Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia

Running title: Modification of sialylation in human AML MDR

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Aberrant cell-surface sialylation patterns have been shown to correlate with tumor progression and metastasis. However, the role of sialylation regulation of cancer multidrug resistance (MDR) remains poorly understood. The present study investigated sialylation in modification on MDR in acute myeloid leukemia (AML). Using mass spectrometry (MS) analysis, the composition profiling of sialylated N-glycans differed in three pairs of AML cell lines. Real-time polymerase chain reaction (PCR) showed the differential expressional profiles of 20 sialyltransferase (ST) genes in the both AML cell lines and bone marrow mononuclear cells (BMMC) of AML patients. The expression levels of ST3GAL5 and ST8SIA4 were detected, which were over-expressed in HL60 and HL60/ADR cells. The altered levels of ST3GAL5 and ST8SIA4 were found in close association with the MDR phenotype changing of HL60 and HL60/ADR cells both in vitro and in vivo. Further data demonstrated that manipulation of these two genes' expression modulated the activity of phosphoinositide-3 kinase (PI3K)/Akt signaling pathway and its downstream target thus regulated the proportionally mutative 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 by Akt small interfering RNA (siRNA) resulted in the reduced chemosensitivity of HL60/ADR cells. Therefore this study indicated that sialylation involved in the development of MDR of AML cells probably through ST3GAL5 or ST8SIA4 regulating the activity of PI3K/Akt signaling and the expression of P-gp and

MRP1.

Keywords: sialylation; MDR; AML; PI3K/Akt signaling; P-gp; MRP1.

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 cell populations in 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 e.g., P-gp and MRP1 belonging to the ATP binding cassette (ABC) family which lead to lower intracellular drug accumulation and thus reduce cellular toxicity of chemotherapeutic agents.[3] Nowadays, many researchers are struggling to adequately evaluate the relationship between glycan alterations and resistance to chemotherapy of cancer cells. However, there is still little information about the role of glycosyltransferases in the development of leukemia MDR in spite of the modification of glycan structures have been observed in drug-resistance leukemia cells.[4, 5]

Cell-surface glycoproteins commonly contain sialic acid (SA) as a monosaccharide located on the nonreducing terminus of glycans. Evidence from both patient histochemical analysis and experimental tumor models demonstrates that altered sialylation involves in a variety of biological processes, including cell-cell communication, cell-matrix interaction, adhesion, and protein targeting.[6,7] Sialyltransferases (ST) is a family of anabolic enzymes, consisting of 20 members that are subjected into three subfamilies. These glycosyltransferases convert sialic acid from cystidine-5-monophospho-N-acetylneuraminic acid (CMP-NeuAc) to

glycoproteins or glycolipids.[8] Alpha-2, 3-sialyltransferases mediate the transfer of sialic acid with an alpha 2, 3-linkage to it with terminal Gal residues (ST3Gal I-VI). Alpha-2, 6-sialyltransferases mediate the transfer of sialic acid with an alpha 2, 6-linkage to it with terminal Gal (ST6Gal I-II) or GalNAc residues (ST6GalNAc I-VI). Alpha-2, 8-sialyltransferases mediate the transfer of sialic acid with an alpha 2, 8-linkage (ST8Sia I-VI) to it with other SA residues.[9]

Although the modification of sialylation in solid tumors has been documented in details, [10, 11] there are not many reports in case of blood cancer. ST3Gal V was found predominantly expressed in childhood acute lymphoblastic leukemia (ALL).[12] High level of ST3Gal III, an enzyme catalyzing Sialyl-Lewis X (SLX) synthesis, produced cellular resistance to Taxol and thus reduced the efficacy of Taxol therapy.[13] Elevated mRNA levels of ST6Gal I and ST3Gal V positively correlated with the high risk of pediatric acute leukemia.[12] Endogenous expression of ST6GalNAc I in CML cell line K562 was associated with the expression of the STn O-Glycan related to a lack of response to chemotherapy.[14] Yamada et al confirmed ST6GalNAc IV as the major enzyme controlling the expression of sialyl-T antigen in leukemia cell lines.[15] ST8Sia I (GD3 synthase) was down regulated in childhood ALL while was up-regulated in adult T-cell leukemia cell lines.[12] Polysialic acid (PSA) formed by ST8Sia II and ST8Sia IV has been found on the unidentified proteins in basophilic leukemia cell lines.[16] In order to inhibit MDR of AML targeting of ST genes could be applied to cancer therapy.

Phosphoinositide 3 kinase (PI3K) /Akt signaling activation is implicated involving

in the progression of a wide variety of neoplasias.[17] Recent studies illustrate that aberrant activation of PI3K/Akt pathway leads to the short-term survival and drug resistance of different types of human neoplasm cells.[18, 19] Activation of Akt is associated with poor prognosis and chemotherapeutic resistance in pediatric B-precursor acute lymphoblastic leukemia.[20] However, little is known regarding the PI3K/Akt signaling pathways on ST-mediated leukemia MDR.

In order to understand the role of ST gene family in regulation of MDR in AML, the present study aimed to investigate the sialylated oligosaccharide alterations and mRNA expression levels of ST genes in three pairs of AML cell lines and in BMMC isolated from the diagnostic AML patients, as well as the possible mechanisms via PI3K/Akt pathway.

RESULTS

MALDI-MS analysis of N-glycan composition profiling from AML cell lines

MALDI-TOF MS analysis was utilized to evaluate the N-glycan composition profiling of HL60 and HL60/ADR cell lines. Fig. 1 showed the MS spectra of N-glycans released from cell membranes and the observed MS signals of the N-glycans (peaks 1-35 in Figure 1a) and the assigned N-glycan signals were summarized in Table 4. The observed signal intensities in the mass spectra were presented as a histogram (Figure 1b), with the estimated monosaccharide composition. High-mannose glycans (peak 4, 6, 9, 13 and 16) were observed in both cell lines (Table 4). The N-glycans detected in HL60/ADR cells showed remarkably different

profiles vs those of HL60 cells. The peaks at 5 and 30 were exclusively detected only in the HL60/ADR cell line. HL60/ADR cells also showed higher incidence of additional significant peaks at 1, 2, 7, 10, 11, 17, 21, 23, 31 and 34 (≥2 fold). Peak 14, 15, 25 and 28 (≥2 fold) clearly showed a significant increase in HL60 cell line. Surprisingly, the most significant increase peaks corresponding to sialylated oligosaccharides were observed at peak 17, 21, 23, 31 and 34 in HL60/ADR cells. The sialylated oligosaccharides observed at peaks 15, 25 and 28 also showed significant increase in HL60 sample.

Differential expression of ST family in three pairs of AML cell lines

Real-time PCR analysis showed that the expression of ST gene family was differed between the three pairs of AML cell lines. A common feature was that the expression levels of ST8SIA4 (5.81 folds), ST6GAL1 (4.56 folds), ST3GAL4 (3.18 folds) and ST6GALNAC4 (3.01 folds) were remarkably high in three MDR cells. In addition, drug sensitive cells showed higher expressional levels of ST3GAL5 (5.19 folds), ST8SIA6 (4.17 folds) and ST3GAL1 (2.72 folds). No statistically significant differences were found in the expression levels of ST3GAL2, ST3GAL3, ST6GAL2, ST6GALNAC1, ST6GALNAC2, ST6GALNAC6, ST8SIA1 and ST8SIA5 mRNA, while ST3GAL6, ST6GALNAC3, ST6GALNAC5, ST8SIA2 and ST8SIA3, were undetectable in both drug-sensitive cells and MDR cells (Figure 2a-e).

Altered expression of ST8SIA4 modulates chemosensitivity of AML cells in vitro and

in vivo

Due to the significant increase of ST8SIA4 mRNA expression in HL60/ADR cells, ST8SIA4 was silenced, by shRNA, so as to elucidate its direct effect on the chemosensitivity of HL60/ADR cells. As shown in Figure 3a.b, the expression level of ST8SIA4 was significantly reduced in HL60/ADR-shRNA transfectants compared with those in the controls both at transcription and protein level. Furthermore, α-2, 8 sialylation level detected by FITC-conjugated Siglec7 on the cell surface, was also found reduced in HL60/ADR-ST8SIA4 shRNA1 cells (Figure 3c).

As depicted in Figure 3d, in addition to adriamycin, HL60/ADR cells were also resistant to other chemotherapeutic drugs including paclitaxel and vincristine. The IC_{50} values for the drugs were greater in the HL60/ADR group than those in the HL60 group (Figure 3d), which indicated that HL60/ADR cells awarded MDR characteristics. The IC50 values for the drugs were significantly less in the HL60/ADR-ST8SIA4 shRNA1 groups than those in the HL60/ADR-control shRNA groups, suggesting that cell proliferation was inhibited and chemosensitivity was remarkably restored (Figure 3d).

Nude mice bearing HL60, HL60/ADR, HL60-control shRNA and HL60/ADR-ST8SIA4 shRNA1 xenografts were used to determine the treatment efficacy of adriamycin by measuring tumor volumes. Figure 3e showed that a significant reduction of mean tumor volume of HL60 tumors was observed, as compared with HL60/ADR groups. The mean tumor volume was significantly less in the HL60/ADR-ST8SIA4 shRNA1 groups than those in the HL60/ADR-control

shRNA groups, and the effect of concomitant application of adriamycin. IHC staining analysis of the tumor sections revealed that the expression of ST8sia IV was decreased in the mouse group treated with ST8SIA4 shRNA1 compared to that in untreated group (Fig. 3f).

To further investigate the effect of ST8SIA4 on chemoresistance, ST8SIA4 expression vector was transfected in HL60 cells and higher expression levels of ST8SIA4 were detected in HL60/ST8SIA4 cells (Figure 4a.b.c.f). The chemoresistance of HL60 cells was increased after the transfection of the ST8SIA4 expression vector in vitro and in vivo (Figure 4d.e).

We evaluated the expression of relevant substrate in the aberrantly expressed ST8SIA4 cell lines. CD147 is N-glycoprotein, which was analyzed by way of immunoprecipitation (IP). A remarkable change of CD147 expression was observed in HL60/ADR-ST8SIA4 shRNA1 and HL60/ST8SIA4 cells compared to those in the control cells. Siglec7 lectin blotting also showed the same tendency, suggesting that CD147 was a specific substrate of ST8SIA4 sialyltransferase (Supplementary Figure S1a, b).

Altered expression of ST3GAL5 regulates chemosensitivity of AML cells in vitro and in vivo

As it was found that the mRNA and protein levels of ST3GAL5 were decreased notably in HL60-ST3GAL5 shRNA transfectants (Figure 5a.b), we targeted ST3GAL5 to determine whether changed expression of ST3GAL5 could influence

1 chemosensitivity of HL60 and HL60/ADR cells. Fluorescence intensity on MAL
2 revealed less α-2, 3 sialylation in HL60-ST3GAL5 shRNA1 cells than that in control
3 cells, since lower fluorescence intensity of lectin was corresponding with lower
4 expression of glycogene (Figure 5c)

5 MTT assay results showed that the IC_{50} values for the drugs were greater in the
6 HL60/ADR group than those in the HL60 group (Figure 5d). The IC_{50} values for the
7 drugs were significantly increased in HL60-ST3GAL5 shRNA1 cells groups
8 compared to those in the HL60-control shRNA groups (Figure 5d).

9 Tumor volumes were measured and compared between the groups with or
10 without adriamycin treatment. Figure 5e displayed that a significant reduction of
11 mean tumor volume of HL60 tumors was observed, as compared with HL60/ADR
12 groups. In the group of mice bearing HL60 tumors, tumor volumes with adriamycin
13 treatment were lower than those without. But in the group of mice bearing
14 HL60-ST3GAL5 shRNA tumors, tumor volumes increased significantly even after
15 adriamycin treatment. Reduced expression level of ST3Gal V in tumor cells of
16 HL60-ST3GAL5 shRNA1 was also validated by IHC staining (Figure 5f).

17 In order to determine whether over-expression of ST3GAL5 could enhance
18 chemosensitivity of HL60/ADR cells, HL60/ADR cells were then transfected with
19 ST3GAL5 expression vector and higher levels of ST3GAL5 were detected in
20 HL60/ADR/ST3GAL5 cells (Figure 6a.b). The chemosensitivity of HL60/ADR cells
21 was increased with the ST3GAL5 expression vector transfection in vitro and in vivo
22 (Figure 6d.e). IHC staining and flow cytometry analysis revealed elevated expression

1 of ST3Gal V in HL60/ADR/ST3GAL5 cells (Figure 6c.f).

2 Futher on, we investigated the expression of relevant substrate in the aberrantly 3 expressed ST3GAL5 cell lines. CD147 and MAL lectin showed no difference in 4 expression among the six groups, suggesting that CD147 was not a relevant substrate 5 of ST3GAL5 sialyltransferase (Supplementary Figure S1c, d).

6

7 Effect of ST3GAL5 or ST8SIA4-activated PI3K/Akt signaling pathway on the 8 expression of P-gp and MRP1

9 Here, we investigated whether ST3GAL5 or ST8SIA4 activated the PI3K/Akt 10 pathway and whether this pathway was involved in ST3GAL5 or ST8SIA4-mediated 11 cell MDR. Western blotting (Figure 7b.c) showed that the levels of the main 12 molecules of PI3K/Akt signaling pathway, P110α (the catalytic subunit of PI3K), 13 phosphorylation Akt at Ser473 and Thr308, and its downstream effector NF-κB were 14 significantly increased in HL60/ST8SIA4 cells and HL60-ST3GAL5 shRNA1 cells, 15 while decreased in HL60/ADR/ST3GAL5 cells and HL60/ADR-ST8SIA4 shRNA1 16 cells (Figure 7a.d). However, there was no change in the total amount of Akt protein. 17 Interestingly, we also revealed the same tendency of PI3K/Akt/mTOR pathway on 18 stimulation with ST3GAL5 or ST8SIA4 (Supplementary Figure S2).

19 Moreover, we investigated whether ST3GAL5 or ST8SIA4 could influence the 20 expression of P-gp and MRP1. Interestingly, flow-cytometric analysis (Figure 7f.g) 21 illustrated that elevated expression levels of P-gp and MRP1 were detected in 22 HL60/ST8SIA4 cells and HL60-ST3GAL5 shRNA1 cells compared to those in the

1 control groups. In addition, HL60/ADR cells expressed lower levels of P-gp and
2 MRP1 with ST8SIA4 suppression and ST3GAL5 overexpression. (Figure 7e.h)
3
4 PI3K/Akt inhibition modulates the chemoresistance of HL60/ADR cells both in vitro
5 and in vivo
6 The effect of PI3K/Akt signaling activity on chemoresistance of HL60/ADR cells was
7 explored by pharmacologic inhibition of the PI3K/Akt pathway. By western blotting,
8 HL60/ADR cells treated with LY294002 or Akt siRNA exhibited apparently
9 decreased expression levels of the main signal molecules of PI3K/Akt pathway
10 (Figure 8a). As seen in Figure 8b, the inhibition of PI3K/Akt pathway resulted in the
11 HL60/ADR cells susceptible to chemotherapy. The similar results were also obtained
12 in *in vivo* chemosensitivity analysis that reduced tumor volumes were detected in
13 mouse group bearing HL60/ADR tumors with impaired PI3K/Akt signaling (Figure
14 8c). Altered expression levels of the main signal molecules of PI3K/Akt pathway in
15 mouse group bearing HL60/ADR tumors with LY294002 or Akt siRNA treatment
16 were also validated by IHC staining, as shown in Figure 8d. Moreover, the inhibitor of
17 PI3K/Akt or silencing Akt reduced the expression of P-gp and MRP1 (Figure 8e).
18
19 Differential expression of ST gene family in AML patients
20 Expression of MDR-related marker, ST gene family presenting in BMMC of AML
21 patients is summarized in Table 4. The frequency of P-gp positivity was 90.8% (69 of
22 76) in the AML patients. The BMMC were first divided into six groups, M2,

M2/MDR, M3, M3/MDR, M5 and M5/MDR, and were evaluated the mRNA expression level of ST gene family. The groups of M2/MDR, M3/MDR and M5/MDR showed significantly high ST3GAL4 (P=0.002; P=0.006; P=0.009), ST6GAL1 (P=0.004; P=0.002; P=0.004), ST6GALNAC4 (P=0.003; P=0.001; P=0.003) and ST8SIA4 (P=0.001; P=0.004; P=0.008) mRNA expression compared to those of the chemosensitive group. In contrast, group of M2, M3, M5 without MDR showed significantly up regulated ST3GAL1 (P=0.032; P=0.002; P=0.025), ST3GAL5 (P=0.001; P=0.001; P=0.003) and ST8SIA6 (P=0.002; P=0.001; P=0.002) expression compared to AML/ADR group. Expression of the other members of ST family showed no difference between the two groups, while ST3GAL6, ST6GALNAC3, ST6GALNAC5, ST8SIA2 and ST8SIA3 were detected slightly.

DISCUSSION

MDR is the major obstacle to the efficiency of chemotherapy in the treatment of leukemia.[21] In this study, we explored the possible mechanism of sialylation modification on MDR in hunman AML cell lines. We also investigated the differential expression of ST family, which was reported to be correlated with clinicopathological characteristics in AML patients.

Recent development in mass spectrometry (MS) technology has fueled high throughput analyses of glycoproteins.[22, 23] MS technology as a novel methodology provides high sensitivity and more rapid glycan analysis.[24] Zhang et al have investigated novel N-glycan changes involved in MDR between leukemia cell line

K562 and adriamycin resistant K562/ADR by MALDI-TOF/MS.[5] To identify sialylated N-glycan associated with MDR of human AML, we used MS method to analyze the composition profiling of N-glycans. We compared the total N-glycans from HL60 and HL60/ADR cell lines, and found dramatic differences in N-glycan profiles between these two groups (Figure 1, Table 4). A major population of N-glycans detected in HL60 and HL60/ADR cells was corresponded to high-mannose structures (peak 4, 6, 9, 13 and 16). The peaks at 5 and 30 were exclusively detected only in the HL60/ADR cell line. Major peaks (peak 1, 2, 7, 10, 11, 17, 21, 23, 31 and 34) corresponded to sialylated oligosaccharides originating from HL60/ADR cells showed a significant increase (≥2 fold). Moreover, peaks 14, 15, 25 and 28 corresponded to sialylated oligosaccharides originating from HL60 cells also showed a significant increase (≥2 fold). Therefore, monitoring of the sialylated N-glycan profile would be an important step in the prevention of tumor MDR and would increase our understanding of MDR mechanisms.

Aberrant expression of sialylated glycans in various cancers is recognized as prognostic factors and potential targets for therapeutic approaches.[25] Malignant cell surface properties are generally changed, which mainly due to altered sialoglycoconjugates expressed on the plasma membrane.[26] The biosynthetic pathway of sialylated glycans in leukemia pathogenesis highlighted the importance of ST in AML. In this study, we revealed the differential expression of ST genes in three pairs of AML cell lines. All MDR cells were characterized by higher levels of ST8SIA4 (5.81fold, Figure 2) while parental cells expressed more ST3GAL5 (5.19 fold),

suggesting that the AML cell lines displayed altered α2, 3- and α2, 8-linked sialylation.

ST3GAL5 encodes Lactosylceramide α-2, 3-sialyltransferase that catalyzes the formation of ganglioside GM3. GM3 is a sialylated trisaccharide antigen richly expressed by a number of tumors and thus becomes an important molecular target in the development of novel diagnostic and therapeutic strategies for cancers.[27] ST8SIA4 encodes N-acetylgalactosaminide α-2, 8-sialyltransferase IV (ST8Sia IV) that catalyzes the polycondensation of α-2, 8-linked sialic acid required for the synthesis of polysialic acid, a modulator of the adhesive properties of neural cell adhesion molecule (NCAM) which is implicated in tumor formation/metastasis.[28] It is of interest to know whether the change of ST3GAL5 or ST8SIA4 confers the alteration of MDR is in leukemia cells. Our former work demonstrated that the expression of ST3GAL5 or ST8SIA4 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 ST3GAL5 or ST8SIA4 led to drug-resistant phenotype changes of HL60 and HL60/ADR cells both in vitro and in vivo (Figure 3, 4, 5, 6). Based on these results we hypothesized that ST3GAL5 or ST8SIA4 might function as a pivotal modulator to MDR in AML.

N-glycans bind proteins, and the combinations of glycan and protein may be important for their function. [29, 30] In this study, we analyzed the expressions of CD147 in AML cell lines to identify specific, relevant substrates of the aberrantly expressed sialyltransferases. CD147 is a highly N-glycosylated immunoglobulin superfamily

transmembrane protein that is composed of two extracellular Ig domains, which contribute to a highly N-glycosylated HG-CD147 (~40–60 kDa) and a low glycosylated form, LG-CD147 (~33 kDa).[31] Our previous results showed that altered N-glycosylation of CD147 was found in K562/ADR cells, and further suggested a link between defective N-glycosylation of K562/ADR cells and drug resistance.[5] In this study, the results confirmed that CD147 was a specific substrate of ST8SIA4 sialyltransferase, rather than a relevant substrate of ST3GAL5 sialyltransferase (Supplementary Figure S1).

PI3K/Akt signaling pathway is reported to be over-activated in AML cells and plays an essential role in proliferation, drug resistance, motility, invasion and inhibition of apoptosis in cancer cells.[32-35] PI3K/Akt inhibition modulates MDR and activates NF-κB in murine lymphoma cell lines.[36] Inhibition of the PI3K/mTOR pathway is a promising therapeutic approach in patients with acute lymphoblastic leukemia.[37] A number of studies have also demonstrated that GM3 gangliosides inhibit phosphorylation of the epidermal growth factor receptor (EGFR) that results in inhibition of PI3K/Akt signaling in varied cell types.[38, 39] NCAM-mediated adhesion leads to activation of various intracellular signal transduction pathways, including the Ras-mitogen activated protein kinase (MAPK) and the PI3K/Akt pathways.[40] In this study we found that a novel mechanism by which MDR of AML cells can be developed and changed, i.e. MDR is induced though activation of the PI3K/Akt pathway by sialylation modification. The resistant cell line HL60/ADR exhibited higher PI3K/Akt activity than the sensitive one, which was in accordance with the

1 MDR phenotype. Suppression of ST3GAL5 or ST8SIA4 markedly modulated the
2 activity of PI3K/Akt pathway and its downstream target NF-κB and mTOR in AML
3 cell lines (Figure 7a-d and Supplementary Figure S2). In addition, inhibition of the
4 PI3K/Akt pathway with LY294002 or Akt gene silencing by siRNA pretreatment
5 reversed chemoresistance of HL60/ADR cells (Figure 8b.c). These results indicated
6 that ST3GAL5 or ST8SIA4-modulated MDR in AML cell lines is, at least in part,
7 PI3K/Akt-dependent.

8 To date, tremendous evidence indicates that tumor cells maintain the MDR
9 through the PI3K/Akt pathway enhancing drug efflux by ATP-binding cassette (ABC)
10 transporters.[41] As two main members of ABC transporters, P-gp and MRP1 are
11 frequently used as markers to screen MDR patients clinically. PI3K inhibitor,
12 LY294002, therefore has therapeutic potential in the treatment of MRP1-mediated
13 drug resistance when combined with doxorubicin, [42] since it is able to block P-gp
14 expression in mouse leukemic cell lines.[43] A recent report in acute myelogenous
15 leukemia has demonstrated that MRP-1 but not P-gp efflux was inhibited by the PI3K
16 inhibitor wortmannin.[41] Moreover, constitutive activation of PI3K/Akt is associated
17 with the expression of ST3GAL5 and ST8SIA4.[38-40] Therefore, a close association is
18 found between the levels of ST3GAL5 and ST8SIA4 and the levels of phosphorylated
19 Akt, as well as P-gp, MRP1 expression in leukemic cells. In this study, we showed
20 that, the levels of P-gp and MRP1 had a proportional relationship with the expression
21 of ST3GAL5 or ST8SIA4 and the activity of PI3K/Akt signaling in HL60 or
22 HL60/ADR cell lines. Since ST3GAL5 or ST8SIA4 was observed as regulatory gene

1 for the activity of PI3K/Akt signaling, we assume that ST3GAL5 or ST8SIA4 might
2 regulate the expression of P-gp and MRP1 through PI3K/Akt pathway, thereby
3 promoting MDR of leukemia cells.

4 Although the clinical outcome of AML has been improved with advancements in
5 chemotherapy, MDR remains a critical challenge for successful treatment. A great
6 number of AML (including M2, M3 and M5) patients were examined and analyzed in
7 the present study, while more than 90% of the AML patients were found resistant to
8 the anticancer drugs. ST3GAL4, ST6GAL1, ST6GALNAC4 and ST8SIA4 were
9 expressed at a high level in BMMC of a significant proportion of M2/MDR,
10 M3/MDR and M5/MDR patients, whereas expression of ST3GAL1, ST3GAL5 and
11 ST8SIA6 were at a high level in BMMC of a number of cases M2, M3 and M5
12 without MDR. Once again the findings from the clinical samples confirm that altered
13 levels of these genes were probably associated with MDR phenotype in AML. On the
14 basis of the above results, it might be possible to utilize differential expressional ST
15 genes as useful leukemia biomarkers for clinical diagnosis of drug resistant leukemia
16 and as potential targets for therapeutic approaches in the future.

17 In summary, our work reveals differential expression patterns of ST genes in
18 three pairs of AML cell lines and in BMMC of the AML patients. Altered ST3GAL5
19 and ST8SIA4 presented the unusual property of association with MDR of AML cells
20 via regulating the PI3K/Akt signaling pathway and the expression of P-gp and MRP1.
21 MDR in AML is a multifactorial phenomenon and targeting these molecules seems to
22 be a feasible approach to clinical diagnosis and treatment of MDR AML.

1 **MATERIALS AND METHODS**

2 Parental AML cell culture

3 Three AML cell lines, including an acute myelogenous leukemia (M2) cell line HL60,
4 an acute promyelocytic leukaemia (M3) cell line NB4, and a leukemic monocytic
5 lymphoma (M5) cell line U937 were obtained from the KeyGEN Company (China).
6 All cell lines were cultured as previously described.[52] Adriamycin (Sigma) was added
7 to parental cell cultures in stepwise increasing concentrations from 0.1μg/ml to
8 5μg/ml for 2 months to develop an adriamycin-resistant (ADR) subline, namely
9 HL60/ADR, NB4/ADR and U937/ADR, correspondingly. To maintain the MDR
10 phenotype, the complete medium of the resistant cell clones were supplemented with
11 1.0 mg/L adriamycin. ADR cells were maintained in complete medium without
12 adriamycin for one week and cells with >90% viability prior to subsequent treatments.

13

14 Samples from leukemia patients

15 76 previously untreated AML patients comprising 53 males and 23 females, with age
16 ranging from 12 to 79 years (median age of 42 years) and 7 healthy donors were
17 included in this study. The diagnosis of AML was based on cytomorphology,
18 cytochemistry, multiparameter flow cytometry, immunology, molecular genetics and
19 cytogenetics, and the leukemic subtypes of AML were determined according to the
20 French-American-British classification as follows: 35, 27 and 14 cases of M2, M3 and
21 M5, respectively. All AML patients who were obtained from June 2010 to May 2012
22 at the First Affiliated Hospital of Dalian Medical University (Dalian, China) provided

written informed consent, and the institutional ethics committees approved the study as well as contents of the written consent. Bone marrow samples were collected at diagnosis and before treatment. BMMC were isolated by Ficoll-Hypaque density gradient centrifugation and were cultured in plastic dishes to remove adherent cells at 37°C for 24 h. Freshly separated non-adherent cells were maintained in modified Dulbecco's medium containing 10% fetal bovine serum, 10 mM β-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL human stem cell factor, 10 ng/mL human interleukin-3, and 10 ng/mL human interleukin-6. Patients' clinical characteristics were given in Table 2.

Membrane protein extract and release of N-glycans from cell membrane proteins

Membrane protein extract has been described previously.[8] For releasing of N-glycans, three 100 μg aliquots of lyophilized cell membrane proteins were first digested with trypsin (10 μg) and chymotrypsin (10 μg) dissolved in 25 mM ammonium bicarbonate (25 μL) at 37°C for 18 h. The digest was left in a water bath (85°C, 5 min) and after cooling N-linked oligosaccharides were released from peptides by treatment with PNGaseF enzyme (2μL; 6U) at 37°C (18 h) followed by Pronase digestion (10 μg) at 37°C (8 h). During the incubation time, the reaction sample was mixed occasionally. The released N-glycans were purified using an Oasis HLB cartridge (60 mg/3ml; Waters) and then were lyophilized.

MS analysis

1 The mass spectra were carried out in reflectron positive ion mode with MALDI-TOF
2 MS ((Bruker Corp., Billerica, MA, USA). To increase sensitivity and provide more
3 informative fragmentation, the released glycans were permethylated and further
4 characterized by MALDI-TOF MS. For the type of MALDI analysis of the
5 permethylated glycans, 2, 5-DHB was used as the matrix. All MS spectra were
6 obtained from Na+ adductions.
7

8 Real Time PCR analysis

9 Total RNA was isolated with Trizol reagents (Gibco BRL, Rockville, MD, USA),
10 treated by QuantiTect Reverse Transcription Kit (QIAGEN, valencia, CA) and
11 QuantiTect SYBR Green PCR Kit (QIAGEN, valencia, CA). The primer pairs for
12 PCR are listed in Table 1. Level of GAPDH mRNA was measured for standardization
13 and calcultingated as $2^{-(Ct_{\text{Target gene}} - Ct_{\text{GAPDH}})}$.
14

15 Western blot analysis

16 Western blot analysis was carried out as described previously.[52] Briefly, samples (20
17 μg protein) were subjected to SDS–PAGE. After transfer of proteins onto the
18 polyvinylidene difluoride membrane, the blots were probed with the following
19 antibodies: rabbit anti-human ST8Sia IV, ST3Gal V, PI3K p110α, p-Akt 308, p-Akt
20 473, Akt, NF-κB antibodies (Abgent, Cambridge, UK, 1:1000 dilution), and then
21 incubated with secondary antibody anti-rabbit-HRP (1:2000 diluted, Santa Cruz
22 Biotech, Santa Cruz, CA). CD147, mTOR, p-mTOR^{ser2448}, p-p70S6K^{Thr389}, and

GAPDH antibody (1:200 diluted, Santa Cruz Biotech, Santa Cruz, CA) was used as a control.

Deregulation of ST3GAL5 or ST8SIA4 by RNAi

RNAi was performed as previously described.[52] The cell cultures were transfected with ST3GAL5 or ST8SIA4 specific shRNA, and scrambled shRNA used as the negative control. ST3GAL5 shRNA or ST8SIA4 shRNA was mixed with Lipofectamine™ 2000 (Invitrogen). Transfer cells were cultured and incubated at 37°C for 6 h, followed by incubation with complete medium for additional 24h. The cell transfection efficiency was 85% by fluorescent microscope and the cell viability was 90 % by trypan blue dye exclusion assay.

Over-expression of ST3GAL5 or ST8SIA4

The human ST3GAL5 and ST8SIA4 coding sequences were obtained from TaKaRa company (Dalian, China) and were inserted into the pEGFP-N2 vector (Invitrogen, Carlsbad, CA) respectively using EcoRI, XhoI sites. Cells were transfected with 5μg 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 ST3GAL5 (HL60/ADR/ST3GAL5) and ST8SIA4 (HL60/ST8SIA4), empty vector (HL60/ADR/mock, HL60/mock) were established. The cell transfection efficiency was 79% and the survival rate was 90%.

In vitro drug cytotoxic assay

Drug resistance was evaluated by MTT assay as previously described.[52] Briefly, Cells (1×10^4) were plated in 96-well plate and allowed to grow for 48 h before the addition of MTT. The spectrometric absorbance was measured at 490 nm by microplate reader (Model 680; Bio-Rad, Hercules, CA).

In vivo chemosensitivity assay

Animal studies were performed as previously described.[52] Briefly, when mice bearing palpable tumors, HL60, HL60/mock, HL60/ST8SIA4, HL60-control shRNA, HL60-ST3GAL5 shRNA-1, HL60/ADR, HL60/ADR/ST3GAL5, HL60/ADR-control shRNA, HL60/ADR-ST8SIA4 shRNA-1 tumor-bearing mice were randomly divided into control and treatment groups (n=6 animals per group). The treatment groups received 7 mg/kg adriamycin i.p. three times a week for 3 weeks, and the control groups received physiological saline alone. The tumor volume was calculated by the following formula: Tumor volume = $1/2(\text{length} \times \text{width}^2)$.

Immunohistochemical (IHC) staining analysis

Visible tumors were removed from the mice and immunohistochemistry was performed on paraffin embedded sections. The slides were dried, deparaffinized, rehydrated. After deparaffinization and blocking of endogenous peroxidase, the slides were labeled overnight at 4°C with antibodies (Abcam, Cambridge, UK) at a dilution

of 1:200. The following staining was performed at 37°C for 60min with secondary streptavidin-HRP-conjugated antibody (Santa Cruz Biotech, Santa Cruz, CA). Finally, the sections were counterstained with hematoxylin and cover-slipped.

Inhibition of the PI3K/Akt signaling

LY294002 (Sigma) or Akt siRNA was used to suppress the activity of the PI3K/Akt signaling in HL60/ADR cells. Briefly, cells (1×10^4 cells per well) were incubated with DMSO, the PI3K inhibitor LY294002 (10 μM) dissolved in DMSO, Akt control siRNA and Akt siRNA, and collected after 24 h.

Flow cytometry analysis

Flow cytometry analysis has been described previously.[52] For surface staining of P-gp and MRP1, aliquots of cells were incubated with fluorescein isothiocyanate (FITC)-anti human P-gp, MRP1 (Abcam, Cambridge, UK) or an isotype control antibody (Santa Cruz Biotech, Santa Cruz, CA). For detection of α-2, 3 andα-2, 8 sialylation, cell lysates were incubated with FITC-MAL or Sig 7 lectin (Sigma, St Louis, MO, USA). After repeated centrifugation at 1000 r/min, labeled cells were resuspended in 0.2 ml PBS and were analyzed with FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data were expressed as mean± SD and Student's t-test was carried out. $P < 0.05$ was

considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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20

21

22 **Figure legends**

1 **Figure 1.** Differential N-glycan composition of HL60 and HL60/ADR cell lines. (a) MALDI-TOF
2 MS spectra of permethylated N-glycans released from HL60 and HL60/ADR cells, respectively.
3 (b) Histograms of relative intensities of the differential glycan signals were observed. The signals
4 indicated with Arabic numerals are summarized in Table 4.
5
6 **Figure 2.** Differential expression of ST gene family in three pairs of AML cell lines. (a-e) The
7 mRNA levels of ST gene family analyzed by real-time PCR. The relative amount of gene
8 mRNA level was normalized to GAPDH level. Three MDR cells expressed higher levels of
9 ST3GAL4, ST6GAL1, ST6GALNAC4 and ST8SIA4 mRNA, while their parental cell types
10 expressed higher levels of ST3GAL1, ST3GAL5 and ST8SIA6 mRNA (*$P<0.05$). Data are the
11 means ± SD of triplicate determinants.
12
13 **Figure 3.** Silence of ST8SIA4 gene facilitates HL60/ADR cells sensitive both in vitro and in vivo.
14 (a) Silencing of ST8SIA4 in HL60/ADR cells was analyzed by RNAi approach. ST8SIA4
15 transcripts were decreased apparently in HL60/ADR cells by shRNA treatment. (b) After shRNA
16 transfection, distinct reduction of ST8SIA4 was observed at protein levels by western blot analysis.
17 (c) Flow cytometry analysis showed α-2, 8 sialylation level detected by FITC-conjugated Siglec7
18 on the cell surface, was also reduced in HL60/ADR-ST8SIA4 shRNA1 cells. (d) Cell
19 chemosensitivity was assessed by cytotoxicity assays. The reported values were the IC_{50} (Mean ±
20 SD) of three independent experiments. IC_{50} represents the drug concentration producing 50%
21 decrease of cell growth. *$P<0.05$ vs HL60/ADR cells. **$P<0.05$ vs HL60/ADR-control shRNA
22 cells. (e) A decrease of mean tumor volume in mice group with HL60/ADR-ST8SIA4 shRNA1

tumors was observed, as compared to the control group. Within HL60/ADR-ST8SIA4 shRNA1 group, a decrease of tumor growth was found in group with ADR, compared with that without ADR (*P<0.05). (f) Reduced regulation of ST8Sia IV was also shown by IHC staining in xenograft tumors derived from HL60/ADR-ST8SIA4 shRNA1 cells (400×). The data are means ± SD of 3 independent assays (*P<0.05).

Figure 4. Overexpression of ST8SIA4 mediates the acquirement of MDR in HL60 cells. After full-length sequences transfection, ST8SIA4 mRNA (a) and protein (b) were increased notably in HL60 cells by real time PCR and western blot. (c) Flow cytometry analysis showed α-2, 8 sialylation level detected by FITC-conjugated Siglec-7 on the cell surface, was also increased in HL60/ ST8SIA4 cells. The chemoresistance of HL60 cells was increased with the ST8SIA4 expression vector transfection in vitro (d) and in vivo (e). (f) Up-regulation of ST8Sia IV was also shown by IHC staining in xenograft tumors derived from HL60/ST8SIA4 cells (400×). The data are means ± SD of 3 independent assays (*P<0.05).

Figure 5. Silence of ST3GAL5 gene increases the chemoresistance of HL60 cells both in vitro and in vivo. After transfection, ST3GAL5 mRNA (a) and protein (b) were reduced notably in HL60 cells. (c) Fluorescence intensity on MAL revealed less α-2, 3 sialylation in HL60-ST3GAL5 shRNA1 cells than that in nontransfection cells. The chemoresistance of HL60 cells was increased with the ST3GAL5 shRNA1 transfection in vitro (d) and in vivo (e). (f) Down-regulation of ST3Gal V was also shown by IHC staining in xenograft tumors derived from HL60-ST3GAL5 shRNA1 cells (400×). The data are means ± SD of 3 independent assays (*P<0.05).

Figure 6. Overexpression of ST3GAL5 gene enhances the chemosensitivity of HL60/ADR cells both in vitro and in vivo. (a-b) Higher levels of ST3GAL5 were detected in HL60/ADR/ST3GAL5 cells. (c) Fluorescence intensity on MAL revealed higher α-2, 3 sialylation in HL60/ADR/ST3GAL5 cells than that in nontransfection cells. The chemosensitivity of HL60/ADR cells was increased with the ST3GAL5 expression vector transfection in vitro (d) and in vivo (e). (f) IHC staining showed an enhanced expression of ST3Gal V in xenograft tumors derived from HL60/ADR/ST3GAL5 cells (400×). The data are means ± SD of 3 independent assays (*$P<0.05$).

Figure 7. Effect of ST8SIA4 or ST3GAL5-activated PI3K/Akt/NF-κB signaling pathway on the expression of P-gp and MRP1. (a-d) Expression of PI3K/Akt/NF-κB signaling molecules were altered at protein levels with ST8SIA4 shRNA1 and ST8SIA4 expression vector transfection in HL60 cells or ST3GAL5 shRNA1 and ST3GAL5 expression vector transfection in HL60/ADR cells. Flow cytometry analysis revealed a higher expression of P-gp and MRP1 in HL60-ST3GAL5 shRNA-1 (g) or HL60/ST8SIA4 cells (f). Decreased expression of P-gp and MRP1 were examined by flow cytometry analysis in HL60/ADR-ST8SIA4 shRNA-1 (e) or HL60/ADR/ST3GAL5 cells (H). The data are means ± SD of 3 independent assays (*$P<0.05$).

Figure 8. PI3K/Akt inhibition modulates the chemosensitivity of HL60/ADR cells both in vitro and in vivo. (a) The HL60/ADR cells were pretreated LY294002 or Akt siRNA. Expression of PI3K/Akt/NF-κB signaling molecules were then examined by western blot analysis. LY294002 or

1 Akt siRNA treatment also alleviated chemoresistance of HL60/ADR cells, revealed by in vitro (b)

2 and in vivo (c). (d) Down-regulation of PI3K/Akt signaling molecules was also shown by IHC

3 staining in xenograft tumors derived from LY294002 or Akt siRNA treatment cells (400×). (e)

4 Flow cytometry analysis showed that suppression of PI3K/Akt signaling resulted in reduced level

5 of P-gp and MRP1. The data are means ± SD of 3 independent assays. $^{*}P<0.05$ vs DMSO

6 treatment cells; $^{\#}P<0.05$ vs control siRNA treatment cells.

7

8

9

a

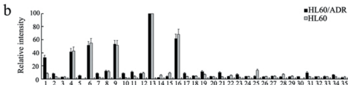
HL60/ADR

HL60

Intensity

m/z

b



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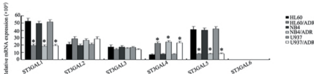
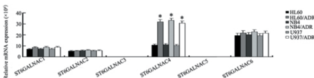
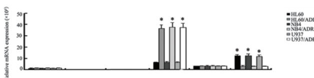


b

c

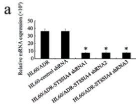


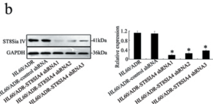
d

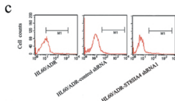


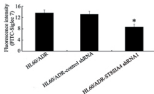
e

Gene	Ratio (≥2-fold)					
	HL60ADR / HL60	HL60 / HL60/ADR	NB4/ADR / NB4	NB4 / NB4/ADR	U937/ADR / U937	U937 / U937/ADR
ST8SIA4	5.81±0.64		5.78±0.61		5.88±0.72	
ST6GAL1	4.56±0.57		4.27±0.52		4.75±0.63	
ST3GAL4	3.18±0.42		3.04±0.46		3.34±0.50	
ST6GALNAC4	3.01±0.48		2.98±0.44		3.11±0.52	
ST3GAL5		5.19±0.66		5.06±0.58		5.24±0.75
ST8SIA6		4.17±0.52		4.06±0.49		4.57±0.52
ST3GAL1		2.72±0.41		2.66±0.37		2.78±0.45



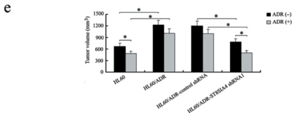


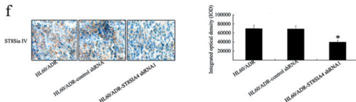


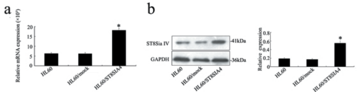


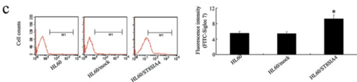
d

Drugs (mg/L)	HL60	HL60/ADR	HL60/ADR-control shRNA	HL60/ADR-ST8SIA4 shRNA1
Adriamycin	0.5922±0.0669 *	74.1203±7.8853	73.9425±7.5385	45.1633±5.4015 **
Paclitaxel	0.2895±0.0386 *	9.4871±0.8519	9.0558±0.9536	5.7719±0.6002 **
Vincristine	1.2118±0.2981 *	65.1139±6.9525	64.8813±6.0750	38.5521±4.2153 **



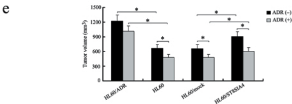


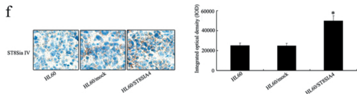


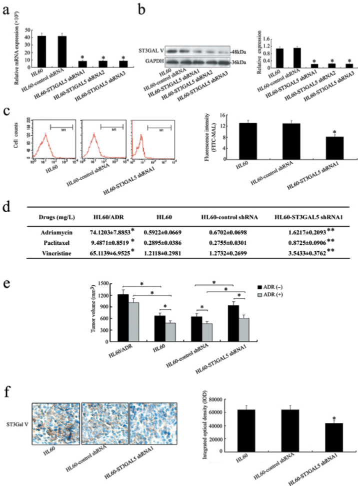


d

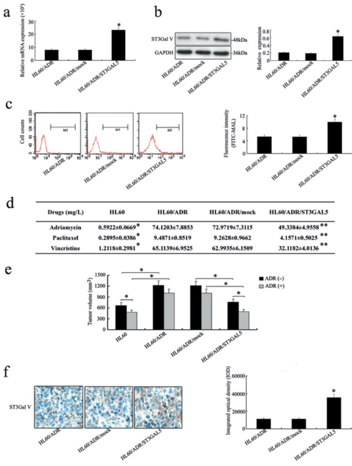
Drugs (mg/L)	HL60/ADR	HL60	HL60/mock	HL60/ST8SIA4
Adriamycin	74.1203±7.8853 *	0.5922±0.0669	0.6107±0.0655	1.4173±0.2232**
Paclitaxel	9.4871±0.8519 *	0.2895±0.0386	0.2549±0.0296	0.7295±0.0896**
Vincristine	65.1139±6.9525 *	1.2118±0.2981	1.2420±0.2456	3.1685±0.3926**



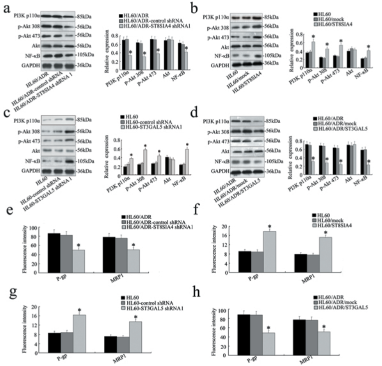


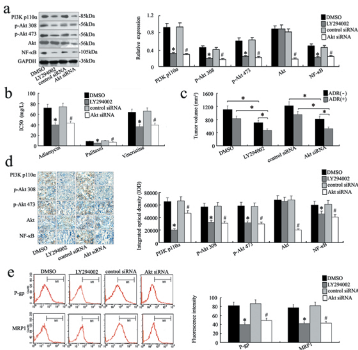


Drugs (mg/L)	HL60/ADR	HL60	HL60-control shRNA	HL60-ST3GAL5 shRNA1
Adriamycin	74.1203±7.8853*	0.5922±0.0669	0.6702±0.0698	1.6217±0.2093**
Paclitaxel	9.4871±0.8519*	0.2895±0.0386	0.2755±0.0301	0.8725±0.0906**
Vincristine	65.1139±6.9525*	1.2118±0.2981	1.2732±0.2699	3.5433±0.3762**



Drugs (mg/L)	HL60	HL60/ADR	HL60/ADR/mock	HL60/ADR/ST3GAL5
Adriamycin	0.5922±0.0669*	74.1203±7.8853	72.9719±7.3115	49.3384±4.9558**
Paclitaxel	0.2895±0.0386*	9.4871±0.8519	9.2628±0.9662	4.1571±0.5025**
Vincristine	1.2118±0.2981*	65.1139±6.9525	62.9935±6.1509	32.1182±4.0136**


a
PI3K p110α
p-Akt 308
p-Akt 473
Akt
NF-κB
GAPDH
-85kDa
-56kDa
-56kDa
-56kDa
-105kDa
-36kDa
HL60/ADR
HL60/ADR-control shRNA
HL60/ADR-ST8SIA4 shRNA1
Relative expression
b
HL60
HL60/mock
HL60/ST8SIA4
c
HL60
HL60-control shRNA
HL60-ST3GAL5 shRNA1
d
HL60/ADR
HL60/ADR/mock
HL60/ADR/ST3GAL5
e
Fluorescence intensity
P-gp
MRP1
f
g
h



a
PI3K p110α
p-Akt 308
p-Akt 473
Akt
NF-κB
GAPDH
85kDa
56kDa
56kDa
56kDa
105kDa
36kDa
DMSO
LY294002
control siRNA
Akt siRNA
Relative expression
PI3K p110α
p-Akt 308
p-Akt 473
Akt
NF-κB
b
IC50 (mg/L)
Adiamycin
Palitaxel
Vincristine
c
Tumor volume (mm³)
ADR(-)
ADR(+)
d
Integrated optical density (IOD)
e
P-gp
MRP1
Fluorescence intensity

Table1 qRT-PCR conditions and primer sequences for analysis of gene expression

Gene	Primers	Amplicon
ST3GAL1	5'-CAGAGATGGACGGTCACT-3'; 5'-CAACTGTGGTTTCTGACG-3'	197bp
ST3GAL2	5'-GTGCCTCCGACTGGTTTG-3'; 5'-GAAGCGGTAGGGGTTCTC-3'	191bp
ST3GAL3	5'-TATGCTTCAGCCTTGATG-3'; 5'-TTGGTGACTGACAAGATGG-3'	164bp
ST3GAL4	5'-ATGTTGGCTCTGGTCCTG-3'; 5'-AGGAAGATGGGCTGATCC-3'	176bp
ST3GAL5	5'-CAAAGCAAGATGAGAAGG-3'; 5'-AAACTTGGGACGACATTC-3'	213bp
ST3GAL6	5'-ATGTCTATTGGGTGGCAC-3'; 5'-CGCACACAGAAAAGGGTG-3'	189bp
ST6GAL1	5'-CTTGTTTTCCTGCTCAGA-3'; 5'-GCAAACAGAAGAAAGACCA-3'	166bp
ST6GAL2	5′-ACGCTGCTGATTGACTCTTCT-3′; 5′-CACATACTGGCACTCATCTAA-3′	160bp
ST6GALNAC1	5'-CTGGTCTTCTTTCTCTTCG-3'; 5'-GTTGAGGGCATTGTTCTCT-3'	192bp
ST6GALNAC2	5'-CTTTGCCCTGTACTTCTCG-3'; 5'-CAGCACTGGAATGGAGAGA-3'	205bp
ST6GALNAC3	5'-GGACAACCTGGTACAAAGT-3'; 5'-TATCTCATTTCCCACCTTC-3'	174bp
ST6GALNAC4	5'-ACCTGCCTGGACCACCACT-3'; 5'-TCGGCACTGTCGATCTCAG-3'	188bp
ST6GALNAC5	5'-TGGACGGATACCTCGGAGT-3'; 5'-GTCTGGTCAATCTGGGAGC-3'	121bp
ST6GALNAC6	5'-ACCTACCCCTCAGCAGACG-3'; 5'-CTTGAGGTTGACAGGTCGG-3'	179bp
ST8SIA1	5'-TACTCTCTCTTCCCACAGG-3'; 5'-GACAAAGGAGGGAGATTGC-3'	149bp
ST8SIA2	5'-GTGGTCTTCCTCATCTTCG-3'; 5'-GAGGAGCCGTTTATTACAAC-3'	140bp
ST8SIA3	5'-ATTCTCTCACCCAGGAACTC-3'; 5'-CAATCCGAACACTATTCTTG-3'	141bp
ST8SIA4	5'-CAAGAACTGAGGAGCACC-3'; 5'-TTTCCAACCTTCTACATTGTG-3'	140bp
ST8SIA5	5'-CCTTTGCCTTGGTGACCT-3'; 5'-CATGGACAGCACCTTCACT-3'	152bp
ST8SIA6	5'-CGGCAAGCAGAAGAATATG-3'; 5'-GCTTTCCACCTCGTAACTC-3'	126bp
GAPDH	5'-CTCCTCCACCTTTGACGCTG-3'; 5'-TCCTCTTGTGCTCTTGCTGG-3'	175bp

Table 2 Clinicopathologic characteristics of the leukemia patients

Patients Demographics	Subcategory	AML(n=76)		
		M2	M3	M5
Gender	Male	24	19	10
-	Female	11	8	4
Age(years)	Median	42	44	38
-	Range	15-79	18-70	12-70
Splenic enlargement	-	25	18	10
Hemoglobin<110.0g/l	-	34	25	12
WBC count (10^9/L)	20-100	19	17	7
	>100	2	0	1
Platelet count (10^9/L)	<300	32	26	14
	>300	3	1	0
P-gp (+)	-	33	24	12
MRP1 (+)	-	31	23	12

Table 3 N-Glycans analyzed in HL60 and HL60/ADR cell lines by MALDI-TOF MS

Peak no.	Observed *m/z*		Composition					
	HL60/ADR	HL60	Hex	HexNAc	Man	GlcNAc	NeuAc	Deoxyhexose
1	1345.65	1345.80	0	0	3	2	0	1
2	1375.63	1375.88	1	0	3	2	0	0
3	1416.77	1416.85	0	1	3	2	0	0
4	1579.73	1579.88	0	0	5	2	0	0
5	1662.11	No	0	2	3	2	0	0
6	1784.09	1784.08	0	0	6	2	0	0
7	1795.07	1795.23	1	1	3	2	0	1
8	1835.77	1836.07	0	2	3	2	0	1
9	1987.92	1988.07	0	0	7	2	0	0
10	2029.19	2029.31	3	1	3	2	0	0
11	2081.23	2081.39	0	3	3	2	0	1
12	2186.30	2186.17	2	1	3	2	1	0
13	2191.95	2192.14	0	0	8	2	0	0
14	2244.39	2244.28	2	2	3	2	0	1
15	2360.07	2360.17	2	1	3	2	1	1
16	2396.00	2396.20	0	0	9	2	0	0
17	2431.00	2431.20	2	2	3	2	1	0
18	2564.05	2564.27	3	1	3	2	1	1
19	2605.04	2605.25	2	2	3	2	1	1
20	2693.03	2693.25	3	3	3	2	0	1
21	2779.10	2779.32	2	2	3	2	1	2
22	2792.09	2792.33	2	2	3	2	2	0
23	2850.68	2850.83	2	3	3	2	1	1
24	2880.07	2880.23	3	3	3	2	1	0
25	2967.68	2967.91	2	2	3	2	2	1
26	3054.15	3054.38	3	3	3	2	1	1
27	3142.26	3142.43	4	4	3	2	0	1
28	3418.41	3418.52	3	3	3	2	2	1
29	3503.03	3503.43	4	4	3	2	1	1
30	3602.85	No	3	3	3	2	3	0
31	3776.20	3777.36	3	3	3	2	3	1
32	3864.92	3864.49	4	4	3	2	2	1
33	3952.49	3952.28	5	5	3	2	1	1
34	4225.52	4226.71	4	4	3	2	3	1
35	4314.81	4314.38	5	5	3	2	2	1

The N-glycans were observed as [M+Na]+.

Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid.

Table 4 Expressional profiles of ST gene family in AML/MDR and AML patients

Gene	Relative mRNA expression (×10³)		P-value	Relative mRNA expression (×10³)		P-value	Relative mRNA expression (×10³)		P-value
	M2	M2/MDR		M3	M3/MDR		M5	M5/MDR	
ST3GAL1	42.875±1.556	25.016±11.098	0.032*	40.529±1.681	23.548±8.422	0.002**	39.941±1,945	21.885±9.647	0.025***
ST3GAL2	23.527±1.093	25.804±5.619	0.576	21.952±1.137	24.116±5.082	0.476	19.336±0.885	22.739±4.871	0.359
ST3GAL3	16.027±1.189	13.426±3.518	0.311	14.332±0.769	12.754±3.016	0.383	14.891±1.026	12.273±2.635	0.202
ST3GAL4	10.052±0.885	17.531±3.081	0.002*	10.976±0.734	18.226±4.115	0.006**	9.549±0.811	17.764±3.626	0.009***
ST3GAL5	31.103±1.187	12.491±6.762	0.001*	29.569±1.994	12.116±7.538	0.001**	28.374±1.863	11.782±6.235	0.003***
ST3GAL6	0.002±0.001	0.003±0.001	0.179	0.006±0.002	0.005±0.003	0.582	0.003±0.001	0.004±0.001	0.214
ST6GAL1	15.469±1.092	32.109±7.387	0.004*	15.126±0.873	30.772±7.588	0.002**	14.285±0.891	28.423±5.439	0.004***
ST6GAL2	8.174±0.648	7.882±2.015	0.841	7.771±0.563	7.126±1.835	0.557	6.591±0.347	7.004±1.862	0.767
ST6GALNAC1	6.248±1.013	7.841±3.152	0.486	6.112±0.435	7.537±2.844	0.402	5.895±0.411	7.105±2.764	0.561
ST6GALNAC2	5.012±0.428	5.526±1.332	0.595	4.693±0.516	4.887±1.653	0.844	4.530±0.511	4.671±1.214	0.877
ST6GALNAC3	0.003±0.001	0.006±0.003	0.173	0.002±0.001	0.005±0.004	0.215	0.003±0.001	0.004±0.002	0.512

ST6GALNAC4	10.293±1.139	26.274±6.928	0.003*	10.185±0.973	25.863±6.761	0.001**	6.086±1.541	23.847±6.528	0.003***
ST6GALNAC5	0.003±0.001	0.002±0.001	0.178	0.002±0.001	0.005±0.003	0.103	0.004±0.001	0.006±0.004	0.508
ST6GALNAC6	17.391±1.034	18.346±3.628	0.716	16.663±0.942	17.365±3.177	0.711	15.387±0.731	16.839±3.284	0.684
ST8SIA1	1.088±0.249	1.236±0.527	0.699	1.006±0.172	1.178±0.461	0.534	0.994±0.131	1.035±0.482	0.909
ST8SIA2	0.005±0.001	0.011±0.003	0.782	0.007±0.002	0.008±0.004	0.677	0.004±0.001	0.006±0.003	0.382
ST8SIA3	0.006±0.002	0.008±0.004	0.492	0.009±0.001	0.013±0.006	0.267	0.005±0.002	0.007±0.003	0.389
ST8SIA4	10.355±1.021	31.657±8.429	0.001*	9.848±1.967	30.582±9.843	0.002**	9.736±0.822	28.754±8.319	0.008***
ST8SIA5	2.557±0.516	2.683±0.544	0.752	2.305±0.374	2.461±0.449	0.571	2.268±0.255	2.359±0.412	0.772
ST8SIA6	9.964±0.931	3.755±2.468	0.002*	10.135±0.942	3.808±2.617	0.001**	10.047±0.661	3.519±2.133	0.002***

*P<0.05 vs M2 patients, **P<0.05 vs M3 patients, ***P<0.05 vs M5 patients.