O-GlcNAcylation promotes migration and invasion in human ovarian cancer cells via the RhoA/ROCK/MLC pathway

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Abstract. O-GlcNAcylation is a dynamic and reversible post-translational modification associated with the regulation of multiple cellular functions. The addition and removal of O-Linked β-N-acetylglucosamine (O-GlcNAc) on target proteins is catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. Accumulating evidence suggests that O-GlcNAcylation is associated with the malignancy of several types of human cancer. To investigate the effect of O-GlcNAcylation on ovarian cancer phenotypes, global O-GlcNAc levels were decreased by OGT silencing through RNA interference and increased by inhibiting OGA activity with Thiamet-G. Transwell assay results demonstrated that OGT silencing inhibited the migration and invasion of SKOV3 and 59M ovarian cells in vitro, while Thiamet-G treatment promoted migration and invasion. Furthermore, a pull-down assay and western blot analysis demonstrated that Thiamet-G treatment enhanced RhoA activity and the phosphorylation of the Rho-associated protein kinase (ROCK) substrate, myosin light chain (MLC), while OGT silencing attenuated RhoA activity and MLC phosphorylation. In addition, RhoA silencing via RNA interference and inhibition of ROCK activity with Y-27632 prevented Thiamet-G-induced increases in cell migration and invasion. These data suggest that O-GlcNAcylation augments the motility of ovarian cancer cells via the RhoA/ROCK/MLC signaling pathway. Therefore, O-GlcNAcylation may be a potential target for the diagnosis and treatment of ovarian cancer.

Introduction

O-GlcNAcylation is a post-translational modification in nuclear and cytoplasmic proteins in which O-linked β-N-acetylglucosamine (O-GlcNAc) monosaccharide is linked to a serine or threonine residue (1). O-GlcNAc cycling is catalyzed by only two enzymes: O-GlcNAc transferase (OGT), which is responsible for the addition of O-GlcNAc to proteins; and O-GlcNAcase (OGA), which is responsible for the removal of O-GlcNAc from proteins (2). O-GlcNAcylation is emerging as a key regulator of cellular biological processes, including transcription, signaling, cell metabolism, morphogenesis, motility, cell cycle and development (3-5). Abnormal O-GlcNAcylation levels are associated with a variety of human diseases including diabetes, cardiovascular disease and neurologic disorders (6,7). An increasing number of O-GlcNAc-modified proteins have been revealed to be closely associated with tumorigenesis and development (8). Multiple oncogene and anti-oncogene products, including p53, c-Myc, c-Jun, c-Fos and retinoblastoma protein, have been demonstrated to be modified by O-GlcNAc (9-11), suggesting that O-GlcNAcylation may be associated with tumorigenesis and development. Aberrant O-GlcNAcylation has been linked to several types of human cancer, including breast (12), lung (13), colon (14), pancreatic (15) and prostate (16) cancer. However, the effect of O-GlcNAcylation on ovarian cancer remains poorly understood.

Ovarian cancer is the fifth most common cause of cancer-associated mortality in females, and exhibits the highest mortality of all gynecological malignancies (17). Aggressive ovarian cancer cells are often able to break away from the primary tumor to invade and spread to other parts of the body, including the lymph nodes, liver and lungs, and the lining of the bladder, bowel and abdomen (18,19). This results in a poor prognosis and a high mortality rate. The malignancy of tumor cells is assessed by measuring the migratory and invasive ability of the cells, so investigations of the molecular mechanisms underlying these abilities may aid the diagnosis and treatment of cancer. In the present study, the effects of O-GlcNAcylation on ovarian cancer cell motility were examined, including migration and invasion, and the underlying molecular mechanism.

Ras homolog family member A (RhoA) is a member of the Rho GT-Pase family associated with actin cytoskeleton rearrangement, regulation of the cell cycle, gene transcription, cell polarity and movement (20). As with other GTPases, RhoA functions through the exchange between two states: The GTP-bound active state and the GDP-bound inactive
state (21). Rhoa-mediated signaling pathways, particularly the Rho-associated protein kinase (ROCK)/myosin light chain (MLC) pathway, are closely associated with diverse biological activities including cytoskeleton reorganization, gene expression, muscle contraction, cell growth and motility (22-27). High Rhoa expression levels and activity have been observed in a variety of human types of cancer (28-32), suggesting that it may be associated with signaling pathways relevant to cancer. In addition, increased Rhoa and ROCK expression levels are more commonly observed in advanced cancer compared with early stage cancer (33). Furthermore, it has been reported (34) that Rhoa silencing significantly suppresses growth, adhesion, migration and invasion of ovarian cancer cells. Therefore, the present study aimed to examine whether Rhoa/ROCK signaling is involved in the regulation of O-GlcNAcylation in ovarian cancer cells, and how this affects their capacity to migrate and invade tissues.

Materials and methods

Cell cultures. Human ovarian epithelial adenocarcinoma SKOV3 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and endometrioid-type ovarian epithelial carcinoma 59M cells were purchased from the European Collection of Authenticated Cell Cultures; Public Health England (Salisbury, UK). SKOV3 cells were cultured in McCoy’s 5A medium (Thermo Fisher Scientific, Inc., Waltham, MA) and 59M cells in Dulbecco’s modified Eagle’s medium ( Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Lonza Group, Basel, Switzerland), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO₂.

RNA interference (RNAi). The sequence of the OGT-targeting small interfering RNA (siRNA) used was 5’-GGATGCTTATATCAATTITAGG-3’. Negative control siRNA oligonucleotides (F: 5’-CCGGTACGGATACCTCAGGAAATTCTCGAGAATTCCTCGGAACGTGTCAGTTTGGT-3'; R: 5’-AATTCAAACACGTGACCGTGGAGAATTCTCGAGACTTCTCGGAATTCCTCGGAGAATTCTCGAGAATTCCTCGGAACGTGTCAGTTTGGT-3') were purchased from Eurogentec (Liège, Belgium). siRNA (1.6 µg) was diluted with Opti-MEM I Reduced Serum Medium (Invitrogen; Thermo Fisher Scientific, Inc.) to a final volume of 100 µl. DreamFect reagent (8 µl; OZ Biosciences, Marseille, France) was diluted with Opti-MEM I Reduced Serum Medium to a final volume of 100 µl. The RNAi solution and transfection reagent were added into 100 µl of 10% Opti-MEM I Reduced Serum Medium (Invitrogen; Thermo Fisher Scientific, Inc.) to a final volume of 100 µl. siRNAs were transfected into SKOV3 and 59M cells by electroporation with the Amaza Nucleofector (Lonza Group) according to the manufacturer’s protocols, then lysed 24 h later and analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis to measure Rhoa mRNA and protein expression levels, respectively.

RT-qPCR. Total RNA in cells was extracted using a total RNA isolation kit (A&A Biotechnology, Gdynia, Poland). cDNA was obtained by reverse transcription of 1 µg of total RNA in a 20 µl reaction using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) and was amplified using a Taqman® Gene Expression Assay (Applied Biosystems; Thermo Fisher Scientific, Inc.), using primers specific for the target proteins. The sequences of the primers were as follows: 5’-CGGGAGCTAGCAGCAAG AGTGAAG-3’ (F) and 5’-GCTTGCAAGACGCTCTCGTA-3’ (R) for Rhoa. 5’-GGCCGGTAAAGTCTGCAAGA-3’ (F) and 5’-GCCAGAGGCGCGAGAA-3’ (R) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The two genes were amplified by a first step of 120 sec at 95˚C, followed by 45 cycles of 30 sec at 95˚C, 30 sec at 60˚C, and 30 sec at 72˚C. mRNA expression of Rhoa was calculated using the formula 2-ΔΔCq (35) and was normalized to GAPDH expression levels. mRNA expression levels in cells transfected with Rhoa siRNA are presented relative to mRNA expression levels in cells with negative control siRNA.

Migration and invasion assays. Cell migration was evaluated by Transwell assay using Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) (36). A total of 600 µl cell culture medium, with or without 5 µM Thiamet-G (an OGN inhibitor) or 50 µM Y-27632 (a ROCK inhibitor) was added in the lower chamber. Culture medium (100 µl) containing 1x10⁵ SKOV3 or 59M cells and 1% FBS, was plated into the upper chamber, with or without Thiamet-G or Y-27632. The cells on the undersurface of the upper chamber were stained with crystal violet 20 h later and were observed using a light microscope. A total of six fields at x100 magnification were selected at random to measure the average cell coverage using ImageJ software version 1.45s (National Institutes of Health, Bethesda, MD, USA). Invasion assays were performed using the same protocol as the migration assay, but the upper face of the polycarbonate membrane in the upper chamber was covered with 1 mg/ml Matrigel (BD Biosciences) and the invasive cells were detected following 24 h incubation.

Western blot analysis. Cells were lysed for 15 min at ice bath using a lysis buffer (1% Triton X-100, 20 mM Tris pH 7.5, 1 mM MgCl₂, 150 mM NaCl, 1 mM NaVO₃, 50 mM NaF, 1.5 mM EDTA, 10% glycerol, 20 mM β-glycerophosphate, 10 µg/ml aprotinin, 1 µM peptatin A) containing 5 µM PUGNAc (an OGA inhibitor; Toronto Research Chemicals, Inc., North York, Canada). Protein samples (50 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany). The membrane was blocked with 5% non-fat dried milk in TBST for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies. Antibodies specific to O-GlcNAcylated (RL2; 1:1,000; Affinity BioReagents, Golden, CO, USA) and
OGT (F-12; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used. RhoA (67B9; 1:1,000), MLC (3672; 1:1,000) and phosphorylated (p-)MLC (3671; 1:1,000) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). GAPDH antibody (sc-25778; 1:2,000) and horseradish peroxidase-linked goat anti-mouse (sc-2005; 1:2,000) and goat anti-rabbit (sc-2004; 1:2,000) IgG secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Development was carried out using an enhanced chemiluminescence western blotting detection reagent (GE Healthcare Life Sciences, Chalfont, UK).

**RhoA activity detection.** RhoA activity was analyzed using Rhotekin Rho binding domain (Upstate Biotechnology; Thermo Fisher Scientific, Inc.) bound to glutathione agarose beads to pulldown the active GTP-bound RhoA form from ovarian cell lysates in lysis buffer (20 mM HEPES, pH 7.5, 0.5% NP-40, 100 mM NaCl, 0.2% deoxycholic acid, 10% glycerol, and 10 mM MgCl₂) supplemented with protease and phosphatase inhibitors (37). GTP-bound RhoA and total RhoA were evaluated by western blot analysis as above, using RhoA antibody (67B9; 1:1,000; Cell Signaling Technology, Inc.).

**MLC phosphorylation detection.** Cells were starved in serum-free medium for 24 h, then incubated at 37°C for 60 min with or without Thiamet-G at 5 μM concentration. The cells were subsequently lysed for 15 min at 4°C in cell lysis buffer [100 mM NaCl, 1 mM Na₃VO₄, 40 mM Na₂P₂O₇, 20 mM NaF, 30 mM HEPES/NaOH (pH 7.4), 1% Triton X-100, 1 mM EGTA, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml aprotinin], and cell lysates were centrifuged for

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**Figure 1.** O-GlcNAcylation in ovarian cancer cells is upregulated in response to ThiaG treatment and downregulated in response to OGT silencing. Protein expression levels were measured by western blotting in SKOV3 (A) and 59M (B) cells. GAPDH was used as an internal reference. ThiaG, Thiamet-G; OGT, O-GlcNAc transferase; Ctrl, control; siOGT, O-GlcNAc transferase small interfering RNA; O-GlcNAc, O-linked β-N-acetylglucosamine; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

**Figure 2.** O-GlcNAcylation regulates (A) migration and (B) invasion in SKOV3 and 59M ovarian cancer cells in *in vitro* Transwell assays. OGT was silenced with siOGT, and upregulated with ThiaG treatment. "P<0.01 vs. ctrl. O-GlcNAc, O-Linked β-N-acetylglucosamine; OGT, O-GlcNAc transferase; siOGT, O-linked β-N-acetylglucosamine transferase small interfering RNA; Ctrl, control; ThiaG, Thiamet-G.
10 min at 4°C. The cell extracts were then used for western blot analysis using MLC and p-MLC antibodies, as above.

Statistical analysis. All experiments were repeated at least three times. SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for analysis, and data were expressed as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Differential regulation of O-GlcNAcylation in ovarian cancer cells. Two human ovarian cancer cell lines, SKOV3 and 59M, were used to determine the involvement of O-GlcNAcylation in ovarian cancer. To alter the O-GlcNAcylation level, cells were transfected with OGT-targeting siRNA or treated with the OGA inhibitor Thiamet-G (5 µM) for 24 h. Western blot analysis revealed that OGT silencing decreased the level of global O-GlcNAc in SKOV3 (Fig. 1A) and 59M cells (Fig. 1B) compared with cells transfected with control siRNA. OGT protein expression levels were also visibly reduced in SKOV3 (Fig. 1A) and 59M cells (Fig. 1B) transfected with OGT siRNA compared with cells transfected with control siRNA. Thiamet-G treatment visibly increased global O-GlcNAc levels in SKOV3 (Fig. 1A) and 59M cells (Fig. 1B) compared with untreated control cells, indicating that it effectively inhibited OGA activity.

O-GlcNAcylation affects ovarian cancer cell migration and invasion. The effect of O-GlcNAcylation on ovarian cancer malignancy was investigated via in vitro analysis of cell...
migration and invasion using a Transwell assay. Transfection with OGT siRNA significantly decreased migration (SKOV3 cells, \(P=0.007\); 59M cells, \(P=0.009\); Fig. 2A) and invasion (SKOV3 cells, \(P=0.006\); 59M cells, \(P=0.008\); Fig. 2B) in OGT siRNA transfected cells compared with control siRNA transfected cells. However, Thiamet-G treatment significantly increased migration (SKOV3 cells, \(P=0.007\); 59M cells, \(P=0.009\); Fig. 2A) and invasion (SKOV3 cells, \(P=0.007\); 59M cells, \(P=0.006\); Fig. 2B) in treated cells compared with untreated controls. This indicates that a positive correlation exists between the intracellular global O-GlcNAcylation level and the motility of ovarian cancer cells.

**O-GlcNAcylation affects the RhoA/ROCK/MLC signal pathway.** It has previously been reported (22-27) that Rho GTPases are associated with cell motility, with RhoA stimulating ROCK and MLC to regulate these cellular events. To determine how O-GlcNAcylation modulates ovarian cancer cell motility, RhoA activity was detected by pull-down assay. The results revealed that Thiamet-G treatment-induced O-GlcNAcylation upregulation visibly enhanced RhoA activity at 3 and 6 h in SKOV3 and 59M cells compared with untreated control cells (Fig. 3A), while downregulation of O-GlcNAcylation induced by OGT silencing visibly reduced RhoA activity in SKOV3 and 59M cells compared with control cells (Fig. 3A). MLC phosphorylation is stimulated by RhoA through ROCK activation (25), so MLC phosphorylation was analyzed by western blotting. The results indicated that O-GlcNAcylation upregulation increased MLC phosphorylation in SKOV3 and 59M cells compared with untreated controls (Fig. 3B), and O-GlcNAcylation downregulation attenuated this phosphorylation in SKOV3 and 59M cells compared with control cells (Fig. 3B). This suggests that the RhoA/ROCK/MLC signal pathway may be closely associated with O-GlcNAcylation and the regulation of motility in ovarian cancer cells.

RhoA silencing reverses O-GlcNAcylation-induced cell motility. To determine whether O-GlcNAcylation affected ovarian cancer cell motility by targeting RhoA/ROCK signaling, RhoA was knocked down by RNAi and interference efficiency was assessed using RT-qPCR and western blot analysis to measure mRNA and protein expression levels, respectively. RhoA mRNA and protein expression levels were effectively decreased in SKOV3 cells transfected with RhoA siRNA compared with control cells (Fig. 4A and B, respectively). RhoA silenced and non-silenced cells were subsequently treated with or without Thiamet-G, and cell migration and invasion were evaluated by Transwell assay. Thiamet-G treatment resulted in a significant increase in migration and invasion compared with control cells in SKOV3 (\(P=0.005\) and \(P=0.006\), respectively; Fig. 4C and D, respectively) and 59M cells (\(P=0.009\) and \(P=0.005\), respectively; Fig. 4C and D, respectively). RhoA silencing significantly attenuated cell migration and invasion in SKOV3 (\(P=0.004\) and \(P=0.006\), respectively; Fig. 4C and D, respectively) and 59M cells (\(P=0.007\) and \(P=0.004\), respectively; Fig. 4C and D, respectively) compared with control cells. No significant difference was observed in migration or invasion between RhoA silenced cells and RhoA silenced cells treated with Thiamet-G (Fig. 4C and D, respectively). These findings suggest that RhoA is involved in the regulation of O-GlcNAcylation in ovarian cancer cell motility.

**Y-27632 inhibited O-GlcNAcylation-induced cell migration and invasion.** Y-27632 is able to effectively inhibit ROCK activity (38) and is often used in the investigation of the ROCK signal pathways (39). Therefore, Thiamet-G treated and untreated SKOV3 and 59M cells were treated with or without 50 µM Y-27632, and cell motility was analyzed. Thiamet-G treatment resulted in a significant increase in migration and invasion compared with control cells in SKOV3 (\(P=0.005\) and \(P=0.008\), respectively; Fig. 5) and 59M cells (\(P=0.007\) and \(P=0.003\), respectively; Fig. 5). Y-27632 treatment significantly inhibited cell migration and invasion in SKOV3 (\(P=0.006\) and \(P=0.004\), respectively; Fig. 5) and 59M cells (\(P=0.007\) and \(P=0.003\), respectively; Fig. 5) compared with control cells. No significant difference was observed in invasion or migration between Y-27632 treated and Thiamet-G+Y-27632 treated cells (Fig. 5). These results suggest that O-GlcNAcylation regulates ovarian cancer cell motility through the RhoA/ROCK signal pathway.

**Discussion**

Ovarian cancer exhibits the highest mortality of all gynecological malignancies due to a high rate of metastasis (17).
Metastasis is the final step in the progression of numerous solid tumors, and previous studies (40-42) have demonstrated that ovarian cancer cells often spread to distant sites including the lung, spleen, liver and bone aspirates, leading to increased complications and higher mortality rates. Therefore, the investigation of mechanisms associated with ovarian cancer is necessary to improve the diagnosis and treatment of ovarian cancer.

A growing body of evidence (12-16) has demonstrated that O-GlcNAcylation is a critical regulator of several human tumors and is associated with anchorage independent growth, proliferation, adhesion, migration and invasion in cancer cells, which are closely associated with tumor cell malignancy (3-5,8). Cellular migration and invasion particularly represent the metastatic ability of tumor cells. In the present study, O-GlcNAcylation upregulation was demonstrated to promote migration and invasion of ovarian cancer cells, whereas O-GlcNAcylation downregulation inhibited migration and invasion. This finding is supported by previous studies (12,13) regarding the involvement of O-GlcNAcylation in breast, lung and colon cancer progression.

High RhoA mRNA and protein expression levels have been reported in several human types of cancer, including bladder (28), gastric (29), breast (30), testicular (31) and ovarian (32) cancer. RhoA expression is also significantly increased in prostate cancer cells compared with normal prostate cells, contributing to aberrant cell growth, and knockdown of RhoA decreases prostate cancer cell viability and motility (43). ROCK affects the growth, formation, migration, invasion and metastasis of tumor cells by modulating cell stress-fiber and intercellular connection formation (28,44-51) and RhoA-mediated signaling pathways, particularly the RhoA/ROCK/MLC pathway, are involved in regulating cell motility (23,24,28). In order to explore the underlying molecule mechanisms behind O-GlcNAcylation modulation of motility in ovarian cancer cells, the present study investigated the RhoA/ROCK/MLC signal pathway. The data demonstrated that O-GlcNAcylation activated the RhoA/ROCK/MLC pathway by stimulating the formation of activated GTP-bound RhoA and MLC phosphorylation. Deficiencies in this pathway, mediated by either RhoA silencing or the inhibition of ROCK by Y-27632, blocked O-GlcNAcylation and induced increased migration and invasion. These results suggest that O-GlcNAcylation modulates motility in ovarian cancer cells by stimulating RhoA/ROCK/MLC signaling. However, RhoA activity is regulated by a variety of proteins. p27 regulates the activation of the RhoA/ROCK/MLC signaling pathway by binding with RhoA, which affects biological functions of the cell (52). p27-Rho is able to activate RhoA and induce invadopodia, thus regulating tumor cell invasion (53). RhoA/ROCK/MLC signaling is also activated by guanine-nucleotide exchange factor-H1 to regulate cell contractility (54). However, whether altered RhoA activity is the result of direct modification or an indirect effect of O-GlcNAc remains to be elucidated, with more study required.

In conclusion, O-GlcNAcylation enhanced RhoA/ROCK/MLC signaling, which promoted the migration and invasion of ovarian cancer cells. This finding suggests valuable novel targets to control metastasis, and lays a theoretical foundation for the diagnosis and treatment of ovarian cancer.

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