Effect of ST3GAL 4 and FUT 7 on sialyl Lewis X synthesis and multidrug resistance in human acute myeloid leukemia

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A B S T R A C T
Sialyl Lewis X (sLeX, CD15s) is a key antigen induced on tumor cell surfaces during multidrug resistance (MDR) development. The present study investigated the effect of α1,3 fucosyltransferase VII (FucT VII) and α2,3 sialyltransferase IV (ST3Gal IV) on sLe X synthesis as well as their impact on MDR development in acute myeloid leukemia cells (AML). FUT7 and ST3Gal IV were overexpressed in three AML MDR cells and bone marrow mononuclear cells (BMMCs) of AML patients with MDR by real-time polymerase chain reaction (PCR). A close association was found between the expression levels of FUT7 and ST3Gal IV and the amount of sLe X oligosaccharides, as well as the phenotype variation of MDR of HL60 and HL60/ADR cells both in vitro and in vivo. Manipulation of these two genes' expression modulated the activity of phosphoinositide-3 kinase (PI3K)/Akt signaling pathway and the expression of P-glycoprotein (P-gp) and multidrug resistance related protein 1 (MRP1), both of which are known to be involved in MDR. Blocking the PI3K/Akt pathway by its specific inhibitor LY294002 or Akt short hairpin RNA (shRNA) resulted in the reduced activity of PI3K/Akt signaling pathway and the expression of P-gp and MRP1.

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1. Introduction

Acute myeloid leukemia (AML), the most common type of leukemia in adults, has the lowest survival rate among all leukemias [1]. It is a clonal malignancy of the hematopoietic system characterized by accumulation of immature cells, population of the bone marrow or peripheral blood [2]. Multidrug resistance (MDR) is a major challenge to the successful treatment of AML. Classic MDR is the consequence of overexpression of transporter proteins belonging to the ATP binding cassette (ABC) family e.g., P-gp and MRP1, which lead to lower intracellular drug accumulation and reduced cellular toxicity of chemotherapeutic agents [3]. Nowadays, many researchers are managing to adequately evaluate the interaction of glycan alterations and resistance to chemotherapy of neoplastic cells so as to understand their pathogenesis. However, there is still little information about the role of glycosyltransferases and relevant glycogenes in the development of AML MDR except the modification of glycan structures has been observed in drug-resistance leukemia cells [4,5].

Glycosylation is one of the most important modifications of proteins and lipids [6]. Alterations in cell surface glycosylation are acknowledged as a hallmark of carcinogenesis which usually leads to the expression of tumor-associated carbohydrate antigens (TACAs) on glycoproteins or glycolipids that decorate cell surfaces [7]. Lewis antigens are functionally important terminal glycan epitopes, which are usually subdivided into two groups: types 1 and 2, depending on whether the terminal galactose is bound to the preceding GlcNAc by α1,3 (β1,3 Gal-T) or α1,4 (β1,4 Gal-T) [8]. All type 1 structures contain a α1,4-Fuc residue on the GlcNAc catalyzed by α1,4 fucosyltransferase (FucT) and α1,3 fucosyltransferase VII (FucT VII) or β2,3 fucosyltransferase (FucT II) [9].

Sialyltransferases (STs) catalyzed the transformation of sialic acid residues from donor substrate CMP-sialic acid to the oligosaccharide side chains of glycoconjugates. Different STs showing cell and tissue tropism are unique in substrate specificities and in types of linkage formed.
2. Materials and methods

2.1. Parental AML cell culture

Three AML cell lines including an acute myeloid leukemia cell line HL60, an acute promyelocytic leukemia cell line NB4, and a leukemic monocytic lymphoma (M5) cell line U937, were obtained from the KeyGen Company (China). All cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco), and 1% penicillin-streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO2. Adriamycin (Sigma, St. Louis, MO, USA) was added to parental cell cultures in stepwise increasing concentrations from 1 μg/mL to 5 μg/mL for 2 months to develop an adriamycin-resistant (ADR) subline, namely HL60/ADR, NB4/ADR, and U937/ADR, respectively. To maintain the MDR phenotype, the complete medium of the resistant cells was supplemented with 2.5 μg/mL adriamycin. Over 80% of ADR cells were susceptible to subsequent treatments if they were maintained in complete medium without adriamycin supplementation.

2.2. Samples from leukemia patients

101 previously untreated AML patients and 7 healthy donors were included in this study. The diagnosis of AML was based on cytomorphology, cytometry, multiparameter flow cytometry, immunology, molecular genetics and cytogenetics, and the leukemic subtypes of AML were determined according to the French–American–British classification as follows: 46, 37 and 18 cases of M2, M3 and M5, respectively. There were 71 male and 30 female with age ranging from 12 to 78 years (median, 42 years). P-gp (+) was observed in 94 of 101 AML patients and MRP1 (+) was observed in 90 and 90 patients. All the participants who were obtained from May 2010 to July 2012 at the First Affiliated Hospital of Dalian Medical University (Dalian, China) provided written informed consent. Both the study and the contents of the written consent were approved by the institutional ethics committees.

2.3. Separation of leukemic blast cells

BMMC were separated by Ficoll-Hypaque density gradient centrifugation from bone marrow or peripheral blood taken at the initial diagnosis and were further cultured in plastic dishes to remove adherent cells at 37 °C for 24 h. Fresh separated non-adherent cells were maintained in modified Dulbecco’s medium containing 10% fetal bovine serum, 10 mM β-mercaptoethanol, 4.5 mM l-glutamine, 50 ng/mL human stem cell factor, 10 ng/mL human interleukin-3, and 10 ng/mL human interleukin-6.

2.4. Real time PCR analysis

ST3GAL3, ST3GAL4, ST3GAL6 and FUT7 mRNA levels of all the cells were determined by real time PCR. Total RNA was isolated with Trizol reagents (Gibco BRL, Life Tech, MD, USA), and cDNA was synthesized using Quantitect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. Real time PCR was carried out on a Roche Lightcycler480 real time PCR system (Applied Biosystems, Foster City, CA). A ST3GAL4 Quantitect SYBR Green PCR Kit (QIAGEN, Valencia, CA), the sequences of upstream and downstream primers were as follows: 5′-TATGTCCTACCTATTGACT-3′ and 5′-TTGGTACTGACTGACAAG-3′ for ST3GAL3; 5′-ATATGTCCTACCTATTGACT-3′ and 5′-AGGAAGATGGCTAGCT-3′ for ST3GAL4; 5′-ATGTCTATTGTCCTGCACT-3′ and 5′-GCCACACAGAGCTG-3′ for ST3GAL6; 5′-CAACAGAAACACGGGTAGAC-3′ and 5′-AACACACAGACCC-3′ for ST3GAL7; 5′-GGCCGCAATCGGAC-3′ and 5′-GCCACACAGAGCTG-3′ for ST3GAL8. Primers for GAPDH, the housekeeping gene, included 5′-TTGGTGACTGACAAG-3′ and 5′-AGGAAGATGGCTAGCT-3′ for ST3GAL4; 5′-ATGTCTATTGTCCTGCACT-3′ and 5′-GCCACACAGAGCTG-3′ for ST3GAL6; 5′-CAACAGAAACACGGGTAGAC-3′ and 5′-AACACACAGACCC-3′ for ST3GAL7. The expression level of target genes was determined relatively to GAPDH and calculated as 2^-ΔΔCt. Target gene expression was normalized to control.

2.5. Western blot analysis

Total cell protein was electrophoresed in 10% SDS-PAGE gel and blotted onto a polyvinylidene difluoride membrane. After being blocked with 5% powdered skim milk for 2 h in phosphate-buffered saline containing 0.1% Tween 20 (PBST), the membranes were incubated with anti-human ST3Gal III (Abcam, Cambridge, UK, 1:1000 dilution), ST3Gal IV (Abcam, Cambridge, UK, 1:1000 dilution), ST3Gal VI (Abcam, Cambridge, UK, 1:1000 dilution), FUT7 (Abgent, Cambridge, UK, 1:1000 dilution), KM93 (recognizing the sLe X, Merck, Temecula, CA, 1:1000 dilution), PI3K p110α (Abcam, Cambridge, UK), PI3K p110α and p-Akt 308 (Abgent, Cambridge, UK), p-Akt 473 (Abgent, Cambridge, UK), p-Akt 308 (Abgent, Cambridge, UK, 1:1000 dilution) and Akt (Abgent, Cambridge, UK, 1:1000 dilution) respectively at 4 °C overnight, and then incubated with secondary antibody anti-rabbit-HRP or anti-mouse-HRP (1:2000 diluted, Santa Cruz Biotech, Santa Cruz, CA). GAPDH antibody (1:200 diluted, Santa Cruz Biotech, Santa Cruz, CA) was used as a control. All bands were detected using ECL western blotting kit (Amersham Biosciences, UK), according to the manufacturer’s instruction.

2.6. Dereglulation of ST3GAL4 or FUT7 by RNAi

HL60 or HL60/ADR cells were incubated in appropriate antibiotic-free medium with 10% fetal bovine serum (Gibco), and were transferred to a 6 well tissue culture incubating in a CO2 incubator maintaining 40–60% confluent at 37 °C. The cell cultures were transfected with ST3GAL4,
FUT7 specific shRNA, and scrambled shRNA used as the negative control. ST3GAL4 shRNA or FUT7 shRNA was mixed with Lipofectamine™ 2000 (Invitrogen). Transfected cells were cultured and incubated at 37 °C for 6 h, followed by incubation with complete medium for additional 24 h. Then cells were harvested for further study. The cell transfection efficiency was 85% by fluorescent microscopy and the cell viability was 90% by trypan blue dye exclusion assay.

2.7. Over-expression of ST3GAL4 or FUT7

The human ST3GAL4 and FUT7 coding sequences obtained from Takara company (Dalian, China) were inserted into the pEGFP-N2 vector (Invitrogen, Carlsbad, CA) at the sites of EcoRI and Xhol. Cells were transfected with 5 μg of target gene expression vector or empty vector (EV) in 100-mm dishes using PolyFect Transfection Reagent (QIAGEN, Valencia, CA) according to the manufacturer's instruction. After 4 weeks of screening, the cell lines stably expressing ST3GAL4 (HL60/ST3GAL4) and FUT7 (HL60/FUT7), and cells with empty vector (HL60/EV) were established. Then cells were collected for gene expression assay and further explorations. The cell transfection efficiency was 79% and the survival rate was 90%.

2.8. In vitro drug cytotoxic assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS kit) (Promega, Madison, WI) was used to determine the chemosensitivity of cell groups with genetic manipulation and pharmacologic inhibition of the PI3K/Akt pathway. The tumor cells (1 × 10⁴) were seeded in 96-well plate and incubated with paclitaxel, vincristine, and adriamycin (Sigma, St. Louis, MO) for 24 to 48 h. The absorbance was measured at 490 nm by microplate reader (Model 680; Bio-Rad, Hercules, CA). The drug resistance was estimated by comparing the IC50 values (drug concentration that inhibits cell growth by 50%) from the treated and control cells.

2.13. Flow cytometry analysis

The tumor cells were washed twice with phosphate buffered saline (PBS) buffer containing 20 g/L bovine serum albumin and then were preincubated with 5% powdered skim milk for 1 h at 37 °C to block nonspecific binding. For surface staining of P-gp, MRP1, AAL lectin, and sLe X, aliquots of the cells (1 × 10⁶) were incubated with fluorescein isothiocyanate (FITC)-anti-human P-gp, anti-MRP1 (Abcam, Cambridge, UK), FITC-AAL lectin (Sigma, St. Louis, MO), or an isotype control antibody (Santa Cruz Biotech, Santa Cruz, CA) at the recommended dilution for 1 h at 4 °C. Flow cytometric analysis of the PI3K/Akt signaling in HL60/ADR cells. Briefly, the tumor cells (1 × 10⁶ cells per well) were incubated in DMSO supplemented with the PI3K inhibitor LY294002 (10 μM), Akt control shRNA and Akt shRNA, and collected after 24 h. Variation in chemosensitivity and gene expression were measured by MTS assay and western blot analysis, respectively. Each experiment was run in triplicate to determine means and SDs.

2.12. Flow cytometry analysis

The tumor cells were washed twice with phosphate buffered saline (PBS) buffer containing 20 g/L bovine serum albumin and then were preincubated with 5% powdered skim milk for 1 h at 37 °C to block nonspecific binding. For surface staining of P-gp, MRP1, AAL lectin, and sLe X, aliquots of the cells (1 × 10⁶) were incubated with fluorescein isothiocyanate (FITC)-anti-human P-gp, anti-MRP1 (Abcam, Cambridge, UK), FITC-AAL lectin (Sigma, St. Louis, MO), or an isotype control antibody (Santa Cruz Biotech, Santa Cruz, CA) at the recommended dilution for 1 h at 4 °C. Flow cytometric analysis of the PI3K/Akt signaling in HL60/ADR cells. Briefly, the tumor cells (1 × 10⁶ cells per well) were incubated in DMSO supplemented with the PI3K inhibitor LY294002 (10 μM), Akt control shRNA and Akt shRNA, and collected after 24 h. Variation in chemosensitivity and gene expression were measured by MTS assay and western blot analysis, respectively. Each experiment was run in triplicate to determine means and SDs.

2.11. Inhibition of the PI3K/Akt signaling

LY294002 (Sigma) or Akt shRNA was applied to suppress the activity of the PI3K/Akt signaling in HL60/ADR cells. Briefly, the tumor cells (1 × 10⁶ cells per well) were incubated in DMSO supplemented with the PI3K inhibitor LY294002 (10 μM), Akt control shRNA and Akt shRNA, and collected after 24 h. Variation in chemosensitivity and gene expression were measured by MTS assay and western blot analysis, respectively. Each experiment was run in triplicate to determine means and SDs.

2.10. Immunohistochemical staining analysis

The slides were mounted with 5% powdered skim milk for 30 min followed by blocking nonspecific binding. For immunohistochemical staining, the sections were counterstained with hematoxylin and cover-slipped. The Image-Pro Plus 4.5 Software (Media Cybernetics, USA) was used to analyze the expression of proteins.

2.9. In vivo chemosensitivity assay

5-week-old nude mice were obtained from the Animal Facility at Dalian Medical University and were fed standard rodent rodent food and water. Approximately, 1 × 10⁶ cells (HL60/mock, HL60/ST3GAL4, HL60/FUT7, HL60/ADR, HL60/ADR-control shRNA, HL60/ADR-ST3GAL4 shRNA, or HL60/ADR-FUT7 shRNA) were subcutaneously injected into the right flank of each immunosuppressed nude mouse. Tumors were palpable when tumor volume of 1 cm³. The tumor volume was calculated by the following formula: Tumor volume = 1/2(length × width²).

2.8. In vitro drug cytotoxic assay

The cell lines stably expressing ST3GAL4 (HL60/ADR-ST3GAL4 shRNA, or HL60/ADR-FUT7 shRNA were injected subcutaneously into the right flank of each nude mouse. Tumors were palpable when tumor volume of 1 cm³. The tumor volume was calculated by the following formula: Tumor volume = 1/2(length × width²).

3.1. ST3GAL4 and FUT7 were responsible for the synthesis of sLe X in three pairs of AML cell lines

To find out the link between expression of glycosyltransferases and expression of sLe X, the expression of sLe X in three pairs of AML cell lines was first evaluated by western blotting. A remarkable increase of ST3GAL4 or FUT7 mRNA expression was observed in three MDR cell lines compared to that in the parental cells (Fig. 1C).

The mRNA expression levels of ST3GAL3, ST3GAL4, ST3GAL6, FUT3, FUT5, FUT6 and FUT7 were determined by real-time PCR analysis, and α2, 3-STs and FucT VII expression levels were determined by western blot analysis for the same AML cell lines. The three MDR cell lines with higher expression of sLe X, also showed a tendency of an increasing expression of ST3GAL3 (3.74 folds) and FUT7 (3.01 folds). Statistical significant differences were not found in the expression levels of ST3GAL3, FUT3, FUT5 and FUT6 mRNA (Supplementary Fig. S1A), while ST3GAL6 was undetectable in both in drug-sensitive parental cells and MDR cells (Fig. 1A, B). Our results indicated a positive association between sLe X synthesis and expression of ST3GAL4 and FUT7 in AML cell lines.
3 fucosylation level was also found reduced in HL60/ADR-ST3GAL4 shRNA-1 or HL60/ADR-FUT7 shRNA-1 cells by detecting FITC-conjugated MAL or AAL on the cell surface (Fig. 2C, D). Following the decreased expression of ST3GAL4 or FUT7, the expression of sLe X was significantly decreased in HL60/ADR-ST3GAL4 shRNA1 and HL60/ADR-FUT7 shRNA1 cells (Fig. 3A, B). On the contrary, the expression of Lewis X or Lewis Y, another glycan structure of the type 2 chains, was significantly increased in HL60/ADR cells treated with ST3GAL4 and FUT7 shRNA (Supplementary Fig. S1B).

After ST3GAL4 or FUT7 shRNA transfection, the inhibition activity to the growth of HL60/ADR was evaluated by adriamycin and vincristine. The IC50 values were significantly decreased in HL60/ADR-ST3GAL4 shRNA1 group compared to the control, suggesting that cell proliferation was inhibited by therapeutic drug when HL60/ADR cells were treated with ST3GAL4 shRNA. Similar results were obtained in HL60/ADR-FUT7 shRNA1 cell group (Fig. 3C).

Nude mice bearing HL60/ADR, HL60-control shRNA, HL60/ADR-ST3GAL4 shRNA-1 and HL60/ADR-FUT7 shRNA-1 xenografts were used to determine the treatment efficacy of adriamycin by measuring tumor volumes. In HL60/ADR and HL60-control shRNA group, there was no significant change in tumor volumes regardless of drug treatment. In HL60/ADR-ST3GAL4 shRNA1 group, the tumor volumes were found reducing significantly in the mouse group after drug treatment (Fig. 3D). The same tendency was also seen in HL60/ADR-FUT7 shRNA1 group. IHC staining analysis of the tumor sections revealed that the expressions of ST3Gal IV, FucT VII and sLe X were decreased in the mouse group treated with ST3GAL4 shRNA1 or FUT7 shRNA1 compared to that in the untreated group (Fig. 3E–G).

3.3. Overexpression of ST3GAL4 or FUT7 gene enhances the sLe X expression and the chemoresistance of HL60 cells both in vitro and in vivo

To further identify the role of ST3GAL4 and FUT7 in the synthesis of sLe X structures, we transfected HL60 cells with ST3GAL4 or FUT7 expression vector to determine the effect of over-expression of these genes on chemoresistance of HL60 cells. Notably the levels of mRNA and protein of ST3GAL4 and FUT7 were detected increasing in ST3GAL4 and FUT7 transfectants (Fig. 4A, B). Fluorescence intensity of the MAL or AAL revealed more α-2, 3 sialylation or α-1, 3 fucosylation in HL60/ST3GAL4 or HL60/FUT7 cells were more than that in control cells, since higher fluorescence intensity of lectins was observed that reflected higher expression of glycome (Fig. 4C, D).
The biosynthesis of sLe X antigen was assessed by western blot and flow cytometry. Higher levels of sLe X were observed in HL60/ST3GAL4 and HL60/FUT7 cells when compared to the control (Fig. 5A, B).

To investigate the possible effects of sLe X expression on the chemoresistance of HL60 cells, MTS assay was performed. The results showed that the IC50 values of two drugs were significantly higher in HL60/ST3GAL4 and HL60/FUT7 cells than in HL60 cells, suggesting a positive association between the sLe X expression and chemoresistance of leukemia cells (Fig. 5C).

The nude mice inoculated with tumor cells HL60, HL60/mock, HL60/ST3GAL4 and HL60/FUT7 were used to measure and compare tumor volumes with or without adriamycin treatment. In the group of mice bearing HL60 tumors, tumor volume was reduced after adriamycin treatment than those without. In the group of mice bearing HL60/ST3GAL4 or FUT7 tumors, tumor volumes increased obviously even after adriamycin treatment (Fig. 5D). High expression of ST3Gal IV, FucT VII and sLe X was illustrated in the tumor cells of HL60/ST3GAL4 or HL60/FUT7 by IHC staining, as shown in Fig. 5E–G. Therefore up-regulation of ST3GAL4 or FUT7 gene in HL60 cells led to increasing resistance to adriamycin chemotherapy.

3.4. Effect of sLe X-activated PI3K/Akt signaling pathway on the expression of P-gp and MRP1

In order to investigate whether sLe X activated the PI3K/Akt pathway and whether this pathway was involved in sLe X-mediated cell MDR, western blotting was applied to measure the levels of the main molecules of PI3K/Akt signaling pathway, P110α (the catalytic subunit of PI3K), phosphorylation Akt at Ser473 and Thr308. Fig. 6A showed that the levels of the three main molecules were significantly decreased in low sLe X expression cells (HL60/ADR-ST3GAL4 shRNA1 cells and HL60/ADR-FUT7 shRNA1 cells). On the contrary, the expression of all of the above-mentioned molecules was obviously increased in sLe X overexpression cells (HL60 cells treated with ST3GAL4 and FUT7 expression vector) (Fig. 6B). However, there was no change in the total amount of Akt protein.

Further on, we investigated whether sLe X influence the expression of P-gp and MRP1. Interestingly, flow-cytometric analysis illustrated that elevated expression levels of P-gp and MRP1 were detected in high sLe X expressing cells compared to those in the control groups (Fig. 6D). In contrast, HL60/ADR cells with sLe X suppression expressed...
low levels of P-gp and MRP1 (Fig. 6C). These data indicated a possible pathogenetic mechanism of MDR development of AML cells.

3.5. PI3K/Akt inhibition modulates the chemosensitivity of HL60/ADR cells both in vitro and in vivo

The effect of PI3K/Akt signaling activity on chemoresistance of HL60/ADR cells was explored by pharmacologic inhibition of the PI3K/Akt pathway. By western blotting, the expression levels of the main signal molecules of PI3K/Akt pathway apparently decreased in HL60/ADR cells treated with LY294002 or Akt shRNA (Fig. 7A). As shown in Fig. 7B, the inhibition of PI3K/Akt pathway resulted in the HL60/ADR cells susceptible to chemotherapy. Accordingly in vivo chemosensitivity analysis revealed that reduced tumor volumes were detected in mice group bearing HL60/ADR tumors with impaired PI3K/Akt signaling (Fig. 7C). Altered expression levels of the main signal molecules of PI3K/Akt pathway were also validated in mouse group bearing HL60/ADR tumors treated with LY294002 or Akt shRNA treatment were also validated by IHC staining, as shown in Fig. 7D.

As well, we revealed the same tendency of ZSTK474, a novel PI3K/Akt pathway inhibitor, in HL60/ADR cells (Supplementary Fig. S2). Moreover, the inhibitor of PI3K/Akt or silencing Akt reduced the expression of P-gp and MRP1 (Fig. 7E). The results implicated a role of PI3K/Akt signaling in regulating P-gp and MRP1 expression and modulating the chemoresistance of HL60/ADR cells.

3.6. ST3GAL4 and FUT7 were responsible for the synthesis of sLe X in BMMC of AML patients

Expression of MDR-related marker, ST3GAL3, ST3GAL4, ST3GAL6 and FUT7 presenting in BMMC of AML patients is summarized in Table 1. The frequency of P-gp positivity was 93.06% (94 of 101) in the HL60/ADR cells both in vitro and in vivo.

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Expression of MDR-related marker, ST3GAL3, ST3GAL4, ST3GAL6 and FUT7 presenting in BMMC of AML patients is summarized in Table 1. The frequency of P-gp positivity was 93.06% (94 of 101) in the HL60/ADR cells both in vitro and in vivo. Distinct reduction of sLe X was observed in HL60/ADR-ST3GAL4 or FUT7 shRNA1 cells by western blotting analysis (A) and flow cytometry analysis (B). (C) Cell chemosensitivity was assessed by cytotoxicity assays. The reported values were the IC50 (mean ± SD) of three independent experiments. IC50 represents the drug concentration producing 50% decrease of cell growth. *P < 0.05 vs HL60/ADR-control shRNA cells. (D) A decrease of mean tumor volume in mice group with HL60/ADR-ST3GAL4 or FUT7 shRNA1 tumors was observed, as compared with that in HL60/ADR group and HL60/ADR-control shRNA group. Within HL60/ADR-ST3GAL4 or FUT7 shRNA1 group, an increase of tumor growth was found in group without ADR, compared with that with ADR (P < 0.05). (E, F, G) Down-regulation of ST3GAL IV, FucT VII or sLe X was also shown by IHC staining in xenograft tumors derived from HL60/ADR-ST3GAL4 or HL60/ADR-FUT7 shRNA1 cells (400×). The data are means ± SD of 3 independent assays (*P < 0.05).
Fig. 5. Overexpression of ST3GAL4 or FUT7 gene enhances the chemoresistance of HL60 cells both in vitro and in vivo. Western blotting analysis (A) and flow cytometry analysis (B) showed a significantly increased expression of sLe X in HL60/ST3GAL4 or HL60/FUT7 cells. The chemoresistance of HL60 cells was increased with the ST3GAL4 or FUT7 expression vector transfection in vitro (C) and in vivo (D). (E, F, G) Up-regulation of ST3Gal IV, FucT VII or sLe X was also shown by IHC staining in xenograft tumors derived from HL60/ST3GAL4 or HL60/FUT7 cells (400×). The data are means ± SD of 3 independent assays (*P < 0.05).
AML patients, mRNA expression levels of ST3GAL4 or FUT7 were measured in the BMMC of AML without MDR and AML/MDR by realtime PCR and flow cytometry analysis of sLe X fluorescence intensity. The groups of M2/MDR, M3/MDR and M5/MDR showed significant higher levels of ST3GAL4 and FUT7 mRNA expression and high fluorescence intensity of sLe X than ones of the chemosensitive group. ST3GAL3 showed no difference in expression levels between the two groups, while ST3GAL6 was detected slightly.

4. Discussion

MDR is the major obstacle to the efficiency of chemotherapy in the treatment of leukemia [27]. In this study, we investigated the association between sLe X expression and clinical-pathological characteristics of AML as well as the possible mechanism of sLe X oligosaccharides on MDR development in human AML cell lines.

Sialylated antigens are expressed on the surface of various human cancer cells and are assumed to play a key role in the process of tumor progression [28]. sLe X is the major terminal carbohydrate structure that binds selectins and interferes with tumor cell rolling and metastasis [29]. The present study unraveled the role of ST3Gal IV and FucT VII in the biosynthesis and the regulation of the expression of sLe X in AML cells. Our observations were in agreement with the previous studies showing that expression of ST3Gal IV in Burkitt lymphoma cell line and FucT VII in H7721 cells led to the production of sLe X [30,31]. Overexpression of sialylated Lewis antigens in AML cells indicated an alteration of the glycosyltransferases expression and activity. The present study showed here that all MDR cells were characterized by high levels of sLe X, ST3Gal4 and FUT7, suggesting that the drug resistant AML cell lines produced altered α2, 3-sialylation and α1, 3-fucosylation. The alternation of the expression of sLe X, ST3Gal4 and FUT7 can be regarded as indicators and functional contributors for tumor MDR.

The question then arises whether and how the alteration of MDR in AML cells is caused by the change of ST3Gal IV, FucT VII or their products. Our former work demonstrated that the expression of ST3Gal4 involved in drug resistance development in chronic myeloid leukemia (CML) cell lines K562 and K562/ADR [5]. The present investigation illustrated that the altered level of ST3Gal4 or FUT7 led to drug-resistant phenotype changes of HL60 and HL60/ADR cells. This conclusion was evidenced by the following observations. (1) Expression level of sLe X was remarkably decreased (Fig. 2E, F), and IC50 values of chemotherapy drugs were significantly decreased in HL60/ADR-ST3Gal4 or FUT7 shRNA-1 cell group. Correspondingly reduced tumor volumes were detected in mice group bearing HL60/ADR-ST3Gal4 or FUT7 shRNA-1 tumors by in vivo chemosensitivity analysis. (2) Overexpression of ST3Gal4 or FUT7 gene enhanced the sLe X expression, further the chemoresistance of HL60 cells both in vitro and in vivo. Based on these results we hypothesized that ST3Gal IV, FucT VII and their product sLe X might function as pivotal modulators in MDR development of AML.

Fig. 6. Effect of ST3Gal4 or FUT7-activated PI3K/Akt signaling pathway on the expression of P-gp and MRP1. (A) Expression of PI3K/Akt signaling molecules was repressed at protein levels with ST3Gal4 or FUT7 shRNA transfection in HL60/ADR cells. (B) Decreased expression levels of PI3K/Akt signaling molecules were determined by western blot in HL60/ST3Gal4 or FUT7 cells. (C) Decreased expressions of P-gp and MRP1 were induced by flow cytometry analysis in ST3Gal4 or FUT7-shRNA-treated HL60/ADR cells. (D) Flow cytometry analysis revealed a higher expression of P-gp and MRP1 in HL60 cells with ST3Gal4 or FUT7 transfection. The data are means ± SD of 3 independent assays (*P < 0.05).
It has been well identified that the PI3K/Akt signaling pathway is over activated in AML cells, which controls the expression and function of numerous proteins that are necessary for tumor cell MDR [32–35]. Activity of PI3K inhibitors (for example, wortmannin and LY294002) in combination with chemotherapy is currently studied in cancer cell lines and animal models [36]. Expression of ST3GAL4 induces c-Met which gives rise to sLe X expression. c-Met, a unique receptor tyrosine kinase (RTK), can activate several crucial cellular growth pathways.

Table 1

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<th>Gene</th>
<th>Relative mRNA expression (×10^3)</th>
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<td>M2</td>
<td>M2/MDR</td>
<td>M3</td>
<td>M3/MDR</td>
<td>M5</td>
<td>M5/MDR</td>
</tr>
<tr>
<td>ST3GAL3</td>
<td>14.885 ± 0.702</td>
<td>0.923</td>
<td>14.239 ± 0.547</td>
<td>0.548</td>
<td>14.627 ± 0.244</td>
<td>0.563</td>
</tr>
<tr>
<td>ST3GAL4</td>
<td>9.488 ± 0.783</td>
<td>0.002**</td>
<td>9.235 ± 0.841</td>
<td>0.010**</td>
<td>9.301 ± 0.833</td>
<td>0.018***</td>
</tr>
<tr>
<td>ST3GAL6</td>
<td>0.005 ± 0.001</td>
<td>0.006</td>
<td>0.005 ± 0.002</td>
<td>0.006</td>
<td>0.004 ± 0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>FUT7</td>
<td>10.187 ± 0.819</td>
<td>0.021†</td>
<td>10.728 ± 0.744</td>
<td>0.040</td>
<td>9.793 ± 0.665</td>
<td>0.041***</td>
</tr>
</tbody>
</table>

Fluorescence intensity (%)

| sLe X    | 47.818 ± 2.691                    | 0.040†  | 45.561 ± 1.603                   | 0.026** | 48.359 ± 1.411                   | 0.041***|

* P < 0.05 vs M2 patients.
** P < 0.05 vs M3 patients.
*** P < 0.05 vs M5 patients.
including the PI3K/Akt pathway [37,38]. Moreover, the upregulation of cell surface sLe X and FucT-VII is mediated by PI3K/Akt signaling pathway [39]. The present study demonstrated that the resistant cell line HL60/ADR presented higher PI3K/Akt activity than the sensitive one, which was in accordance with the MDR phenotype. Altered expression of ST3GAL4 or FUT7 markedly modulated the activity of PI3K/Akt pathway in human AML cell lines. In addition, inhibition of the PI3K/Akt pathway with Akt-specific inhibitor LY294002, or Akt gene silencing by shRNA pretreatment reversed chemoresistance of HL60/ADR cells. Our results together with the previous findings, explored a possible mechanism of MDR in AML cells that drug resistance might develop and vary via the PI3K/Akt pathway activated by sLe X overexpression. sLe X-modulated AML cell MDR was, at least in part, PI3K/Akt-dependent.

To date, tremendous evidence indicates that tumor cells maintain the MDR through the PI3K/Akt pathway enhancing drug efflux by ATP-binding cassette (ABC) transporters [40]. PI3K inhibitor, LY294002, therefore has therapeutic potential in the treatment of MRPI-mediated drug resistance when combined with doxorubicin [41], since it is able to block P-gp expression in mouse leukemic cell lines [42]. As two main members of ABC transporters, P-gp and MRPI are frequently used as markers to screen MDR patients clinically. In addition, it has been well demonstrated that sLe X could affect EGFR dimerization [43], which activates distinct downstream signaling such as the PI3K/Akt pathway, and constitutive activation of PI3K/Akt is associated with the expression of ST3GAL4 and FUT7 [37–39]. Therefore, a close association is indicated between the levels of sLe X, ST3GAL4 and FUT7 and the levels of phosphorylated Akt, as well as P-gp, MRPI expression in AML cells. In this study, we showed that the levels of P-gp and MRPI had a proportional relationship with the expression of ST3GAL4 and FUT7 and the activity of PI3K/Akt signaling in HL60 or HL60/ADR cell lines. Since ST3GAL4 or FUT7 was observed as regulatory gene for the expression of sLe X, which regulated the activity of PI3K/Akt signaling, we assume that ST3GAL4 or FUT7 regulates the expression of sLe X inducing PI3K/Akt pathway, consequently promoting MDR of AML cells.

Although the clinical outcome of AML has been improved with advancements in chemotherapy, MDR remained a critical challenge for successful treatment. Our previous research showed that B4GALT1 expression of sLe X, which regulated the activity of PI3K/Akt signaling, we found that altered levels of these genes were probably associated with MDR phenotype in AML. On the basis of the above results, the implementation of ST3GAL4 or FUT7 as biomarkers for clinical diagnosis and prognosis of MDR of AML and as potential target for therapeutic applications in the future.

In conclusion, our data demonstrated that expression of ST3GAL4 and FUT7 could regulate sLe X oligosaccharide synthesis and sLe X-mediated drug efflux by inhibiting the PI3K/Akt pathway and the expression of P-gp and MRPI. Seeking for agents that simultaneously inhibit ST3Gal IV and FUT7 might be a promising strategy for clinical diagnosis, prognosis prediction and treatment of MDR of AML.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

 Supplementary data associated with this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.06.014.

References


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