Silencing GnT-V reduces oxaliplatin chemosensitivity in human colorectal cancer cells through N-glycan alteration of organic cation transporter member 2

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Abstract. Organic cation transporter member 2 (OCT2) is an N-glycosylated transporter that has been shown to be closely associated with the transport of antitumor drugs. Oxaliplatin, a platinum-based drug, is used for the chemotherapy of colorectal cancer (CRC). However, oxaliplatin resistance is a major challenge in the treatment of advanced CRC. The aim of the present study was to better understand the mechanism underlying the chemosensitivity of CRC cells to oxaliplatin. The present study describes a potential novel strategy for enhancing oxaliplatin sensitivity involving the glycosylation of this drug transporter, specifically the modification of β-1,6-N-acetylglucosamine (GlcNAc) residues by N-acetylglucosaminyltransferase V (GnT-V). The results revealed that the downregulation of GnT-V inhibited the oxaliplatin chemosensitivity of CW-2 cells. Furthermore, the knockdown of GnT-V caused a marked reduction in the presence of β-1,6-GlcNAc structures on OCT2 and decreased the localization of OCT2 in the cytomembrane, which were associated with a reduced uptake of oxaliplatin in wild-type and oxaliplatin-resistant CW-2 cells. Overall, the study provides novel insights into the molecular mechanism by which GnT-V regulates the chemosensitivity to oxaliplatin, which involves the modulation of the drug transporter OCT2 by N-glycosylation in CRC cells.

Introduction

Colorectal cancer (CRC) is one of the most common types of malignant tumor, with an increasing rate of incidence worldwide (1). According to cancer statistics for China, the incidence and mortality rates of CRC are third and fifth, respectively, among all types of cancer, which indicates that CRC is a major cause of cancer-associated deaths in China (2). At present, surgical resection followed by oxaliplatin-based chemotherapy is among the most frequently used therapeutic strategies (3,4). However, the long-term administration of oxaliplatin is associated with drug resistance, which has been a major barrier to the efficacy of CRC treatment (5,6). Thus, improving chemosensitivity to oxaliplatin is an urgent requirement for successful CRC therapeutic intervention.

Chemoresistance frequently compromises the function of antitumor drug transporters (7,8). Studies have revealed that the level of the transporter organic cation transporter member 2 (OCT2) is critical for determining the uptake of platinum compounds, particularly oxaliplatin (9,10). OCT2 is N-glycosylated, and although previous studies have demonstrated that N-linked glycosylation is important for the transport function of OCT2 (11), systemic and complete information is not yet available on the association of N-glycans with chemosensitivity.

The enzyme N-acetylglucosaminyltransferase V (GnT-V), encoded by the gene MGAT5 (12), catalyzes the formation of β-1,6 branched complex N-glycans (13). Previous studies have shown that aberrant N-glycans expressed by tumor cells are associated with chemotherapeutic drug sensitivity (14,15). Despite research advances regarding the effects of glycosylation on chemosensitivity, the role of GnT-V in CRC development and chemoresistance remains poorly understood.

Our previous study demonstrated that GnT-V promotes the chemosensitivity of bladder cancer cells to gemcitabine (16); however, the mechanism underlying the effects of β-1,6-N-glycosylation on chemosensitivity was not defined. In the present study, in order to further investigate the potential effects of GnT-V on chemosensitivity in CRC cells, shRNA-mediated GnT-V silence was accomplished in CW-2 and CW-2/R (oxaliplatin-resistant) cells, through which
the alteration of oxaliplatin cytotoxicity was systematically explored. Furthermore, N-glycan branches, distribution and transport activity of OCT2 were observed to identify the target substrate of GnT-V. The present results are expected to provide insights into the molecular mechanisms underlying the oxaliplatin chemosensitivity in CRC.

Materials and methods

Reagents and antibodies. Oxaliplatin and cimetidine were purchased from Sigma-Aldrich (Merck KGaA). Zosuquidar was obtained from MedChemExpress. DMSO and DAPI were purchased from Beyotime Institute of Biotechnology. Rabbit anti-OCT2 monoclonal antibody (cat. no. ab179808), mouse anti-MGAT5 (GnT-V) monoclonal antibody (cat. no. ab87977), rabbit anti-β-actin polyclonal antibody (cat. no. ab119716) and goat polyclonal secondary antibody to rabbit IgG (Alexa Fluor® 555; cat. no. ab150078) were purchased from Abcam. Goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (cat. no. 7074) and goat anti-mouse IgG HRP-conjugated (cat. no. 7076) antibodies were obtained from Cell Signaling Technology, Inc.

Cell culture. Human CRC cell lines were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. CW-2 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.), and HT29 and HCT 116 cells were cultured in McCoy’s 5A medium (Biological Industries). Each growth medium was supplemented with 10% fetal bovine serum (Beijing Solarbio Science & Technology Co., Ltd.) and 1% penicillin/streptomycin, and all cells were grown at 37˚C with 5% CO₂. The oxaliplatin-resistant CW-2 (CW-2/R) and HT29 (HT29/R) cell lines were developed by exposure to increasing concentrations of oxaliplatin, as previously described (17).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cultured cells using RNAsiso Plus (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Then, 1 µg total RNA was used for reverse transcription with HiScript II Q RT SuperMix (Vazyme Biotech Co., Ltd.) according to the manufacturer’s protocol. The ChamQ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd.) was used for qPCR. Primer sequences were as follows: MGAT5, 5'-ATCATGCAAATT ATGCCCCAATC-3' (forward) and 5'-GGTGCTGCTCAA CCACCAA-3' (reverse); GAPDH, 5'-CATGAGAAGTAT GACAACGCCT-3' (forward) and 5'-AGTCTTCCACG ATACCAAA-3' (reverse). PCR conditions were: 10 min at 95˚C, followed by 40 cycles of 10 sec at 95˚C and 30 sec at 60˚C. Fold change was calculated by determining the ratio of mRNA levels to control values using the Δ threshold cycle (Cq) method (2^(-ΔΔCq)) (18).

Western blot and lectin blot analysis. Total protein was isolated from cultured cells with a Qproteome Mammalia Protein Prep Kit (Qiagen China Co., Ltd.) according to the manufacturer’s protocol. Protein concentration was measured using BCA Protein Assay reagent (Beyotime Institute of Biotechnology). Then, 10-30 µg protein/lane was separated using 10% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were then blocked for 2 h at room temperature with 5% non-fat dried milk. The membranes were then incubated with anti-OCT2 (dilution 1:1,000), anti-GnT-V (dilution 1:200) and anti-β-actin antibodies overnight at 4˚C. For lectin blot assay, blocked membranes were incubated with biotinylated phytohemagglutin-L (PHA-L) lectin (dilution 1:400; Vector Laboratories, Inc.) for 1 h at room temperature. Subsequent to washing three times (15 min per wash) with Tris-buffered saline and 0.05% Tween-20, the blots were incubated with HRP-conjugated secondary antibodies (1:5,000) for 2 h at room temperature. Results were visualized using an ECL Kit (Cytiva) and the signal intensity was measured using a VersaDoc™ Imaging system (version 4.0; Bio-Rad Laboratories, Inc.).

Chemosensitivity assay. To evaluate chemosensitivity to oxaliplatin, cell death and cell viability assays were performed following protocols previously described (16). In brief, 3,000 to 4,000 cells per well were seeded in 96-well plates and treated with oxaliplatin (ranging from 0.375-24 µg/ml). For the functional detection of OCT2 and P-gp, cells were incubated at 37˚C in the medium with cimetidine (100 µM) and zosuquidar (5 µM) for 1 h before exposure to oxaliplatin (0.2 µg/ml for CW-2 group and 2 µg/ml for CW-2/R group). Plates were incubated for 72 h at 37˚C. The percentage of dead cells was measured using the trypan blue method and cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology).

Stable transfection and cell line selection. Short hairpin RNAs (shRNAs) for the knockdown of GnT-V (shRNA#1 and 2) were designed and inserted into the pGPU6/GFP/Neo vector by Suzhou GenePharma Co., Ltd. Non-targeting shRNA sequences were used as a negative control (NC). For transfection, (5x10⁵ cells/well) were seeded into 6-well culture plates at 60-70% confluence. After 24 h, vectors (10 µg) for the knockdown of GnT-V (shRNA#1 and 2) were mixed with 4.5 µl Attractene Transfection Reagent (Qiagen China Co., Ltd.) in 100 µl serum-free RPMI-1640 medium at room temperature for 15 min. Then, the transfection complexes were added to each well with fresh medium (containing serum and antibiotics) and incubated at 37˚C with 5% CO₂ for 48 h. Polyclonal stable cell lines were isolated following fluorescence-activated cell sorting using a FACSCalibur instrument (BD Biosciences).

Colony formation assay. To analyze the sensitivity of cells to oxaliplatin, a colony formation assay was performed as described previously (19). Cells were plated at 50% confluence (1.0x10⁶ cells in a 10-cm dish) and treated with oxaliplatin (0.2 µg/ml for CW-2 group and 2 µg/ml for CW-2/R group) for 12, 24 and 48 h. Cells were then trypsinized and diluted in 6-well plates (1,000 cells/well). After plating, cells were grown in oxaliplatin-free medium for 2 weeks. Colonies were stained with 1% crystal violet at room temperature for 30 min. Photographic images of the dishes were captured.

Lectin precipitation. Cells were harvested in RIPA lysis buffer (Beyotime Institute of Biotechnology), containing
protein inhibitor (1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin; Beyotime Institute of Biotechnology). The total cell lysates were centrifuged at 12,000 x g for 15 min at 4°C. In total, 300 μg cell lysates were incubated with 30 μl PHA-L conjugated agarose beads (Vector Laboratories, Inc.) at 4°C overnight. The resulting β-1,6-glycosylated protein-lectin-agarose complexes were collected by centrifugation (13,000 x g for 30 sec at 4°C) and then washed with lysis buffer. Next, the glycoprotein/lectin conjugates were resolved by 10% SDS-PAGE and immunoblotted for OCT2 by the aforementioned western blot analysis method.

**Immunofluorescence staining.** Cells (1x10^5) were seeded in 6-well dishes and cultured for 48 h. Cells were then washed twice with PBS and fixed with acetone for 10 min at room temperature. Next, the cells were blocked with 3% BSA-PBS (Beyotime Institute of Biotechnology) for 1 h at room temperature following permeabilization with 0.1% Triton X-100 for 5 min. Cells were then incubated with OCT2 (1:200) antibody for 2 h at room temperature, followed by Goat Anti-Rabbit (Alexa Fluor® 647) secondary antibody (1:200; Abcam; cat. no. ab150079) incubation for 1 h at room temperature. DAPI was used to stain the cell nuclei at room temperature for 3 min. Fluorescent signals were detected using a fluorescence confocal microscope (Olympus Corporation; magnification, x400).

**Drug transportation analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).** Cells and cultured medium from each cell group (CW-2 and CW-2/R cells transfected with shRNA#2 or NC) were harvested after exposure to 1 µM oxaliplatin for 0.5, 1 and 1.5 h at 37°C. The cells were washed twice with PBS and then crushed in 120 μl ice ddH₂O by an ultrasonic crusher for 10 sec. 120 μl methyl alcohol was added to the cell lysates. Supernatant was extracted after the lysates were centrifuged at 15,000 x g at 4°C for 15 min. Then, 10 μl each sample was injected into an LC-MS/MS system (Agilent HP1200; Agilent Technologies, Inc.). The chromatographic separation was performed on a Hypersil BDS-C18 column 150 mm x 4.6 i.d., 5 μm (Dalian Elite Analytical Instruments Co., Ltd.) at room temperature. The mobile phase consisted of acetonitrile and water with 0.1% formic acid (70:30, v/v) for digoxin at a flow rate of 0.5 ml/min. The ionization was conducted using a Turboionspray interface in positive ion mode for bestatin and in negative ion mode for digoxin. Multiple reactions monitoring mode was utilized to detect the compound of interest. The selected transitions of m/z were m/z 779.0 to 649.0 for digoxin, m/z 309.1 to 120.3 for bestatin and m/z 370.4 to 288.1 for cilostazol. The intracellular oxaliplatin concentration was measured in each sample according to a standard protocol (20).

**Statistical analysis.** Data were analyzed using SPSS software 16.0 (SPSS, Inc.). Results are expressed as the mean ± SEM. Each experiment was repeated at least three times and analyzed by either a Student's t-test or one-way ANOVA followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*High GnT-V is associated with a good response to oxaliplatin in CRC cells.* To begin the exploration of the oxaliplatin sensitivity of CRC cells, the GnT-V expression levels of three different human CRC cell lines were determined using RT-qPCR (Fig. 1A) and western blotting (Fig. 1B). Among the three CRC cell lines, CW-2 cells exhibited the highest expression of GnT-V and the highest cytotoxic response to oxaliplatin (Fig. 1C). We hypothesized that if GnT-V activity is important for the chemosensitivity of CRC cells, alteration of endogenous GnT-V expression levels should occur when cells are treated with oxaliplatin. To investigate this hypothesis, cells were exposed to 1.5 μM oxaliplatin for 24 or 48 h. As shown in Fig. 1D, this short-term oxaliplatin treatment did not lead to changes in the GnT-V level in either CW-2 or HT29 cells. Furthermore, two stable oxaliplatin-resistant cell lines (CW-2/R and HT29/R) were generated by exposure to continuous oxaliplatin treatment. These stable resistant cell lines were then subjected to RT-qPCR to quantify MGAT5 gene expression. As shown in Fig. 1E, a marked and significant increase of MGAT5 expression was observed in oxaliplatin-resistant cells as compared with the respective wild-type CW-2 and HT29 cells. Consistent with this increased MGAT5 expression, increased GnT-V protein levels and robust enrichment of β-1,6-oligosaccharide levels were observed in the resistant cell lines by western blotting and lectin blotting, respectively (Fig. 1F). These results suggest that GnT-V affects the chemosensitivity of CRC cells to oxaliplatin.

*Downregulation of GnT-V reduces the sensitivity of CW-2 cells to oxaliplatin.* GnT-V expression was stably knocked down in the CW-2 and oxaliplatin-resistant CW-2/R cell lines using shRNA. Successful knockdown was verified by western blotting, which showed decreased GnT-V levels in the cells transfected with shRNA#1 and 2 as compared with the wild-type and negative control (NC) cells. Correspondingly, reduced GnT-V activity in the shRNA#1 and 2 cells compared with the NC cells was confirmed by oligosaccharide analyses in cells stained with PHA-L, a lectin that specifically binds to surface β-1,6-N-acetylgalactosamine (GalNAc) branches (Fig. 2A and B). To evaluate the role of GnT-V in chemosensitivity, NC and shRNA#2 transfected cells were exposed to different concentrations of oxaliplatin for 48 h, and then cell viability was determined by CCK-8 assay. As shown in Fig. 2C, the knockdown of GnT-V increased the viability of oxaliplatin-treated CW-2 cells, even at the highest concentration of oxaliplatin (16 μg/ml) compared with the NC group, and a similar result was observed in the CW-2/R group. Moreover, a colony formation assay was performed (Fig. 2D and E). Notably, oxaliplatin-treated shRNA#2 transfected cells exhibited increased survival and clonogenic potential compared with the NC controls. These results suggest that GnT-V knockdown attenuates the chemosensitivity of cells to oxaliplatin.

β-1,6-N-glycan branches on OCT2 may affect cytotoxic response to oxaliplatin. OCT2 has been indicated to be an independent factor that affects the success of oxaliplatin-based chemotherapy in CRC (10). We hypothesized that the N-glycans of OCT2 may play an important role in the chemosensitivity
of CRC cells to oxaliplatin. To investigate this hypothesis, first, OCT2 expression levels were determined by western blotting. As shown in Fig. 3A and B, the expression level of OCT2 protein was almost unchanged in CW-2 and CW-2/R cells following GnT-V knockdown. To confirm the role of OCT2 in chemosensitivity to oxaliplatin, transfected cells were treated with cimetidine, an inhibitor of OCT2 activity, before exposure to oxaliplatin (21). Notably, the pretreatment of CW-2 and CW-2/R cells with cimetidine significantly increased cell viability, suggesting that the reduction of OCT2 activity reduced the cytotoxic effects of oxaliplatin (Fig. 3C).

To further investigate the association between GnT-V activity and OCT2 N-glycosylation, a PHA-L precipitation experiment was performed. The results indicate that the presence of β-1,6-N-glycan branches on OCT2 was decreased in the GnT-V knockdown group compared with the NC group (Fig. 3D). This suggests that N-glycosylated OCT2 may be a potential substrate of GnT-V and could play a part in the modulation of the chemosensitivity of CRC cells to oxaliplatin.

The possible involvement of another substrate, P-glycoprotein (P-gp), a membrane glycoprotein known to regulate drug sensitivity in cancer cells was also evaluated (22,23). Cells were treated for 24 h with the P-gp inhibitor zosuquidar in combination with oxaliplatin. However, in contrast to OCT2, zosuquidar had no effect on oxaliplatin sensitivity (data not shown).
GnT-V knockdown changes the distribution and function of OCT2. The abnormal N-glycosylation of transporters has been shown to alter their localization and activity in cells (24). Therefore, the intracellular distribution of OCT2 in CRC cells was examined using confocal immunofluorescence microscopy. A decreased localization of OCT2 was observed in the cytomembrane of the CW-2 and CW-2/R/shRNA cells with GnT-V knockdown compared with the respective NC groups (Fig. 4A and B). Subsequently, the transport activity of OCT2 was detected by LC-MS/MS. The uptake of oxaliplatin in the CRC cells exhibited no significant difference between all groups at an early stage (<0.5 h incubation). However, after another 1 h, the concentration of oxaliplatin in the CW-2/R cells was lower than that in the respective non-resistant CW-2 cells, and the oxaliplatin concentration decreased significantly in the GnT-V knockdown groups when compared with respective wild-type CW-2 and CW-2/R groups (Fig. 4C), indicating that OCT2 transport activity was reduced when GnT-V was knocked down. Together, these data imply that GnT-V might affect chemosensitivity to oxaliplatin via regulation of the oligosaccharide branches on OCT2.

Discussion

Glycosylation participates in various biological processes, including cell adhesion, signal transduction and receptor activation (25-27). Previous studies suggest that N-glycosylation serves an important role in tumor chemosensitivity (28-30). The present study identified GnT-V as a regulator of the
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The results demonstrated that higher endogenous GnT-V expression was positively associated with increased sensitivity to oxaliplatin in CRC cells. Additionally, oxaliplatin-resistant CRC cells exhibited elevated levels of GnT-V expression relative to those in the parental oxaliplatin-naïve cells, while short-term oxaliplatin treatment did not increase GnT-V levels. Therefore, it is speculated that GnT-V might serve an important role in the chemosensitivity of CRC cells.

CRC is a common malignant tumor of the digestive tract, and its mortality rate is the second highest among tumor-associated deaths worldwide (31). Oxaliplatin is one of the most commonly used chemotherapeutics after surgical resection due to its improved safety profile and lack of cross-resistance with cisplatin (32). Unfortunately, intrinsic (or de novo) and acquired oxaliplatin resistance are considered to be major challenges in the treatment of CRC (33). Therefore, elucidating the underlying mechanisms of the resistance and exploring biomolecules that can reliably predict the response to oxaliplatin are clinical priorities. The results of the present study demonstrated that the downregulation of GnT-V reduced the sensitivity of CW-2 and oxaliplatin-resistant CW-2/R cells to oxaliplatin.

Chemosensitivity to oxaliplatin depends, at least in part, on the concentration of the drug inside cells. Therefore, facilitating increases in intracellular oxaliplatin accumulation through membrane transporters is an important mechanism for enhancing the efficacy of oxaliplatin. An investigation into the drug transporter expression of OCT2 was performed in the present study. The results showed almost no change in OCT2 expression in CW-2 and CW-2/R cells following GnT-V knockdown. Therefore, we speculate that a possible mechanism by which OCT2 affects the sensitivity of CRC cells to oxaliplatin in associated with its N-glycosylation.

OCT2 is a drug transporter with three N-glycosylation sites in the long extracellular loop between transmembrane domains 1 and 2. Changes in N-glycosylation can markedly affect the stabilization and localization of cell membrane glycoproteins, thus regulating cell differentiation, signal transduction and cell behaviors (34,35). The enzyme GnT-V can affect the biological function of glycoproteins via the modulation of β-1,6-GlcNAc branches (36,37). To date, studies have shown that the expression and N-glycosylation of OCT2 are able to increase oxaliplatin intake (38,39). The present study explored the potential mechanisms underlying OCT2-mediated chemosensitivity in CRC by focusing on the β-1,6-GlcNAc branches of OCT2. OCT2 was identified as a functional target of GnT-V in the regulation of chemosensitivity. It was found that the number of β-1,6-N-glycan branches decreased on OCT2 by silencing GnT-V expression, and led to an apparent translocation of OCT2 from the cell membrane to the cytoplasm in both CW-2 and CW-2/R cells.

Figure 4. β-1,6-N-glycan branches on OCT2 alter the distribution and function of OCT2 in CRC cells. (A) and (B) The distribution of OCT2 was observed using immunofluorescent staining, and the fluorescence intensity was visualized using a fluorescence microscope (magnification, x400). Less OCT2 accumulated on cell membranes in the GnT-V knockdown cells compared with the negative control cells. (C) The transport activity of OCT2 was detected using liquid chromatography-tandem mass spectrometry. The uptake of oxaliplatin in CRC cells represents OCT2 activity. Data are presented as the mean ± SEM from three independent assays. *P<0.05. OCT2, organic cation transporter member 2; CRC, colorectal cancer; GnT-V, N-acetylglucosaminyltransferase V; shRNA#2, short hairpin RNA for knockdown of GnT-V; OXA, oxaliplatin.
It is likely that N-glycan modifications may have an impact on other proteins that affect drug chemosensitivity. As a well-known drug resistance-associated protein, P-gp is a heavy N-glycosylated transmembrane transporter that can pump anticancer drugs out of cells in a reverse concentration gradient, ultimately mediating drug resistance (40,41). Notably, the differential β-1,6-glycosylation of P-gp by GnT-V was not observed in the present study (data not shown). Intriguingly, preliminary experiments suggested that blocking the activity of P-gp by zosuquidar (a specific P-gp inhibitor) increased the survival of cells when treated with oxaliplatin and eliminated the effect of P-gp on the oxaliplatin resistance of CRC cells (data not shown). Therefore, further study is needed to investigate this.

In summary, the present study demonstrated for the first time that OCT2 is a target substrate of GnT-V, and that its distribution and function were affected by the downregulation of GnF-V. The collective results presented in this study add to the body of evidence pointing to an important function for GnT-V-mediated β-1,6-glycosylation in the resistance of CRC to oxaliplatin chemotherapy. Therefore, the present study may provide an experimental basis for clinical individualized chemotherapies.

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Availability of data and materials

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

Authors’ contributions

All authors contributed to study conception. The study was designed by JF and ZS. Material preparation, data collection and analysis were performed by XC, SF, XL and XD. The first draft of the manuscript was written by XC and ZS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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