Knockdown of β3GnT8 reverses 5-fluorouracil resistance in human colorectal cancer cells via inhibition the biosynthesis of polylactosamine-type N-glycans

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Abstract. Aberrant glycosylation is known to be associated with cancer chemoresistance. β-1,3-N-acetyl-glucosaminyltransferase (β3GnT)8, which synthesizes polylactosamine on β1-6 branched N-glycans, is dramatically upregulated in colorectal cancer (CRC). 5-Fluorouracil (5-FU) resistance remains a major obstacle to the chemotherapy of CRC. However, little is known with regard to the correlation between 5-FU resistance and the expression of β3GnT8 in CRC. In this study, a 5-FU-resistant cell line (SW620/5-FU) was generated, and 50% inhibition concentration (IC50) of 5-FU was determined by MTT assay. Flow cytometry and lectin blot analysis were performed to detect the alteration of polylactosamine structures. Quantitative RT-PCR and western blot analysis were used to identify and evaluate candidate genes involved in the synthesis of polylactosamine in SW620/5-FU cells. We found polylactosamine chains were significantly increased in SW620/5-FU cells. Inhibition of the biosynthesis of polylactosamine by 3'-azidothymidine (AZT) was able to reduce 5-FU tolerance. Further studies showed that β3GnT8 expression was also upregulated in 5-FU-resistant cancer cells, and knockdown of β3GnT8 by RNA interference reversed 5-FU resistance through, at least partly, by suppressing the formation of polylactosamine. In conclusion, the alteration of β3GnT8 in CRC cells correlates with tumor sensitivity to the chemotherapeutic drug and has significant implication for the development of new treatment strategies.

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Introduction

Colorectal cancer (CRC) is the second most common cause of death among cancer patients in the developed world and the third most common malignancy in the United States (1). Other than surgery, treatment for CRC patients relies primarily on chemotherapy, especially the patients with advanced CRC. In clinic, application of 5-fluorouracil (5-FU) makes a great contribution to the improvement of the life quality and overall survival of CRC patients (2). A group of patients also suffer cancer recurrence or metastasis when the standard treatment has been completed or even during the course of post-surgical chemotherapy, suggesting a development of 5-FU resistance. Drug resistance, whether intrinsic or acquired, is believed to cause treatment failure in >90% of patients with metastatic cancer (3). Clearly, if drug resistance could be overcome, the impact on survival would be highly significant. Altered regulation of nucleotide metabolism, amino acid metabolism, cytoskeleton organization, transport, and oxygen metabolism have been reported to confer 5-FU resistance (4). For example, high-level expression of hENT1 correlated with poor clinical response to 5-FU among CRC patients (5). BAX downregulation could contribute as an important factor during cell resistance to 5-FU in colon cancer (6). Although some progress has been achieved in the past 50 decades, much more efforts are still needed to resolve 5-FU resistance in CRC.

Glycosylation is one of the most abundant post-translational modifications found on more than half of all secreted and cellular proteins. Most protein glycosylation is either Asn-linked or initiated by O-linked GalNAc added to Ser or Thr (7). Glycans on glycoproteins mediate a dynamic protein state, involving folding, quality control, secretion and catabolism. N-glycans are also related to tumor progression and metastasis as well as to immune system activity, and their potential relationship to chemoresistance has recently been examined (8). Zhang *et al* showed that N-glycomic alterations were associated with adriamycin resistance in human leukemia (9). Increased levels and defective N-glycosylation

of multidrug resistance-associated proteins (MRPs) in ovarian carcinoma cells resistant to oxaliplatin have also been reported (10). In addition, N-glycans bearing a β-1-6-linked GlcNAc branch are consistently elevated in concert with increased expression of Mgat5, and direct correlation has been made between Mgat5 overexpression and enhanced drug resistance (11). Swainsonine, an inhibitor of N-glycan biosynthesis, could reduce 5-FU tolerance of CRC cells (12). Taken together, these studies indicate the existence of differences between sensitive and resistant cells in the content and composition of N-glycans.

Polylactosamine is a linear carbohydrate polymer composed of alternating GlcNAc and Gal residues involved in cellular functions ranging from differentiation to metastasis (13). It can be incorporated into either N-linked or mucin-type O-linked glycans (Fig. 1). It is well known that β-1-6-branched N-glycans serve as most preferred sites for addition of polylactosamine (14). Polylactosamine is synthesized by the alternative action of a β-1,4-galactosyltransferase (β4GalT) and a β-1,3-N-acetylglucosaminyltransferase (β3GnT) (15). β3GnT8, which was the most recently identified enzyme among the β3GnTs, is involved in the biosynthesis of polylactosamine on tetraantennary (β1,6-branched) N-glycans. Ishida *et al* showed that most of the cell lines established from CRC expressed higher levels of the β3GnT8 transcript (16). Recently, β3GnT8 has been reported to be associated with cancer chemoresistance (9). Therefore, it is of interest to clarify the relationship between β3GnT8 expression and drug resistance in CRC.

In this study, a 5-FU-resistant CRC cell line was established from the parental cell line SW620, and the role of β3GnT8 in alteration of 5-FU resistance and the possible pathways involved were investigated by RNA interference-based approaches.

Materials and methods

Cell line generation. Human SW620 CRC cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) in a humidified atmosphere with 5% CO_2 at 37˚C. To establish the drug-resistant cell subline SW620/5-FU, SW620 cells were exposed to stepwise increasing 5-FU (Sigma, St. Louis, MO, USA) concentrations from 10 to 100 μg/ml. SW620/5-FU cells were incubated for 1 week in drug-free medium prior to their use in each experiment.

MTT assay. Cells were seeded in 96-well plates at a density of $5.0x10^3$ cells/ml. After treatment with the indicated methods, medium was removed and 50 μl of MTT (Sigma) was added to each well. Then the cells were incubated in the dark at 37˚C for an additional 4 h. The reaction was stopped by the addition of 150 μl DMSO (Sigma) and the absorbance of samples at 570 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The chemosensitivity of 5-FU was expressed as 50% inhibition concentration (IC50). Cell viability was assessed after 48 h of exposure to 10-100 μg/ml of 5-FU. The IC50 value calculation was performed using GraphPad Prism 5.0 software.

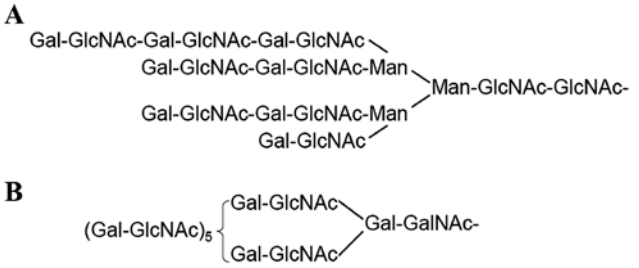

Figure 1. Typical structures of polylactosamine-type glycans. (A) N-glycans and (B) O-glycans.

Plasmid transfection and RNA interference. The pSilen-Circle-Si-β3GnT8 plasmid was constructed by our Lab and identified by digestion with restriction enzymes *Xho*I and *Eco*RI (MBI Fermentas, Vilnius, Lithuania) (17). Plasmid DNA was purified as described in the EndoFree plasmid purification handbook (Qiagen, Ltd., Crawley, UK). For transfection studies, SW620/5-FU cells were plated at a density of $2xl0^5$ cells/well in 6-well plates and incubated for 24 h. The cells were then transfected with 2-4 μg of plasmid DNA using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). As the negative control, the same amount of empty vector, pEGFP-c1, was also transfected. Gene silencing effect was confirmed by western blot analysis and qPCR at 24 h post-transfection.

Quantitative RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). Then the isolated RNA was quantified by spectrophotometry (optical density 260/280 nm). Reverse transcription to cDNA was conducted using the Superscript First Strand synthesis system (Invitrogen). All PCR reactions were carried out on an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR-Green Real-Time PCR Master Mix kit (Toyobo, Osaka, Japan) according to the manufacturer's instruction. PCR conditions used were: denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec and elongation at 72˚C for 30 sec. Primers of all genes are listed in Table I. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GADPH as the reference gene.

Western blot analysis. Harvested cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT, 0.1% SDS, 1 mM PMSF and placed in ice for 30 min. After centrifugation for 15 min at 4˚C, the supernatant was collected. Then proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% fat-free milk for 1 h at room temperature, the membranes were incubated with the primary antibody overnight at 4˚C followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The proteins were visualized using an ECL detection kit purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Rabbit anti-human β3GnT8 affinity pAb was purified in our laboratory (18). GADPH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Table I. Sequences of the primers used for quantitative RT-PCR.

Primer name	Sequences (5'→3')
β3GnT1	F: AACACTGGACTTGGATATGG
	R: TCACATATAGCATCTCATCTG
β3GnT2	F: ATACTGGAACCGAGAGCAAG
	R: TCAGGTTCGCAGTAGTTCAG
β3GnT3	F: TATGTGCCAGAGGTGGTGAC
	R: ACATACCCAGGAAGACATCAT
β3GnT4	F: TCAAGTCACAGCCTGGTCAC
	R: TCATCAAACTCCCTACTCTCAT
β3GnT7	F: CTACTGCTATGGAATGAGAC
	R: AGCTATTTATCTTACTTCTGTT
β3GnT8	F: GTCGCTACAGTGACCTGCTG
	R: GTCTTTGAGCGTCTGGTTGA
P-gp	F: TTGCTGCTTACATTCAGGTTTCA
	R: AGCCTATCTCCTGTCGCATTA
GADPH	F: CCAACCGCGAGAAGATGA
	R: CCAGAG GCGTACAGGGATAG

β3GnT, β-1,3-N-acetylglucosaminyltransferase; F, forward; R, reverse; P-gp, P-glycoprotein.

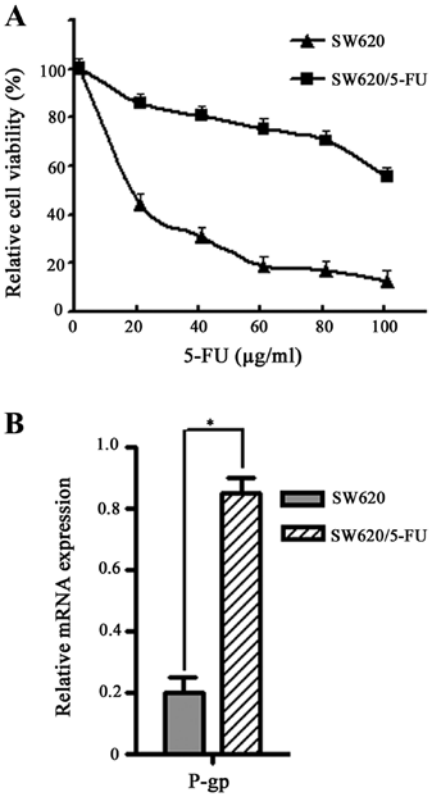

Figure 2. Establishment of a 5-fluorouracil (5-FU)-resistant cell line. (A) SW620/5-FU and SW620 cells were exposed to 5-FU at various concentrations for 48 h, and cell viability was evaluated by MTT assay. (B) mRNA expression level of P-glycoprotein (P-gp) was examined by quantitative RT-PCR. Data were recorded as mean ± SD of three independent experiments. *P<0.05 compared with parental cells.

Lectin blot analysis. SDS-PAGE and electrophoretic transferring were performed in the same manner as described for western blot analysis. After blocking with Carbo-Free Blocking Solution (Vector Labs, Burlingame, CA, USA), the membranes were incubated with 2 μg/ml of biotinylated *Lycopersicon esculentum* agglutinin (LEA) (Sigma) for 1 h. Reactive bands were detected with a diluted HRP-conjugated streptavidin (Sigma), and then visualized using ECL system (GE Healthcare, Pittsburgh, PA, USA).

Analysis of lectin labeling by flow cytometry. Cells were collected and washed three times with PBS. The cell density was adjusted to $2x10^6$/ml, stained with 10 μg/ml FITC-LEA (Sigma) in PBS (contain 0.5% BSA and 0.05% sodium azide) at 4˚C for 1 h, then washed three times with PBS. The fluorescence intensity of the stained cells was measured with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and analyzed with CellQuest.

Apoptosis analysis. Cells were harvested and fixed in cold 80% ethanol overnight at 4˚C and double stained with Annexin V-FITC and PI (both from Sigma) for 30 min at room temperature in dark. Stained cells were passed through a nylon-mesh sieve to remove cell clumps. Apoptotic cells were detected using flow cytometry within 1 h (Becton-Dickinson). Cells in the lower right quadrant represented early apoptosis and in the upper right quadrant represented late apoptotic cells.

Cell cycle analysis. A certain number of cells were trypsinized and fixed with 80% ethanol at 4˚C overnight prior to being stained with PI using freshly prepared staining solution. The distribution of cells in the different phases of the cell cycle was measured by flow cytomety. The percentage of cells in G1 phase, S phase, and G2/M phase was analyzed using standard ModiFit and CellQuest software programs.

Statistical analysis. All values are expressed as mean ± SD from triplicate experiments. Independent t-test was performed for comparison of data from independent samples. P<0.05 was considered significant.

Results

Generation of a 5-FU-resistant cell line. The 5-FU-resistant cell line SW620/5-FU was established from its parent cell line (SW620) by exposure to 5-FU in stepwise increase concentrations from 10 to 100μg/ml over a period of 3 months. Then cells were exposed to various concentrations of 5-FU for 48 h and cell viability was measured by MTT assay. We found SW620/5-FU cells were ~10-fold more resistant to 5-FU as compared with the parental cells (IC50, 128 vs. 13 μg/ml) (Fig. 2A).

P-glycoprotein (P-gp) is a plasma membrane glycoprotein often involved in the resistance of cancer cells towards multiple anticancer agents (19). To further confirm the 5-FU-resistant phenotype, mRNA expression of P-gp in SW620/5-FU cells was examined by quantitative RT-PCR. As shown in Fig. 2B, high level of P-gp mRNA was detected in the SW620/5-FU

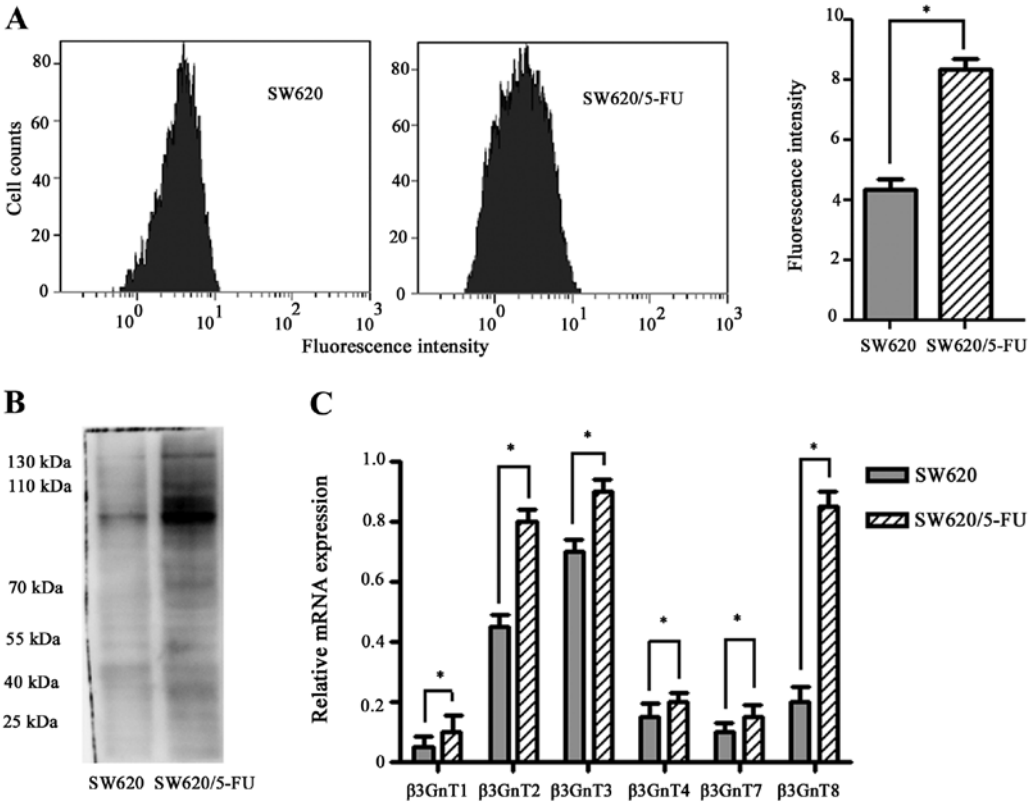

Figure 3. Analysis of polylactosamine and β-1,3-N-acetylglucosaminyltransferases (β3GnTs) expression in SW620/5-FU cells. (A) Results of the flow cytometric analysis. Histograms of fluorescence intensities of cells with specific carbohydrate expression as determined. (B) Results of the lectin blot analysis. (C) mRNA expression levels of β3GnTs were examined by quantitative RT-PCR. Data were recorded as mean ± SD of three independent experiments. *P<0.05 compared with parental cells.

cells. It confirmed that a 5-FU-resistant cell line was successfully constructed, and can be used for the successive experiments.

Increased expression of polylactosamine chains and β3GnT8 in SW620/5-FU cells. Polylactosamine chains can be specifically identified by LEA (20). To determine the alteration of polylactosamine in SW620/5-FU cells, each cell group was bound with LEA. As shown in Fig. 3A, the mean fluorescence intensities of LEA-labeling cells of SW620/5-FU and SW620 were 8.38±0.15 and 4.25±0.19, respectively. Significant differences were seen between the drug-sensitive and -resistance cells (P<0.05). Next, the separated glycoproteins were transferred onto PVDF membranes. Upregulation of polylactosamine chains was also observed in SW620/5-FU cells as detected by lectin blot analysis (Fig. 3B).

β3Gn-T1, -T2, -T3, -T4, -T7, and -T8 have been shown to possess the ability to synthesize polylactosamine chains (20). To identify and evaluate candidate genes involved in polylactosamine synthesis in SW620/5-FU cells, quantitative RT-PCR analysis was performed. We found β3Gn-T1, -T2, -T3, -T4, -T7, and -T8 were both highly expressed in SW620/5-FU cells(P<0.05) (Fig. 3C). However, the change of β3GnT8 mRNA expression was more obvious than other β3GnTs (i.e., >4-fold higher). These data indicated that overexpression of β3GnT8 may be responsible for the increased levels of polylactosamine chains in SW620/5-FU cells.

Effects of 3'-azidothymidine (AZT) on chemosensitivity of SW620/5-FU cells. To explore whether polylactosamine was associated with 5-FU resistance, AZT was used. AZT is a thymidine analogue that is able to inhibit the synthesis of polylactosamine (21). SW620/5-FU and its parent cells showed similar sensitivity to AZT (Sigma). From both cell lines >90% of cells were killed by treatment with 120 μM of AZT, whereas 80% of cells survived at 20 μM AZT (data not shown). Therefore, we applied 5 μM AZT, at which concentration no cytotoxicity was observed. As shown in Fig. 4A and B, AZT treatment resulted in decreased polylactosamine in SW620/5-FU cells as determined by flow cytometry and lectin blot analysis. Then SW620/5-FU cells were pre-treated with AZT for 24 h before exposed to various concentrations of 5-FU for 48 h. MTT assay showed that pre-treatment with AZT reduced the IC50 value against 5-FU of the resistant cells (72 vs. 128 μg/ml) (Fig. 4C). These results further confirmed that the inhibition of polylactosamine was able to reverse 5-FU resistance.

Knockdown of β3GnT8 inhibits the formation of polylactosamine in SW620/5-FU cells. To investigate the potential activity of elevated expression of β3GnT8 in SW620/5-FU cells, pSilenCircle-Si-β3GnT8 plasmid targeting β3GnT8 was used to transfect SW620/5-FU cells. Then β3GnT8 mRNA and protein expression was detected, respectively, by quantitative RT-PCR and western blot analysis. As shown in Fig. 5A and B, β3GnT8 expression

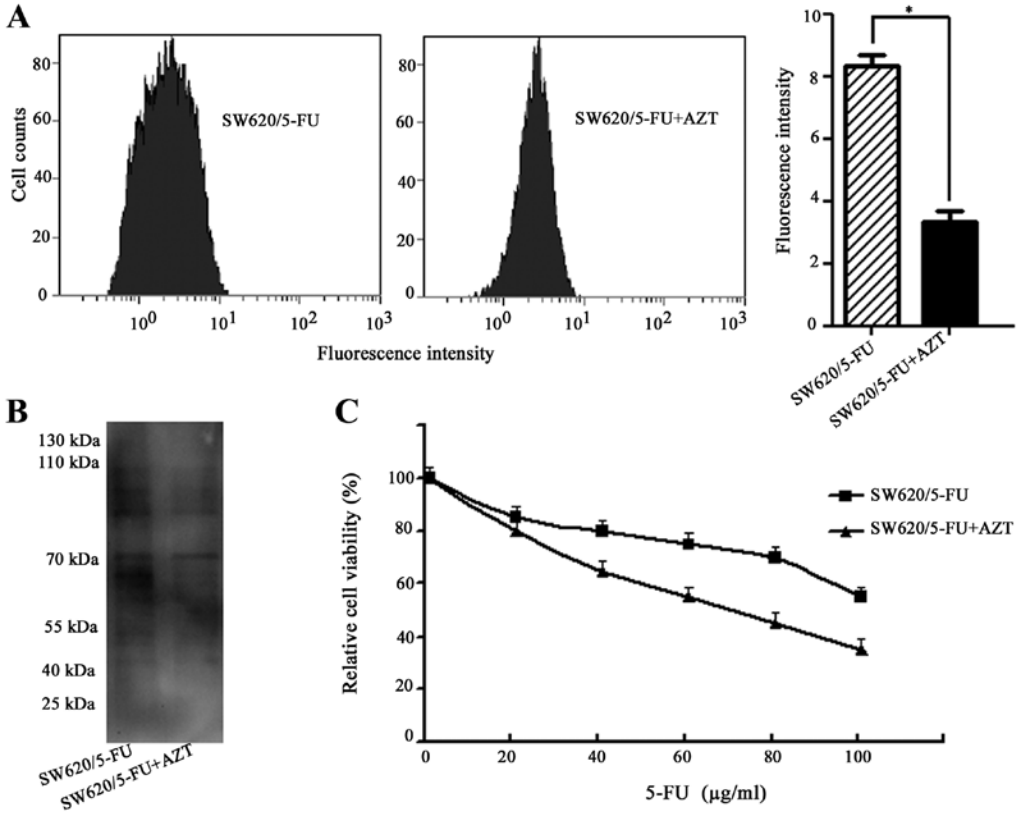

Figure 4. Effects of 3'-azidothymidine (AZT) on the synthesis of polylactosamine and cell viability in SW620/5-FU cells. (A) Results of the flow cytometric analysis. Histograms of fluorescence intensities of cells with specific carbohydrate expression. (B) Results of the lectin blot analysis. (C) Cell viability was determined by MTT assay. Data were recorded as mean ± SD of three independent experiments. *P<0.05 compared with SW620/5-FU cells.

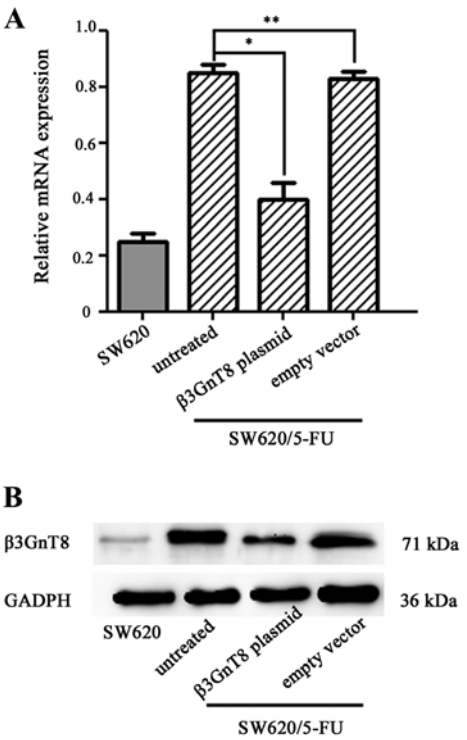

Figure 5. The expression of β-1,3-N-acetylglucosaminyltransferase (β3GnT8) in different groups. SW620/5-FU cells were transfected with pSilenCircle-Si-β3GnT8 plasmid or empty vector. β3GnT8 mRNA and protein expression levels were analyzed by (A) quantitative RT-PCR and (B) western blot analysis, respectively. Data were recorded as mean ± SD of three independent experiments. *P<0.05, **P>0.05 compared with untreated SW620/5-FU cells.

was significantly inhibited (P<0.05) in the SW620/5-FU cells transfected with pSilenCircle-Si-β3GnT8 plasmid, whereas no significant inhibitory effect was observed in the untreated cells and cells treated with empty vector (P>0.05).

To evaluate whether β3GnT8 knockdown could modify polylactosamine-type N-glycans, each cell group was also bound to FITC-LEA. Fig. 6A and B showed that β3GnT8 knockdown resulted in a decrease of fluorescence intensity compared with untreated cells and cells treated with empty vector (P<0.05). In addition, the result of lectin blot analysis revealed that knockdown of β3GnT8 by pSilenCircle-Si-β3GnT8 plasmid led to the downregulation of polylactosamine levels in SW620/5-FU cells (Fig. 6C). These results clearly proved that β3GnT8 contributes to development of 5-FU resistance in CRC cells via regulating the N-glycosylation profile in terms of polylactosamine chains.

Reversal of 5-FU resistance by knockdown of β3GnT8. Having demonstrated that β3GnT8 overexpression may participate in the resistance to 5-FU, we sought to determine whether decreased β3GnT8 expression would render CRC cells more sensitive to 5-FU. Then SW620/5-FU cells were transiently transfected with pSilenCircle-Si-β3GnT8 plasmid or empty vector, followed 24 h later with the 5-FU treatment for a further 48 h, before cell viability was measured using the MTT assay. As shown in Fig. 7A, IC50 value of 5-FU in SW620/5-FU cells was significantly (P<0.05) reduced while β3GnT8 expression was inhibited, suggesting that inhibition of β3GnT8 may increase drug sensitivity.

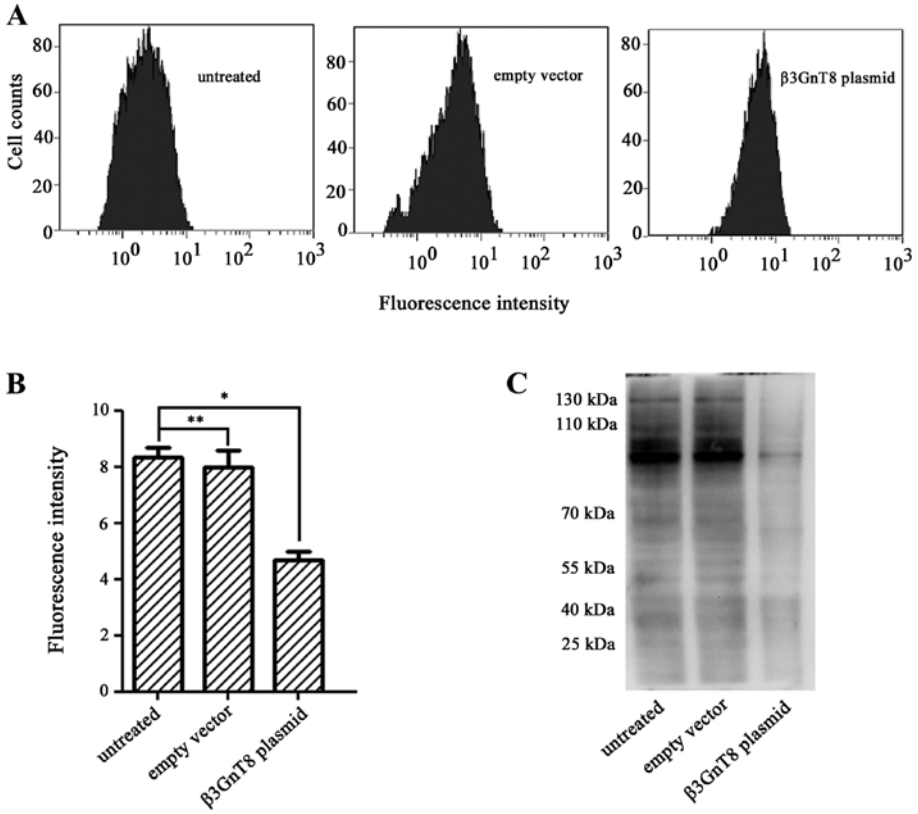

Figure 6. Effects of β-1,3-N-acetylglucosaminyltransferase (β3GnT8) knockdown on the synthesis of polylactosamine in SW620/5-FU cells. (A) Results of the flow cytometric analysis. (B) Histograms of fluorescence intensities of cells. (C) Results of the lectin blot analysis. Data were recorded as mean ± SD of three independent experiments. *P<0.05, **P>0.05 compared with untreated SW620/5-FU cells.

Flow cytometry assays were further performed to evaluate potential effects of β3GnT8 knockdown on the apoptosis and cell cycle arrest. Cells were exposed to 128 μg/ml 5-FU for 48 h. As shown in Fig. 7B, no significant difference in the proportion of apoptotic cell was observed between the untreated cells and cells treated with empty vector when exposed to 5-FU (P>0.05). However, notable apoptosis was found in SW620/5-FU cells exposed to 5-FU, when β3GnT8 was knocked down (P<0.05). In addition, knockdown of β3GnT8 alone could also induce a slight increase in apoptosis in SW620/5-FU cells. Furthermore, no significant difference in the cycle distribution was observed between the untreated cells and cells treated with empty vector when exposed to 5-FU (P>0.05) (Fig. 7C). On the other hand, when β3GnT8 expression was inhibited, significant reduction in cells in G1 phase and the accumulation of cells in S and G2/M phases was observed in SW620/5-FU cells exposed to 5-FU (P<0.05).

Discussion

Long-term chemotherapy unavoidably leads to drug resistance and this has become a major challenge to the triumph of chemotherapy. Over the past 50 years, despite its many advantages, clinical applications of 5-FU have been greatly limited due to drug resistance. The overall response rate for advanced CRC of 5-FU alone is still only 10-15% and the combination of 5-FU with other anti-tumor drugs has merely improved the response rates to 40-50% (4). Although previous studies have reported various mechanisms of 5-FU resistance, there are many 'unknowns' that need further clarification. In the present study, SW620/5-FU cells were generated by exposure to a gradually increasing 5-FU concentration. Higher IC50 value against 5-FU of the resistant cells were found compared to its parental cells. Drug-resistant biomarker such as P-gp was also highly expressed. Such resistance was stable upon the removal of 5-FU and was maintained for a considerable period of time. Thus, it can be considered that SW620/5-FU cells are a useful model for the investigation of 5-FU resistance in CRC.

Cell surface glycans are a class of sophisticated biomolecules related to cancer development and progression, and their analysis is of great significance for early cancer diagnosis and treatment. Colon cancer cells frequently express glycans at different levels or with fundamentally different structures than those observed on normal cells. For example, aberrant O-glycans, such as Galβ1, 3GalNAc (T antigen), were commonly found in colon cancer (22). Structures containing a bisecting GlcNAc were found to be decreased in the colorectal tumor, whereas sulfated glycans, paucimannosidic glycans, and glycans containing a sialylated Lewis type epitope were shown to be increased in tumor tissues (23). Rencently, aberrant changes in N-glycans have been shown to be associated with drug resistance (9,24). Swainsonine, an inhibitor of N-glycan biosynthesis, could reduce 5-FU tolerance in the multistage resistance of CRC cells (12). Therefore, monitoring of the N-glycan profile in CRC would be an important step in the prevention of side-effects and would increase our understanding of 5-FU resistance mechanisms. It is well known

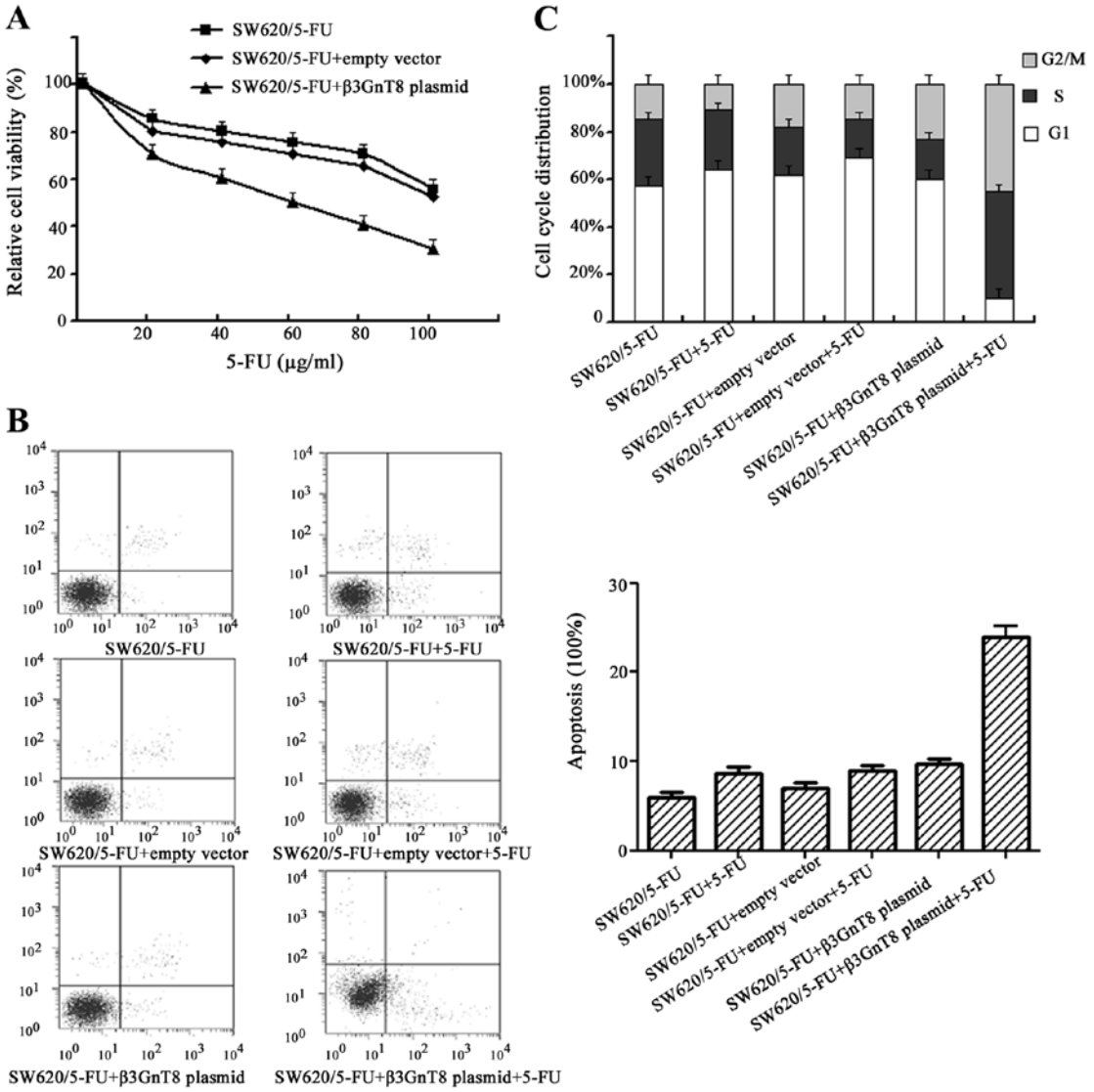

Figure 7. Effects of β-1,3-N-acetylglucosaminyltransferase (β3GnT8) knockdown on cell viability, apoptosis and cell cycle. SW620/5-FU cells were transiently transfected with pSilenCircle-Si-β3GnT8 plasmid or empty vector for 24 h, then cells were exposed for another 48 h at indicated concentrations of 5-fluorouracil (5-FU). (A) Cell viability was determined by MTT assay. (B) Apoptosis and (C) cell cycle was detected by flow cytometry. Data were recorded as mean ± SD of three independent experiments.

that lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin that recognize and reversibly bind to glycans without altering their covalent structure. LEA lectin, obtained from *Lycopersicum esculentum*, has specific affinity for polylactosamine sugar residues (25). In this study, the alteration of polylactosamine in SW620/5-FU cells was detected by flow cytometry and lectin blot analysis assays. The flow cytometry analysis is an effective approach to measure the linkage of FITC-lectin to cell surface carbohydrate not only qualitatively but also quantitatively (26). This study clearly showed that LEA signal was significantly upregulated in SW620/5-FU cells. It suggested that polylactosamine chains were associated with 5-FU resistance in cancer cells.

Polylactosamine is a fundamental structure of glycans carried on N- and O-glycans (27). Polylactosamine preferentially adds to β1-6GlcNAc linked antennae attached to the trimannosyl core of complex-type N-glycans. There are a number of reports regarding the functions and distributions of polylactosamine-type N-glycans. For example, some cancer cells such as U937 (human T-lymphoma) and MKN45 (human gastric cancer) cells specifically express polylactosamine-type N-glycans and such glycans were often modified with fucose and sulfate residues (28). Togayachi *et al* have reported that polylactosamine on N-glycans was a putative immune regulatory factor presumably suppressing excessive responses during immune reactions (27). Common glycoproteins expressing polylactosamine-type N-glycans on matched patient primary and metastatic melanoma cells always showed different glycan profiles (29). In a study on CRC cell lines, highly metastatic cell lines were found to synthesize more N-glycans that contain polylactosamine than poorly metastatic cell lines (30). A highly fucosylated polylactosamine type N-glycan was also expressed on CRC SW1116 cells (31). Therefore, it is interested to clarify the relationship between polylactosamine-type N-glycans and 5-FU resistance in SW620/5-FU cells. AZT was the first approved antiviral for the treatment of human immunodeficiency virus (32). It has been reported that AZT could inhibit the biosynthesis of

highly branched N-glycans and polylactosamine chains in melanoma cells (21). Synergistic antitumor effect of AZT in combination with 5-FU in human CRC cell lines was also observed (33). Here, we found AZT pre-treatment resulted in a reduction in the amount of polylactosamine chains, although the mechanism by which this occurs is not yet clear. We also found that the inhibition of polylactosamine by AZT was able to reverse 5-FU resistance in SW620/5-FU cells. To the best of our knowledge, this study is the first revealing the expression patterns of polylactosamine-type N-glycans in 5-FU-resistant cancer cells and the correlation with reversal of resistance. Furthermore, we made some effort to explore the possible mechanisms, our preliminary results are promising.

Aberrant glycosylation is associated with differential expression of enzymes such as glycosyltransferase and glycosidases (34). The aberrant expressions of the enzymes in turn cause cancer cells to produce glycoproteins with specific cancer-associated aberrations in glycan structures. Eight members in the β3GnT family (β3GnT1-T8) have been identified thus far, and their activities have been characterized. Several of the enzymes, β3Gn-T1, -T2, -T3, -T4, -T7 and -T8, have been shown to mediate polylactosamine synthesis (20). It is worth noting that β3GnT2 showed the strongest activity for polylactosamine synthesis in initial *in vitro* experiments (35). By contrast, β3GnT3 and β3GnT4 were found to have very weak polylactosamine synthase activity (15). β3GnT8, which has been cloned by our and another groups, is the most recently identified enzyme among the β3GnTs (16,36). It has been reported that β3GnT2 and β3GnT8 can form a complex with enhanced enzymatic activity (37). However, the presence of β3GnT8 can stimulate the activity of β3GnT2. Overexpression of β3GnT8, but not β3GnT2, may induce an increase in polylactosamine-type N-glycans in malignant tumor cells (20). Herein, as an alternative strategy, we hypothesized that β3GnT8 is responsible for the synthesis of polylactosamine-type N-glycans in SW620/5-FU cells. We found β3Gn-T1, -T2, -T3, -T4, -T7, and -T8 were both highly expressed in SW620/5-FU cells. As expected, our results demonstrated the change of β3GnT8 mRNA expression was more obvious than other β3GnTs. Based on the findings of our study and other reports, we thought overexpression of β3GnT8 should contribute to development of drug resistance in cancer cells, and knockdown of β3GnT8 may restore the sensitivity to anticancer agents.

To investigate the correlation between β3GnT8 and 5-FU resistance in SW620/5-FU cells, the expression of β3GnT8 was downregulated by pSilenCircle-Si-β3GnT8 plasmid. We found β3GnT8 knockdown led to the downregulation of polylactosamine levels in SW620/5-FU cells. In addition, IC50 value of 5-FU in SW620/5-FU cells was significantly reduced while β3GnT8 expression was inhibited. When β3GnT8 was knocked down by RNA interference, we also observed inhibition of cell proliferation and increase in apoptosis in cells with exposure to 5-FU, indicating a reversal of 5-FU resistance by β3GnT8 knockdown. Although the reports focusing on β3GnT8 and drug resistance remain limited and preliminary, some correlations between overexpression of β3GnT8 and drug resistance (9,11) encouraged us to presume that β3GnT8 should be involved in the development of drug resistance.

In conclusion, we confirmed that β3GnT8 expression was upregulated in 5-FU-resistant cancer cells and that the knockdown of β3GnT8 reversed the 5-FU resistance through, at least partly, suppression the biosynthesis of polylactosamine-type N-glycans. Thus, β3GnT8 is a potential molecular target to overcome anticancer drug resistance in CRC. Whether or not there are other signal transduction pathways involved, and the elucidation of the underlying mechanisms are warranted.

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