Silencing β-linked N-acetylglucosamine transferase induces apoptosis in human gastric cancer cells through PUMA and caspase-3 pathways

TI WEN¹, KEZUO HOU¹, ZHI LI¹, LU LI², HANJIE YU³, YUNPENG LIU¹, YANGGUANG LI²,4 and ZHINAN YIN⁴

¹Department of Medical Oncology, The First Hospital of China Medical University, Heping, Shenyang, Liaoning 110001, P.R. China; ²Wisconsin Institutes for Medical Research, University of Wisconsin, Madison, WI 53705, USA; ³Laboratory for Functional Glycomics, College of Life Sciences, Northwest University, Beilin, Xi’an, Shaanxi 710069; ⁴State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin 300071, P.R. China

Received May 11, 2015; Accepted July 27, 2015

DOI: 10.3892/or.2015.4276

Abstract. β-linked N-acetylglucosamine (GlcNac) is a monosaccharide that is catalyzed by O-GlcNAc transferase (OGT) to bind serine or threonine hydroxyl moieties of numerous nuclear and cytoplasmic proteins. Recent studies have shown that O-GlcNAcylation is elevated in various cancer types, which is associated with oncogenesis and tumor progression. However, whether OGT is expressed and/or plays a role in gastric cancer is unknown. In the present study, we used qPCR to determine that OGT mRNA levels are significantly elevated in gastric cancer tissues compared with that in corresponding adjacent tissues. In addition, in vivo silencing of OGT in nude mice suppressed tumor proliferation and decreased tumor burden. Furthermore, in vitro OGT knockdown induced more cell apoptosis through increasing PUMA and caspase-3 expression. We used a glycan-binding protein gene microarray to identify potential downstream target genes of OGT, and found that apoptosis-related genes such as galectin and HBEGF were decreased after OGT suppression, suggesting that OGT silencing induces apoptosis in gastric cancer tissues. We concluded that OGT plays a key role in gastric cancer proliferation and survival, and could be a potential target for therapy.

Introduction

O-GlcNAcylation (O-GlcNAc) is the covalent attachment of β-D-N-acetylglucosamine (GlcNAc) sugars to serine or threonine residues of nuclear and cytoplasmic proteins. The post-translational O-GlcNAc modification is reversible (1) and plays a critical role in regulating a wide panel of cellular processes, such as apoptosis, cell stress responses and signal transduction. O-GlcNAc transferase (OGT), an enzyme that transfers GlcNAc from uridine diphosphate (UDP) to serine/threonine residues of target proteins (2,3), induced elevated expression of O-GlcNAc in tissues.

OGT and O-GlcNAcylation plays an important role in normal biological process, aberrant regulation contributes to the development of wide range of diseases, including cancer. OGT and O-GlcNAcylation is elevated in breast cancer cell lines and tissues, particularly in metastatic lymph nodes. Also it has been demonstrated that O-GlcNAcylation could promote breast cancer tumorigenesis and metastasis (4,5). In colon and lung cancer, O-GlcNAcylation and OGT are also upregulated, compared with that in the corresponding adjacent tissues. Additionally, it demonstrated that O-GlcNAcylation enhanced cell growth and invasion, and may play important roles in lung and colon cancer formation and progression (6).

In laryngeal cancer, OGT and O-GlcNAcase (OGA) mRNA level was related to larger tumor size, nodal metastases, higher grader and tumor size, their protein level showed a trend of more advanced tumors to be more frequently OGT and OGA positive, suggesting that O-GlcNAcylation may have an effect on tumor aggressiveness (7).

Gastric cancer is the fourth most common cancer worldwide and the second most frequent cause of cancer-related death (8,9), with ~27% 5-year survival rate (10). Although elevated OGT levels were reported in various epithelial cancers, it remains unclear whether OGT is upregulated and how OGT exerts its function in gastric cancer. In the present study, we show that OGT mRNA levels are upregulated in human gastric cancer compared with that in adjacent tissues. Additionally, OGT function is analyzed. Silencing OGT inhibits BCG-823
cell proliferation in vitro, and reduces tumorigenicity in vivo. We demonstrated that OGT silencing induces more cell apoptosis by increasing PUMA and caspase-3 protein levels. Also, we screened the potential targets of OGT using GBP array, our data suggest that silencing OGT promotes apoptosis, which may also be through galectin and HBEGF.

Materials and methods

Sample. Human cancerous and adjacent normal parts of 7 gastric specimens were obtained from the First Hospital of China Medical University as frozen tissues. All tissue specimens were obtained with informed consent, and all investigations were approved by the local Ethics Committee. Each sample was divided into 2 parts, one for histopathological examination and the other was stored at -80°C for protein extraction.

Cell culture. Gastric cancer cell line BCG-823 was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS) in a 5% CO₂ atmosphere at 37°C.

Knockdown (KD) of OGT by shRNA. shRNAs targeting OGT were synthesized and inserted into pG-Pu6/GFP/Neo vector by GenPharm Co. (Shanghai, China). A scrambled sequence provided by GenPharm was used as a negative control (shNC). The shRNA target sequence of OGT is GGATGCTTATATCAGGAT (4). The shRNA was transfected into BGC-823 cells in 6-well culture plates using Lipofectamine™ 2000 (Invitrogen). Then, the first-strand cDNA was used to amplify reverse-transcribed using a First Strand cDNA Synthesis kit manufacturer's protocol. For each RNA sample, 1 µg was divided into 2 parts, one for histopathological examination and the other was stored at -80°C for protein extraction.

Reverse transcription (RT)-PCR and quantitative real-time PCR (qRT-PCR). Total RNA of tissues or cells was extracted using the RNeasy Mini kit (Qiagen), according to the manufacturer's protocol. For each RNA sample, 1 μg was reverse-transcribed using a First Strand cDNA Synthesis kit (Invitrogen). Then, the first-strand cDNA was used to amplify genes of interest with gene specific primers. The number of PCR cycles was optimized for each gene to ensure linear amplification. Gene-specific primers are as follows: OGT forward, 5'-TTGGCTTCTTGTGACATCTCAT-3' and OGT reverse, 5'-TTATCTTCTACCGACCGGCC-3'; galectin-2 forward, 5'-TCTGTTGGGACACACTTCTTCA-3' and reverse, 5'-TTATCTTTAATTGAGAAGAGGA; HBEGF forward, 5'-CTCACCTTTTGGTTTCTTAA-3' and reverse, 5'-GGAACTCACTTTCCCTTGTGTC-3'; GAPDH forward, 5'-ATGGGGAAGGTGAAGGTCG-3' and GAPDH reverse, 5'-GGAACTCACTTTCCCTTGTGTC-3'; galectin-2 forward, 5'-TTGCCTTCTGTGCATCCTCAT-3' and OGT reverse, 5'-TTGCCTTCTGTGCATCCTCAT-3'. Amplification. Gene-specific primers are as follows: OGT forward, 5'-ATGGGGAAGGTGAAGGTCG-3' and GAPDH reverse, 5'-GGAACTCACTTTCCCTTGTGTC-3'; galectin-2 forward, 5'-TTGCCTTCTGTGCATCCTCAT-3' and OGT reverse, 5'-TTGCCTTCTGTGCATCCTCAT-3'. Gene expression was normalized using GAPDH.

The amplification program consisted of one cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative quantity (RQ) of gene expression was normalized to GAPDH and performed using the 2ΔΔCt method.

Western blotting. Western blotting was performed as previously described (11). Briefly, total protein extracts of different cell lines were prepared using RIPA buffer (Beyotime). Then, 20 μg of total proteins for each sample was separated by SDS/PAGE (10% gels) and transferred to PVDF membrane (Millipore). The membranes were blocked in 5% skimmed milk in PBST for 2 h at room temperature. After blocking, the membrane was probed with primary antibodies against the proteins of interest. Finally, the proteins were further detected using the horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescence HRP substrate kit (Millipore). The primary rabbit anti-OGT and anti-O-GlcNAcylation were from Abcam. Caspase-3 and PUMA were from Cell Signaling. Mouse anti-β-actin (clone 6G3) was purchased from Tianjin Sungene.

Cell counting. Cells were treated with DMSO/OGT inhibitor (Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; Santa Cruz). Cell number was measured with an automatic cell counter according to the manufacturer's instructions. Briefly, cells were harvested and suspended, and mixed with equal volume of 0.4% trypan blue. Cell suspension (10 μl) was loaded onto TC20 system (Bio-Rad) counting slides, and the number of viable cells was quantified on a TC20 automated cell counter (Bio-Rad).

Xenograft. BALB/c nude mice (4 weeks old) were purchased from the laboratory animal center of the Academy of Military Medical Sciences. The maintenances and experimental animal procedures were approved by the Animal Ethics Committee of China Medical University. BALB/c nude mice (4 weeks old) were randomly assigned to two groups (7 mice/group). BGC-823 cells (shOGT and shNC) were trypanosed and resuspended at a final concentration of 1x10⁶ cells/ml in phosphate-buffered saline (PBS). Then, 100 μl of cells were injected subcutaneously into the right flank of the mice. Tumor growth was monitored twice every week and recorded by measuring tumor length and width daily for 4 weeks, and tumor volume was calculated using the formula: 1/2 (length x width²). After the experiments, mice were sacrificed and tumors weighed.

Apoptosis analysis. Cell apoptosis was performed using the allopheocyanin (APC)-Annexin V and 7-amino-actinomycin D (7-AAD) staining kit (Tianjin Sungene) following the manufacturer's instructions. Briefly, stable cell line (shOGT and shNC) was plated into a 6-well plate for 72 h with or without 5-fluorouracil (5-FU), cells were then trypsinized and washed twice with ice-cold PBS and then resuspended in binding buffer from the kit. APC-Annexin V and 7-AAD were added into the flow tube. Finally, flow cytometric analysis was performed within 1 h using FACS Calibur (BD Biosciences).

Glycan-binding protein (GBP) gene microarray. Total RNA was extracted as described above. RNA integrity was assessed by agarose gel electrophoresis and spectrophotometric analysis. tcRNA was obtained by linear amplification and labeled with Cy3/Cy5. Samples were hybridized to the array.
Data were analyzed by GenePix Pro 3.0. Differential expression analysis was cut-off at fold-change of ±1.3. Heatmap was produced using the R program.

Statistical analysis. Data are expressed as means ± SEM. Student's t-test was used to evaluate the significance of differences between sample means obtained from three independent experiments. Statistical significance was defined as P<0.05.

Results

OGT is upregulated in gastric cancer. Highly expressed OGT in several types of cancer has been reported, except in gastric cancer. In order to determine OGT expression in gastric cancer, initial analysis was performed comparing OGT expression levels between cancerous and paired adjacent non-cancerous tissue mRNA derived from the same patient with gastric cancer. Seven gastric cancerous and adjacent tissues were used. Quantitative PCR results revealed differential expression, although OGT mRNA was detectable in the normal tissues, OGT is overexpressed in all cancerous tissues in comparison to the normal groups (Fig. 1).

OGT KD in BGC-823 cells decreases cell proliferation. To investigate the function of OGT in gastric cancer, we used RNA interference strategy to knock down endogenous OGT gene. Stable cells of control and OGT silencing group were constructed with that in G418 selection. The efficiency of inhibition of OGT was confirmed by qPCR and western

Figure 1. mRNA levels of OGT in gastric cancer and the corresponding non-cancer tissues were determined by qPCR. The OGT mRNA expression measured by qPCR increased significantly from non-cancer to cancer tissues.

Figure 2. OGT silencing suppresses gastric cancer growth in vitro. (A) OGT silencing efficiency was measured by qPCR and western blotting. mRNA and protein levels were significantly reduced after OGT knockdown. (B and C) Cell growth curves of OGT expressed, and the unexpressed cells by cell counting and MTT assay. (D) Growth curve after treatment with DMSO/OGT inhibitor.
blotting. OGT mRNA and protein level were significantly reduced compared with that in control cells (Fig. 2A). We further analyzed whether the O-GlcNAcylation expression was changed after OGT silencing. As expected, silencing OGT led to significant reduction in global O-GlcNAcylation compared with control cells. Therefore these stable cells were used for following functional experiments. Proliferation of OGT KD cells was first assessed by cell counting and MTT assay. Both results showed that OGT silencing inhibited cell growth (Fig. 2B and C). In addition, to confirm this result, inhibitor specifically targeting OGT was used. Cell counting assay showed similar results (Fig. 2D). Taken together, these results indicated that OGT is required for gastric cancer cell proliferation.

Silencing OGT inhibits tumor progression in vivo. To examine the effects of OGT on tumorigenicity of BGC-823 cells in vivo and explored the therapeutic potential of OGT gene silencing in BGC-823, we compared the tumor growth in immunocompromised nude mice after shNC/shOGT cell inoculation. Tumor size was monitored twice weekly by cationic palpation. At the end of the experiments, animals were sacrificed and the tumors weighed. As shown in Fig. 3A, silencing OGT could significantly suppress tumor growth. The tumors harvested
from the OGT KD group also weighed less (Fig. 3B), these suggesting that OGT could be a therapeutic target in gastric cancer.

**Suppression of OGT induces cell apoptosis.** To investigate whether the inhibition of proliferation in OGT KD cells was due to cell apoptosis, we used APC-Annexin V/7-AAD double staining kit followed by FACS analysis. As shown in Fig. 4A, percentage of APC-Annexin V+/7-AAD− (early apoptosis) and APC-Annexin V+/7-AAD+ (late apoptosis) cells markedly increased after OGT silencing, which indicated that OGT has an anti-apoptosis role in gastric cancer cells. In addition, OGT reversed anti-chemotherapeutic drug-mediated apoptosis. If shOGT/shNC cells were treated with 5-FU, silencing OGT could increase apoptosis, which suggested elevated level of OGT in gastric cancer may be one of the drug-resistant mechanisms.

**Silencing OGT induces cell apoptosis through upregulation of PUMA and caspase-3.** As silencing OGT promoted cell apoptosis, PUMA and caspase-3 were detected by western blotting. Data showed that PUMA and cleaved caspase-3 were significantly upregulated after OGT silencing. In order to examine the downstream genes of OGT, GBP gene microarray was performed. Ninety genes were assessed in this array, they were well known as glycan-binding protein genes based on the database of functional glycomics and uniprot. RNA from shNC/shOGT was investigated. As shown in Fig. 5B, many apoptosis-related genes were changed after OGT silencing. Of these, HBEGF were most significantly reduced, whereas galectin 2 and galectin 7 were increased after OGT suppression. qPCR was used for validation, as shown in Fig. 5C. These genes have apoptotic-related function indicating that OGT KD may target these proteins to modulate PUMA and caspase-3.

**Discussion**

O-GlcNAc is the covalent addition of a GlcNAc moiety to serine/threonine residues of cytosolic and nuclear proteins. OGT could transfer GlcNAc form UDP-GlcNAc to substrate proteins, whereas O-GlcNAcase (OGA) could remove GlcNAc. For O-GlcNAcylation and OGT, the expression level has been examined in various types of cancer, including breast (4,5,12), prostate (13), lung, colorectal (6), liver (14,15) and non-solid cancers such as chronic lymphocytic leukemia (15). However, the role of OGT in gastric cancer was not reported.

The present study was designed to investigate the expression and function of OGT in gastric cancer. We observed the elevated expression of OGT at mRNA level in gastric cancerous tissues compared with that in adjacent tissues. Silencing OGT decreased cell proliferation both in vitro and in vivo due to apoptosis induction. The reduction of OGT results in pro-apoptosis effect in the presence or absence
of 5-FU. In addition, PUMA and caspase-3 were increased after OGT KD. Furthermore, the GBP array results showed expression change of various apoptosis-related genes after OGT KD, pointing to a tumor genesis function of OGT in gastric cancer.

In order to determine the level of OGT in gastric cancer, the mRNA level in 7 cases of gastric cancer and adjacent non-cancer tissues was measured by qPCR. The results showed that OGT is overexpressed in all cancer tissues when compare with that in non-cancerous tissues. Due to the limit number of samples, the protein level and whether OGT is associated with different stages and patient survival are unknown, which need to be further studied.

Several studies have shown that O-GlcNAcylation plays a key role in cell growth, division and invasion. In ES cells, OGT deletion is lethal (16), and OGT tissue-specific mutation results in the loss of O-GlcNAcylation in specific tissues and OGT tissue-specific deletion is lethal (16), and OGT tissue-specific mutation plays a critical role in cell growth, division and invasion. In ES cells, OGT KD was activated by survival signals (21). To determine which critical apoptosis proteins were involved in OGT KD inducing apoptosis, we used a glycan-binding protein gene array downstream of OGT. The results showed many genes were altered after OGT KD, pointing to a tumor genesis function of OGT KD in drug-resistance.

Whereas, OGT KD also increased the sensitivity of 5-FU on BCG-823 suggesting that cancer cell could use the anti-apoptosis effect of OGT in drug-resistance.

In summary, the present study has shed light on the expression and function of OGT in gastric cancer. Our data showed that OGT is overexpressed in gastric cancer tissues. Silencing OGT inhibited cell growth in vitro and in vivo, due to inducing cell apoptosis. Importantly, we showed that PUMA and caspase-3 were upregulated after OGT silencing, which is the mechanism of OGT suppression-induced apoptosis. In addition, we found various candidate genes downstream of OGT. Further studies need to be carried out, to investigate whether these genes were associated with apoptosis in OGT KD cancer cells. Collectively our results suggested that OGT is an oncogene in gastric cancer.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (no. 31300743), the Fund of the Education Department of Liaoning province (no. L2012278) and Science and Technology Plan Project of Liaoning Province (no. 2011404013-1, 2012225001, 2014225013).

References


