT-qPCR data. The threshold cycle threshold (CT) was used to record gene expression. The PCR conditions were as follows: DNA denaturation at 95°C, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The CT was determined by monitoring the incorporation of SYBR Green I (Takara Biotechnology) into the amplified product with fluorescence detection. The expression of *CK8* was normalized according to that of *gapdh*. The following specific primers were designed: *gapdh* (XM_001256799.2) forward, 5'-GTGAAGGTCGGAGTCAACG-3', reverse, 5'-TGAGGTCAATGAAGGGGTC-3', *ogt* (XM_017029908.1) forward, 5'-CGGGAATCACCCTACTTCACACC-3', reverse, 5'-CCGCCATCACCTTCACTCGAAA-3', *oga* (XM_017015586.1) forward, 5'-TCCCCAGAGATGTCCATGCAAG-3', reverse, 5'-TCCTTTGGGTCCATGCTCGTA-3'.

Western Blotting

According to the instructions in the protein extraction kit, the entire process was carried out on ice. The protein concentration in the sample was detected using the QuantiPro BCA assay kit instructions (KeyGen Biotech). The protein was subjected to 10% polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred to a polyvinylidene fluoride membrane, and a 1:1,000 dilution of rabbit antihuman O-GlcNAc antibody was used. The primary antibody was left at room temperature for 3 h, and then TBST was added. All antibodies were diluted with TBST and washed three times with TBST for 10 min. The membrane was incubated with 1:2,500 horseradish peroxidase-conjugated antibody for 1 h at 4°C. The membrane was then placed in an imaging system (Bio-Rad) and ECL luminescent solution was added.

Short, Interfering RNA (siRNA) Knockdown Experiments

The SCC6 cells and CNE-E1 cells were seeded in 6-well plates. For the knockdown experiments, siRNA targeted the *ogt* and *oga* gene (*ogt*-siRNA and *oga*-siRNA; 200 nmol/well) and a negative control siRNA were purchased from GenePharma. The SCC6 cells and CNE-E1 were transfected with the si-OGT and si-OGA Xfect RNA Transfection Reagent (TaKaRa). The transfection efficiency was determined with RT-qPCR.

Cell-Counting Kit-8

The cells were seeded in 96-well plates at 100 μL per well (n = 10^3 cells) and cultured for 24 h before 10 μL of a Cell-Counting Kit-8 (CCK-8) solution was added to each well. The plates were incubated for 4 h.

Absorbance (OD) at 450 nm was measured.

Transwell Invasion Assay

A permeable filter of a Transwell system (Corning Inc) was used to study the invasion capacity of cells. The inside compartment of the Transwell inserts was coated with Matrigel (BD Biosciences) at 37°C for 3 h and then blocked with 1% PBS solution for 30 min at room temperature. The SCC6 and CNE-E1 cells (10^5/well) were loaded in the upper chamber in a culture medium for 24 h. Cell migration to the other side of the membrane was induced by 20% FBS-containing medium in the lower chamber. The cells were fixed in methanol for 30 min and stained with 0.5% Crystal Violet for 20 min. After the cells on the upper side of the top chamber were gently removed, the migrated cells were photographed and counted using ImageJ software (National Institutes of Health).

Wound Healing Assay

SCC6 or CNE-E1 cells were seeded in 6-well plates for 24 h after pretreatment (knockdown or overexpression of O-GlcNAc). The cells were then wounded by removing a 700 μm-wide strip with a standard 200 μL pipette tip. Wound healing was quantified by measuring the migratory distance of cells.

Statistical Methods

The results were graphically depicted as the mean ± standard deviation. One-way ANOVA was performed (SPSS 20.0 for Windows) to detect statistically significant differences. A *P*-value < 0.05 was considered statistically significant.

Results

Expression of O-GlcNAc Levels in NIP and SNSCC

We detected the expression of O-GlcNAc by immunohistochemistry in both NIP and SNSCC. The expression of O-GlcNAc-modified protein in NIP and SNSCC was higher than that in the control group, and the SNSCC group had the highest. As shown in Fig. 1A and B, we found that O-GlcNAc-modified protein was hardly expressed in the control group, but it was expressed higher in NIP, the expression was highest in SNSCC, the expression level increased significantly. The *ogt* mRNA levels in NIP had a consistent trend with O-GlcNAc. *ogt* mRNA levels were significantly higher than control, which also revealed the fact (Fig. 1C). Western blotting results showed that the expression of O-GlcNAc was consistent with the immunohistochemical results. The expression in NIP and SNSCC was higher than that in the control group, and the expression was highest in SNSCC (Fig. 1D).

Expression Levels of Ogt and Oga Genes in SCC6 and CNE-E1 Cells

The expression levels of *ogt* and *oga* genes were detected by RT-qPCR in SCC6 cells (less malignant head and neck squamous cell) and CNE-E1 cells (moderately malignant head and neck squamous cell). The

results showed that the expression of *oga* gene in SCC6 cells was significantly higher than that in the CNE-E1 cells. Expression of the *ogt* gene is the opposite trend, as shown Fig. 2A. It meant that the expression of O-GlcNAc in moderately malignant head and neck squamous cell carcinoma was higher than that in the less malignant form.

After 24 h of transfection with si-OGA and si-OGT, the SCC6 and CNE-E1 cells were observed to have good staining properties under the fluorescence microscope. RT-qPCR showed that o si-OGA and si-OGT were lower at the gene levels than was the control group, and the results were statistically significant ($P < 0.05$; Fig. 2B).

Effect of O-GlcNAc on Reproducibility of SCC6 and CNE-E1 Cells

To explore the mechanism of O-GlcNAc in NIP, OGA and OGT expression in the SCC6 and CNE-E1 cells were reduced. CCK-8 was used to detect the effect of different expression levels of si-OGA and si-OGT on the proliferation of the SCC6 and CNE-E1 cells. The results of CCK-8 showed that proliferation of the SCC6 and CNE-E1 cells were inhibited at the 24th and 48th h after inhibition of OGT, and the results were statistically significant ($P < 0.05$). The OD value of the si-OGA group was significantly increased with the passage of time in the SCC6 and CNE-E1 cells, meaning that si-OGT inhibited SCC6 and CNE-E1cell proliferation and si-OGA promoted cell proliferation (Fig. 3).

Effect of O-Glcnac on Migration and Invasiveness of SCC6 and CNE-E1 Cells

The migration and invasion of cells was detected by wound healing assay and transwell invasion assay. In the SCC6 cells, the si-OGA group promoted migration and invasiveness compared with the si-NC group. In the CNE-E1cells, the si-OGT group inhibited the migration and invasiveness of the SCC6 cells compared with that of the si-NC group. These results suggested that O-GlcNAc enhanced cell migration and invasiveness (Fig. 4A and B).

Discussion

The NIP is one of the more common benign tumors of the nasal cavity and sinuses[9]. The incidence of NIP accounts for about 0.5–4% of primary nasal tumors. The recurrence rate is 25–74%, and the malignancy rate ranges from 5–15%[10]. The surface of the NIP is not smooth or granular, the color is pink or gray-red, and the texture is hard[11]. Although it is a benign tumor pathologically, its biological behavior is malignant, with characteristics of frequent recurrence, destructive growth, and malignant change; it is considered mostly a junctional tumor[4, 12]. The NIP occurs mostly in men aged 50–60, and unilateral disease is more common[2, 13]. The most common sites of NIP are on the outer wall of the nasal cavity and the inner wall of the maxillary sinus, accounting for about 30–84%[14]. The etiology and pathogenesis of NIP are still unknown, and there are many related theories, such as chronic inflammation, viral infection, environmental factors, allergies, genetic factors, and tumor-suppressing genes[15, 16]. Our aim is to explore the pathogenesis of NIP and provide a corresponding theoretical basis for its early treatment. In this paper, immunohistochemical and western blotting were used to detect the level of O-

GlcNAc in NIP, SNSCC, and normal mucosa to analyze the tumor cell proliferation activity, which provided experimental evidence for the pathogenesis of NIP and postoperative targeted therapy and follow-up.

O-GlcNAc glycosylation is a dynamic protein modification process, which is widely involved in cell life, gene transcription, protein translation, and signal transduction[6, 7, 17]. More and more evidence showed that the abnormal modification of glycosylation of O-GlcNAc is closely related to the occurrence and development of tumors, and the role and mechanism of O-GlcNAc glycosylation in NIP have not been reported[18–21]. We speculate that O-GlcNAc glycosylation takes part in the development of NIP. This article aimed to study the regulatory effect of O-GlcNAc glycosylation on cells in the NIP, the specific molecular mechanism of O-GlcNAc glycosylation to regulate NIP, and to clarify the effect of O-GlcNAc glycosylation on the biological behavior of NIP cells. This will provide a new perspective for the basic research of NIP and new ideas for clinical treatment of NIP[22, 23].

First, we collected clinical samples of NIP and SNSCC, and detected the protein expression levels of O-GlcNAc by immunohistochemistry and Western blot. The results showed that the protein expression levels of O-GlcNAc in NIP and SNSCC were higher than those in the control group. The expression level of GlcNAc protein was higher than that of NIP, indicating that O-GlcNAc is closely related to the malignancy of the tumor. O-GlcNAc glycosylation is a dynamic protein modification process, and the addition and removal of modification groups are completed by OGT and OGA, respectively. So, we tested the expression level of *ogt* mRNA, and the results showed that the expression of *ogt* mRNA was also positively correlated with the tumor's malignancy. This result was consistent with the higher expression of O-GlcNAc in NIP and SNSCC than in the control group.

To better prove the mechanism of O-GlcNAc in NIP, we selected both low-grade (SCC6) and moderately malignant head and neck tumor cells for in vitro experiments (CNE-E1). We detected the expression of *ogt* mRNA and *oga* mRNA in SCC6 and CNE-E1 cells. Our results showed that compared with CNE-E1, the expression of *oga* mRNA in SCC6 was higher than that of CNE-E1, while the expression of *ogt* mRNA in CNE-E1 was higher. This result repeatedly proved that O-GlcNAc was closely related to the malignancy of the tumor. To better detect the mechanism of O-GlcNAc in NIP proliferation, invasion, and metastasis, we transfected si-OGA and si-OGT into SCC6 and CNE-E1 cells. Our results showed that transfection of si-OGA promoted the proliferation, invasion, and metastasis of SCC6 and CNE-E1 cells. Instead, transfection of si-OGT inhibited these functions. The above results prove that O-GlcNAc takes an important part in the transformation of NIP into SNSCC.

Conclusions

In summary, O-GlcNAc played an important role in the occurrence and development of NIP, and O-GlcNAc plays an important role in the deterioration of NIP into SNSCC. When we used genetic modification to reduce cell O-GlcNAc glycosylation, the cell's proliferative, invasive, and metastatic abilities were reduced. After increasing the glycosylation of O-GlcNAc, its cell proliferative, invasive, and metastatic abilities were increased. Therefore, O-GlcNAc has the potential of becoming a new target for the treatment of NIP.

Declarations

Acknowledgements

We thank the Special Fund for Liaoning Provincial Program for Top Discipline of Basic Medical Sciences.

Authors' contributions

MD and NZ carried out the studies, participated in collecting data, and drafted the manuscript. YK and HK performed the statistical analysis and participated in its design. XY, JG, XH, and YX helped to draft the manuscript. All authors read and approved the final manuscript.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Natural Scientific Grants of China (grant no.31971209), by the Liaoning Provincial Key R&D Program (2019020048-JH2/10300017).

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Figures

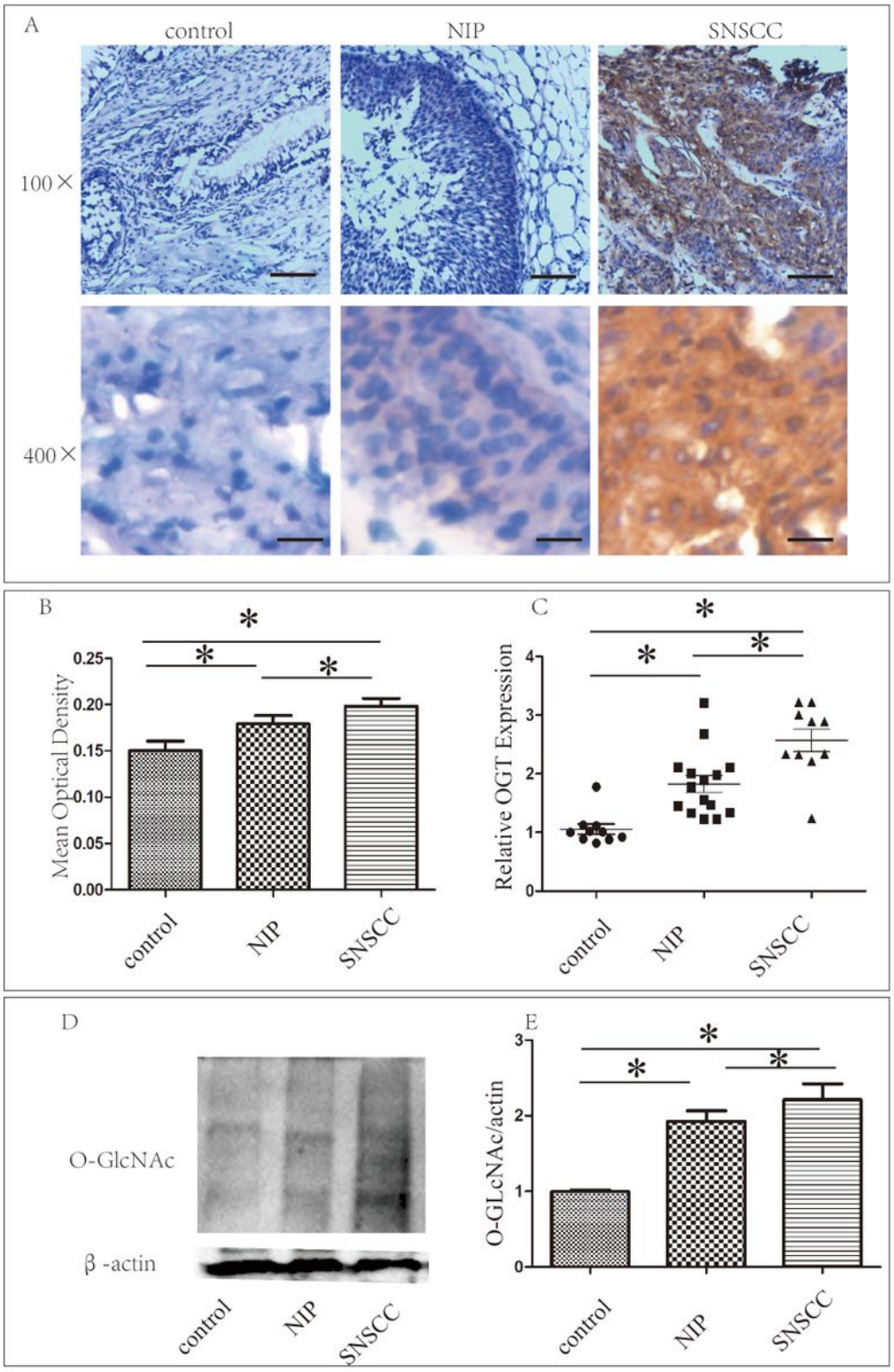

Figure 1

Expression of O-GlcNAc in NIP. (A) Micrographs of immunostaining for O-GlcNAc in NIP and SNSCC (10×, 40×). (B) Statistical analysis of immunohistochemistry. (C) Analysis of ogt level in the NIP and SNSCC tissues by RT-qPCR. (D) O-GlcNAc level was measured by Western blot in the NIP and SNSCC tissues. *P-value <0.05.

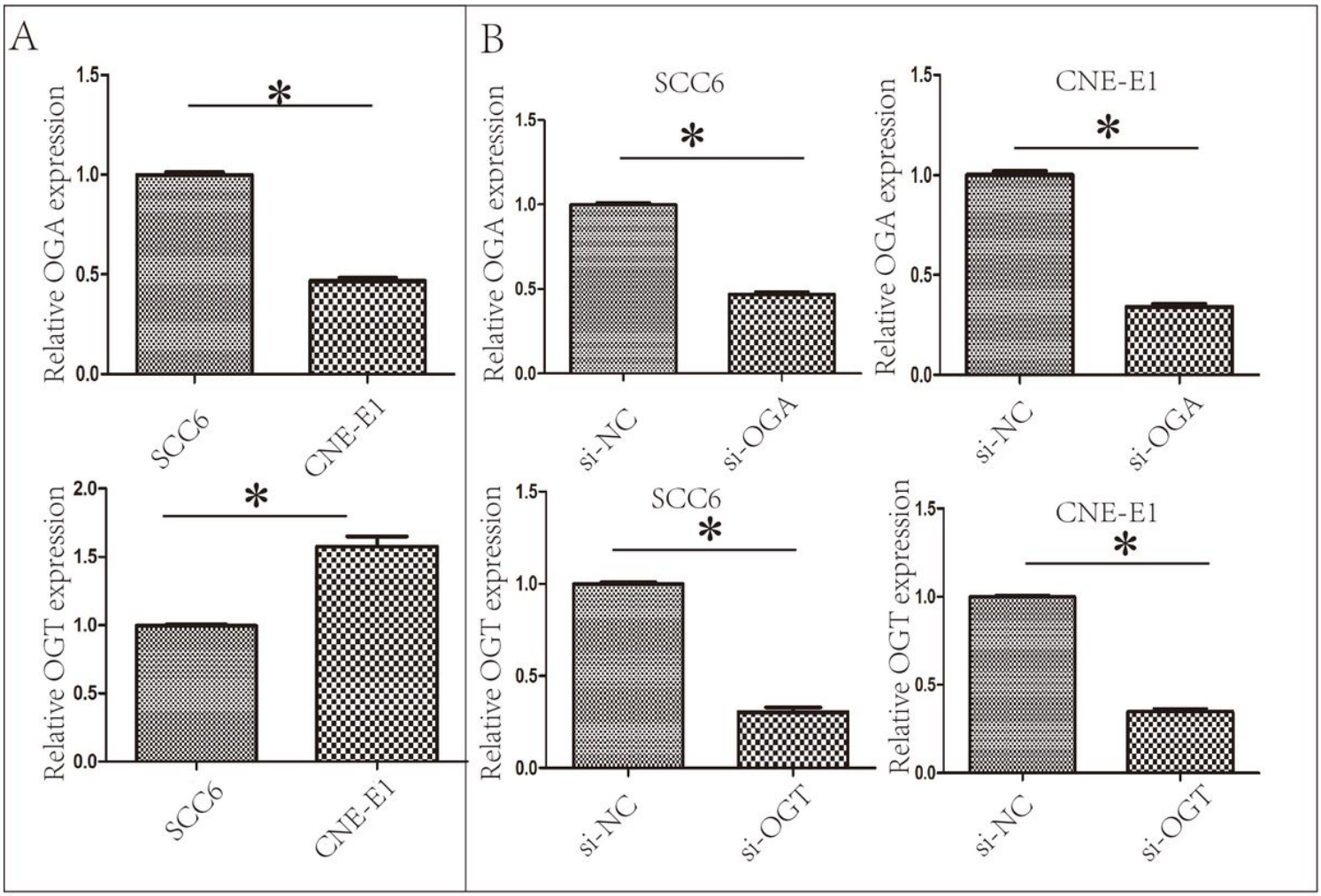

Figure 2

Expression levels of ogt and oga genes in SCC6cells and CNE-E1 cells. (A) Analysis of oga and ogtmRNA expression in SCC6 cells and CNE-E1 cells by RT-qPCR. (B) RT-qPCR analysis was introduced to detect oga and ogtmRNA expression after 24 h transfection. *P-value <0.05.

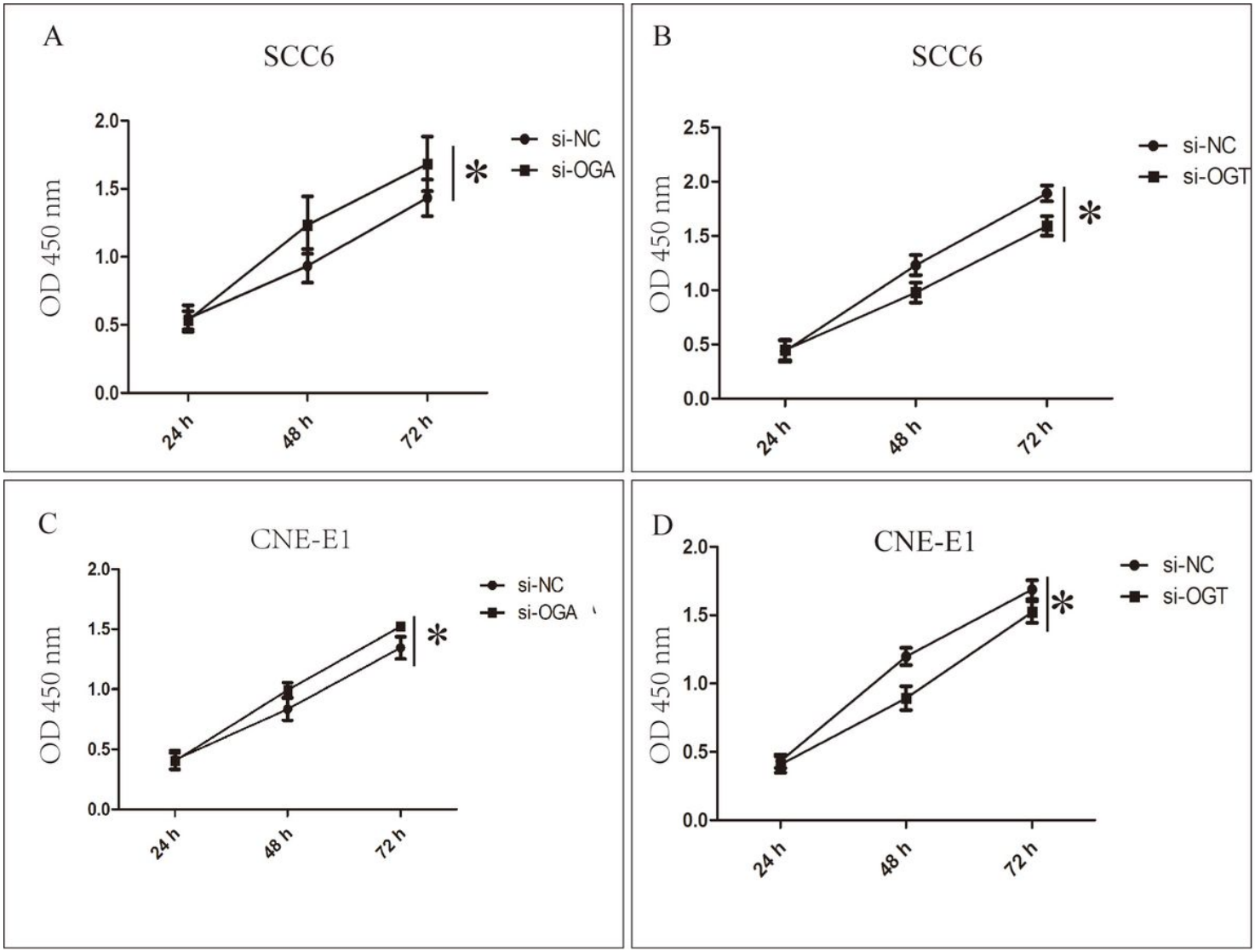

Figure 3

Effect of O-GlcNAc on proliferative ability of SCC6 and CNE-E1 cells. CCK8 results showed that si-OGT inhibited SCC6 and CNE-E1cell proliferation, and si-OGA promoted cell proliferation. *P-value <0.05.

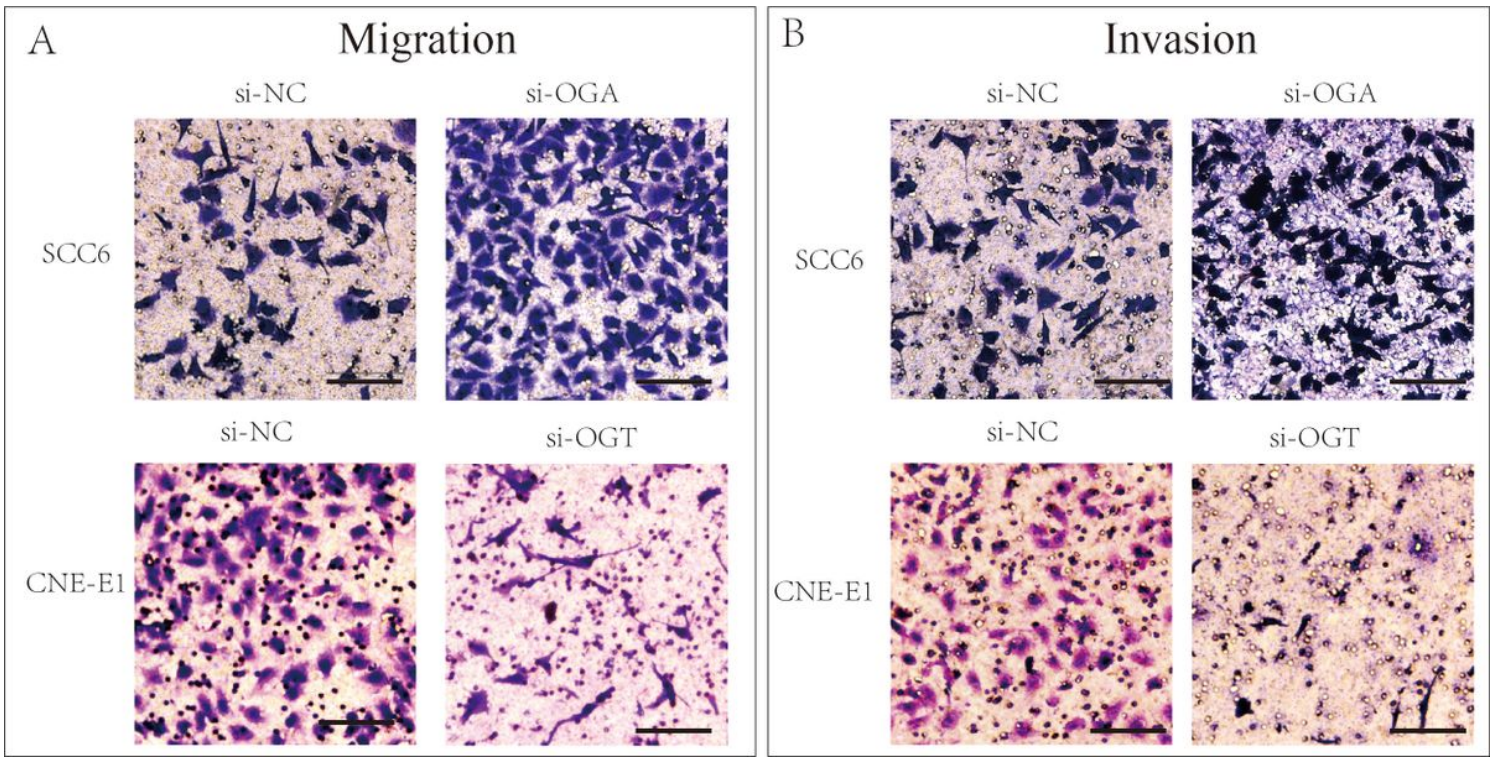

Figure 4

Effect of O-GlcNAc on migration and invasive ability of SCC6 and CNE-E1 cells. (A) Migration of cells was detected by wound healing assay (20×). (B) Invasion of cells was detected by Transwell invasion assay (20×).

Supplementary Files

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- TheresultofWesternblot.pdf