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Down regulation of membrane-bound Neu3 constitutes a new potential marker for childhood acute lymphoblastic leukemia and induces apoptosis suppression of neoplastic cells

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Membrane-linked sialidase Neu3 is a key enzyme for the extralysosomal catabolism of gangliosides. In this respect, it regulates pivotal cell surface events, including trans-membrane signaling, and plays an essential role in carcinogenesis. In this report, we demonstrated that acute lymphoblastic leukemia (ALL), lymphoblasts (primary cells from patients and cell lines) are characterized by a marked down-regulation of *Neu3* in terms of both gene expression (−30 to 40%) and enzymatic activity toward ganglioside GD1a (−25.6 to 30.6%), when compared with cells from healthy controls. Induced overexpression of *Neu3* in the ALL-cell line, MOLT-4, led to a significant increase of ceramide (+66%) and to a parallel decrease of lactosylceramide (−55%). These events strongly guided lymphoblasts to apoptosis, as we assessed by the decrease in Bcl2/Bax ratio, the accumulation of Neu3 transfected cells in the sub G0–G1 phase of the cell cycle, the enhanced annexin-V positivity, the higher cleavage of procaspase-3. Therefore, the reduced expression of Neu3 in ALL could help lymphoblasts to survive, maintaining the cellular content of ceramide below a critical level. Interestingly, we found that Neu3 activity varied in relation to disease progression, increasing in clinical remission after chemotherapy, and decreasing again in patients that relapsed. In addition, a negative correlation was observed between *Neu3* expression and the percentage of the ALL marker 9-*O*AcGD3 positive cells. Consequently, Neu3 could represent a new potent biomarker in childhood ALL, to assess the efficacy of therapeutic protocols and to rapidly identify an eventual relapse.

Acute lymphoblastic leukemia (ALL) is a malignant transformation of lymphoblasts, representing the single commonest type of cancer in pediatric population. With current treatment protocols, the threat of relapse remains, and about 20% patients in remission may harbor residual leukemic blasts defined as minimal residual disease (MRD).[1] The sophisticated technical expertise required to detect MRD constrains its overall clinical acceptance. Therefore, identification of new biochemical markers is still in demand, whose varied expression could be utilized for monitoring individual chemotherapeutic response and predicting impending relapse.

Sialic acids generally found as the terminal sugar of glycan chain attached to cell surface glycoproteins and glycolipids as well as secreted glycoconjugates.[2,3] Frequent *O*-acetylations at positions C-7, C-8 or C-9 of sialic acids generate a family of *O*-acetylated sialoglycoconjugates[4] and associated with many

Key words: acute lymphoblastic leukemia (ALL), sialidase Neu3, apoptosis, 9-O-acetylated GD3, gangliosides

Abbreviations: ALL: acute lymphoblastic leukemia; BM: bone marrow; CML: chronic myelogenous leukemia; Cy5: cyanin 5; DAB: 3:3-diamino benzidine; FACS: fluorescence-activated cell sorter; FCS: fetal calf serum; FITC: fluorescein isothiocyanate; GD3: ganglioside GD3; GM1: ganglioside GM1; HPTLC: high performance thin-layer chromatography; HRP: horse radish peroxidase; IgG: immunoglobulin G; Mab: monoclonal antibodies; MRD: minimal residual disease; MU: 4-methylumbelliferone; 4-MU-NeuAc: 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; Neu5,9Ac2GPs: 9-O-acetylated sialoglycoproteins; 9-O-AcGD3: 9-O-acetylated ganglioside GD3; PB: peripheral blood; PBMC: peripheral blood mononuclear cells; PBS: phosphate buffer saline; PE: phycoerythrin; SD: standard deviation; SEM: standard error of mean; THF: tetrahydrofuran; TLC: thin-layer chromatography

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diseases[5–8] including cancer cells.[9–12] They play an important role in modulating various biological and pathological processes including adhesion, signaling, differentiation, apoptosis and malignancy.[12–20]

Earlier we have demonstrated enhanced *O*-acetylation of sialic acid producing a few ALL-associated 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$GPs)[12,21–23] and glycolipids[19] on lymphoblasts and erythrocytes,[24] not on normal peripheral blood mononuclear cells (PBMC) or other hematological disorders. In addition, many tumors are characterized by a different composition of glycosphingolipids, particularly gangliosides, *i.e.*, sphingolipids containing sialic acid.[25] On these bases, several sialoglycoconjugates,[26–28] their antibodies[29,30] and enzymes[31] involved in their metabolism could reasonably be used as suitable markers in ALL and to identify MRD.

Recently, an ever-increasing interest has been devoted to the study of sialidases in diseased condition. Sialidases cleave sialic acid residues from glycoconjugates and therefore are key enzymes in the catabolism of glycoproteins and glycolipids. In mammalian cells, they have been so far classified into 4 groups (Neu1, Neu2, Neu3 and Neu4) differing in their subcellular localization and enzymatic property.[32] These 4 sialidases behave differently during carcinogenesis.[33] In particular, the membrane-associated sialidase, Neu3 because of its unique localization in membrane lipid rafts and strict substrate specificity toward gangliosides[34] is involved in the regulation of cell growth and differentiation *via* modulation of cell surface gangliosides. Alterations of Neu3 expression have been demonstrated to play a pivotal role in carcinogenesis.[35,36]

Here, we demonstrated that; (a) a significant decrease in membrane-associated sialidase Neu3 expression in lymphoblasts from peripheral blood (PB) and bone marrow (BM) of patients and ALL-cell lines in comparison to healthy PBMC (b) a negative correlation could be traced between Neu3 expression and the severity of the disease; (c) the expression of Neu3 in BM could be followed to monitor the progression of the disease and the response to a clinical treatment and (d) Neu3 overexpression through transfection guided lymphoblasts toward apoptosis, suggesting that reduced Neu3 in ALL may help neoplastic cells to survive.

Material and Methods

Chemicals

2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-NeuAc), 4-methylumbelliferone (MU) and 3,3′-diaminobenzidine (DAB) were from Sigma (St. Louis, MO). [3-^{3}H]sphingosine was from Perkin Elmer (Waltham, MA); [^{3}H]GD1a were prepared by dichloro-dicyano-benzoquinone/sodium boro[^{3}H]hydride method followed by reverse phase HPLC purification.[37] Anti-Neu3 antibody was the kind gift from Dr. Stamatos M. Nicholas, University of Maryland Medical Center, Baltimore, MD. All PE and Cy5 conjugated lineage-specific monoclonal antibodies were from BD Pharmingen (San Diego, CA).

Patient samples

The study involved clinically confirmed ALL patients at diagnosis of disease (10 males, 5 females, median age: 6 years, range: 0.8–16 years). A few children ($n = 10$) were longitudinally monitored during chemotherapy. The diagnosis was established by cytological examination of the bone marrow (BM) smears according to the French-American-British Group recommendations, belonging to L1 or L2.

Each sample was immunophenotyped by FACS analysis using PE and Cy5 conjugated commercially available Mabs.[22,26] They were B-(CD19$^+$ and CD10$^+$, $n = 11$) and T-(CD3$^+$, CD7$^+$, $n = 4$)-ALL. Additionally, FITC-conjugated affinity purified lectin, Achatinin-H[38] was used for detection of the ALL-associated biomarker, namely Neu5,9Ac$_2$-GPs on lymphoblasts of these patients for clinical confirmation.[11,12,26–28] High level of anti-Neu5,9Ac$_2$-GPs antibodies in the sera further confirmed their diagnosis.[29,30]

The samples were collected at Kothari Medical Sciences, sent to the Indian Institute of Chemical Biology. The samples were assayed at diagnoses, clinical remission and during clinical relapse. Normal age matched healthy individuals ($n = 10$) of both sexes served as controls.

PBMC from venous blood (3–4 ml) and/or lymphoblasts from BM of patients were separated by density centrifugation (Ficoll-Hypaque, Amersham Pharmacia, Sweden), washed thoroughly at 4°C and processed within 2–12 hr of collection. The Institutional Human Ethical Committee had approved the study and samples were taken with the consent of the donors, patients, or their parents or guardians.

Cell culture

B-(ALL-PO, REH) and T-(MOLT-4) ALL cell lines were cultured in RPMI-1640 medium supplemented with heat-inactivated FCS [10% (v/v)], L-glutamine (0.002 M), antibiotics and antimycotics (Medium A).[39]

Transient transfection of Neu3 in MOLT4 cells

MOLT4 cells were plated at 50% confluence in a 6-well plate in Medium A. They were transfected with *Neu3* cDNA inserted in the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) by the transfection reagent, FuGENE 6 (Roche, Germany) according to the manufacturer's instructions. The following day (after 18 hr of transfection, referred as time 0 in the results section), the transfection mixture was removed and replaced with fresh medium. Neu3 level was detected using PE-anti-Neu3 antibody by flow cytometer, as an indicator of efficiency of transfection. Neu3 transfected and mock-transfected cells were further cultured for 48 hr and the effects on cell death and cell cycle were monitored.

Preparation of cytosol, membrane and lysosomal fractions

Cells were washed with cold phosphate buffer saline containing 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH-7.4 (PBS), resuspended in cold PBS with protease inhibitors (1 μg/ml pepstain A, 10 μg/ml apoprotinin, 10

Table 1. Primers used for gene expression

Gene	Forward primer	Reverse primer
β-actin	5′CGACAGGATGCAGAAGGAAG3′	5′ACATCTGCTGGAAGGTGGA3′
Neu3	5′TGAGGATTGGGCAGTTGG3′	5′CCCGCACACAGATGAAGAA3′
Neu1	5′CCTGGATATTGGCACTGAA3′	5′CATCGCTGAGGAGACAGAAG3′
Neu2	5′AGAAGGATGAGCACGCAGA3′	5′GGATGGCAATGAAGAAGAGG3′
Neu4	5′TGAGCTCTGCAGCCTTCC3′	5′GTTCATGGACCGGTGCTC3′

μg/ml leupeptin) and lysed by sonication (3 pulse for 8 sec and giving break for 1 min). The supernatant obtained after centrifugation at 800*g* for 10 min at 4°C corresponded to crude cell extract[31] and was subsequently centrifuged at 100,000*g* for 30 min at 4°C. Supernatant was used as cytosol.[39] The pellet containing the membrane fraction was dissolved in ice cold PBS. Protein was estimated by the method of Lowry.[40]

Additionally, lysosomal membranes were prepared by homogenizing these cells (1×10^8) in 0.25 M sucrose in 20 mM Tris/HCl (pH 7.2) in a glass homogenizer.[41] Suspension was spun at 800*g* for 10 min at 4°C and the supernatant was diluted in 0.25 M sucrose in 0.4 M KCl (pH 7.0) and span at 11,000*g* for 20 min at 4°C. The pellet was resuspended in 0.025 M sucrose and allowed to swell for 30 min on ice and spun at 11,000*g* for 20 min at 4°C. Finally, supernatant was spun at 100,000*g* for 1 hr and pellet was suspended in PBS. In this preparation, β-galactosidase activity was ~2-fold higher over the crude homogenate and negligible activity was observed in cytosolic fraction indicating that lysosomes had not been disrupted.

Sialidase assay

The sialidase activity in cytosol, membrane and lysosomal fractions was determined by using a fluorimetric assay with 4-MU-Neu5Ac (30 nmole) as a substrate, at pH 4.6 for membrane (20 μg) and lysosomal fractions (20 μg) and at pH 5.6 for cytosol (100 μg)[34] and a radiochemical assay by using 60 nmole GD1a (containing 0.04 μCi [^{3}H]GD1a) at pH 4.6 for membrane fractions.[34]

The kinetics (K_M and V_{max}) of sialidases (20 μg, as source of enzyme) was studied by incubating them with various concentration of 4-MU-Neu5Ac and [^{3}H]GD1a separately at 37°C for 1 hr in sodium acetate buffer (50 mM, pH 4.6 for membrane and lysosomal sialidases, pH 5.6 for cytosolic sialidase). Enzymatic activity was expressed as μmoles or nmoles of product per hour/mg protein.[31]

Genetic expression of Neu3

Semi-quantitative RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) and treated with an RNase free DNase I (Invitrogen) following the manufacturer's instruction. First strand cDNA was synthesized by ImProm-II-Reverse transcription system (Promega, Madison, WI) according to manufacturer's protocol.

Semi-quantitative RT-PCR was performed on PTC-100, (MJ Research, GMI Ramsey, MN) using PCR Kit (Invitrogen). Expression of β-actin housekeeping gene was used to determine the cDNA yield and to normalize PCR product. The primers used are reported in Table 1. The PCR reactions were cycled 40 times after initial denaturation (95°C, 10 min) with the following parameters: denaturation at 94°C, 30 sec; annealing at 58°C, 30 sec and extension at 72°C, 30 sec; followed by 72°C, 4 min.

Real time PCR. Quantitative analysis was performed by real-time PCR using a LightCycler rapid thermal cycler system (Bio-Rad-Richmond, Richmond, CA) with SYBR Green Jump Start Ready mix (Sigma), following the manufacturer's instruction. The specificity of the reactions was confirmed by melting curve analysis. β-actin was used as an internal control.

Flow cytometry

The expression of Neu3 was determined by flow cytometry. Lymphoblasts were initially permeabilized, fixed by BD Cytofix/Cytoperm™ solution and washed with BD Perm/Wash buffer (BD Pharmigen), according to the manufacturer's protocol. Cells were incubated with rabbit anti-Neu3, diluted in BD Perm/Wash buffer, for 1 hr at 4°C followed by probing with PE-anti-rabbit IgG antibodies, fixed in paraformaldehyde (1%) and analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). The data were analyzed with the CellQuestPro software (Becton Dickinson).

For 9-*O*-AcGD3 detection, cells (1×10^6) were stained with anti-9-*O*-AcGD3 (1:5 dilution) Mab on ice for 1 hr in dark, washed and probed with FITC-anti-murine IgM for 30 min on ice.[19] The cells were then washed, fixed in 1% paraformaldehyde and acquired by flow cytometer.

Determination of cellular sialic acid

Quantification of sialic acids (%) both in the membrane and cytosolic fractions (25 μg) from normal PBMC ($n = 5$) and ALL-cell lines was performed fluorimetrically by oxidizing the samples and processed using the acetylacetone method.[42] The relative fluorescence intensity [$\lambda_{max(excitation\ =\ 410nm)}$/$\lambda_{max(emission\ =\ 510nm)}$] of each sample was measured against reagent blanks on a Hitachi F-4010 spectrofluorimeter (Tokyo, Japan). The sialic acid content was determined from a standard curve obtained using pure sialic acid.

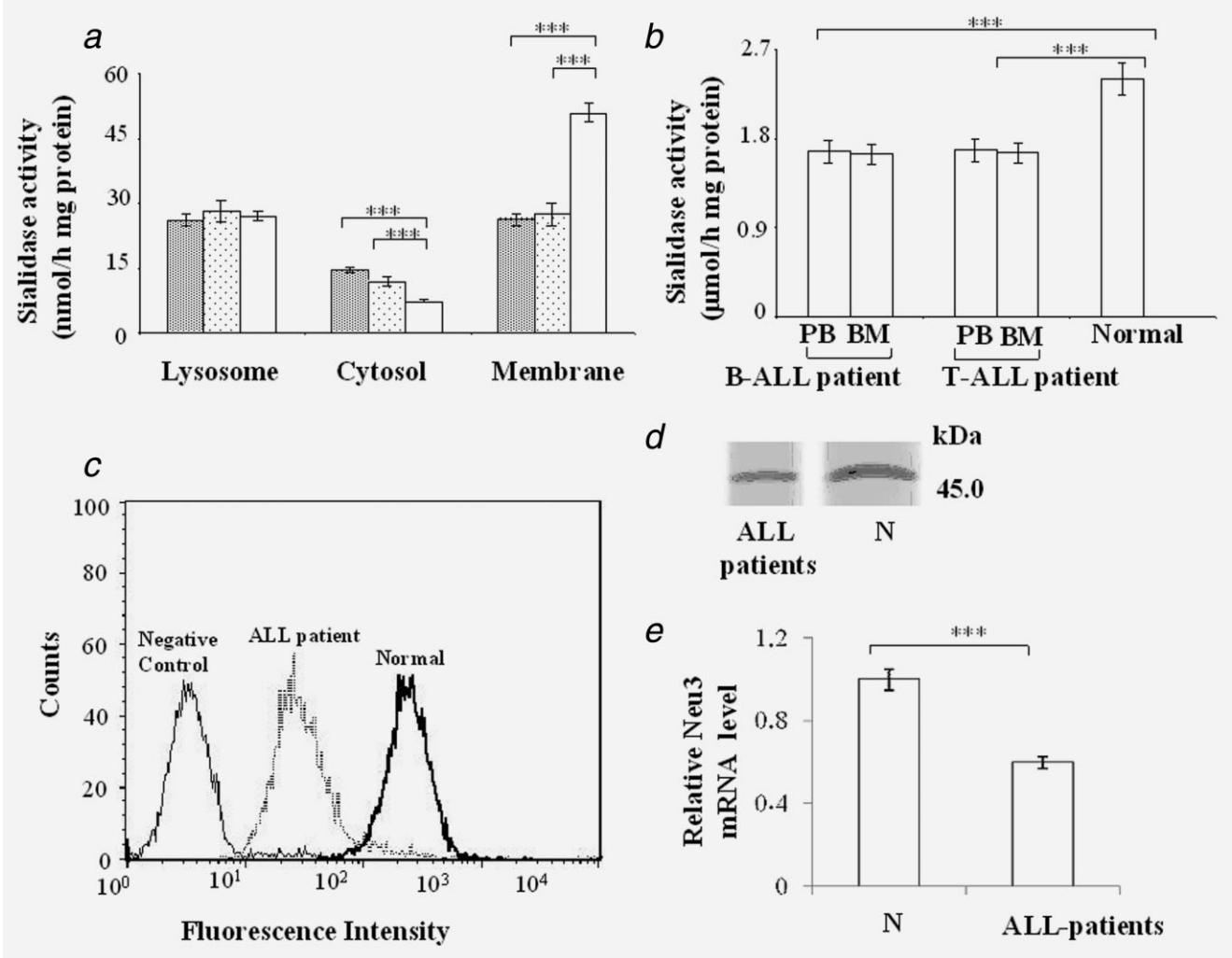


Figure 1. Sialidase expression in primary cells from clinically confirmed B- and T-ALL patients. (*a*) Sialidase activity in the lysosomal, cytosolic and membrane fractions from patients with B-(▨) ($n = 11$) and T-ALL (▩) ($n = 4$), at presentation of disease, was measured by fluorimetric method using MU-Neu5Ac as substrate.[34] Fluorescence emission was measured on Fluorimeter (Perkin Elmer, LS55) with excitation at 365 nm and emission at 450 nm. The enzyme activity of normal PBMC (□) has been shown for comparison. Significance: ***$p < 0.001$. (*b*)Sialidase activity in the membrane fractions from lymphoblasts of peripheral blood (PB) and bone marrow (BM) of B-($n = 11$) and T-($n = 4$) ALL patients was measured by radiometric method taking [^{3}H]GD1a as substrate.[34] The lipids were extracted and analyzed by radio-TLC. The enzyme activity of PBMC from normal PB has been shown for comparison. Significance: ***$p < 0.001$. (*c*) Primary cells from a representative B-ALL patient, at presentation of disease, and PBMC of a normal individual were incubated with rabbit anti-Neu3 antibodies and detected by using PE conjugated anti-rabbit IgG antibody, as described in "Material and methods." Cells were washed, fixed and permeabilized, and the presence of Neu3 was detected by FACS analysis. Comparison was shown as mean fluorescence intensity. (*d*) The presence of Neu3 in the primary cells of a representative B-ALL patient, at presentation of disease, and PBMC of a normal individual was analyzed by Western blot analysis using anti-Neu3 antibody. (*e*) The genetic expression of *Neu3* was measured by quantitative real-time PCR in normal PBMC ($n = 10$) and ALL patients ($n = 15$). The data was normalized against the genetic expression of β-actin. Significance: ***$p < 0.001$.

Radio labeling of gangliosides and neutral glycolipids by [3-^{3}H]sphingosine

[3-^{3}H]sphingosine dissolved in methanol was transferred into a sterile glass tube and dried under a nitrogen stream. The residue was then dissolved in prewarmed (37°C) culture medium to obtain a final concentration of 3×10^{-8} M (corresponding to 0.4 μCi/well). The cells (2×10^6) were incubated in presence of [3-^{3}H]sphingosine for 2 hr pulse, followed by a 24 hr chase, a condition warranting a steady-state metabolic condition. The sphingolipid pattern was determined.[34]

Analysis of Neu3-transfected cells for apoptosis by flow cytometry

Neu3-transfected cells were centrifuged, washed twice in PBS, suspended in annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with FITC-

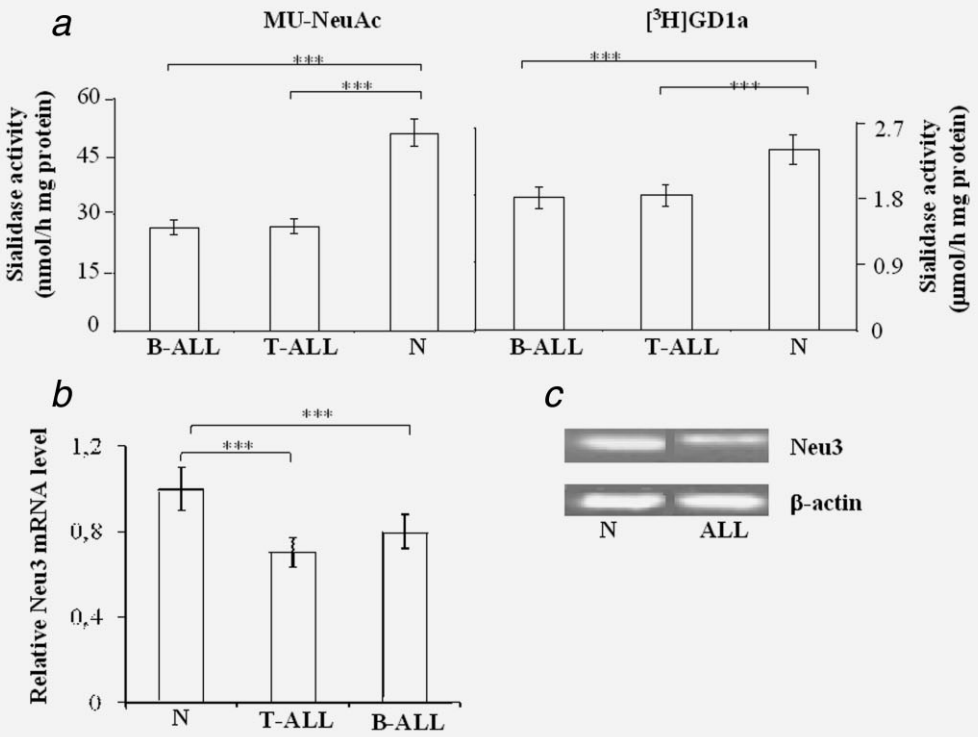


Figure 2. Membrane sialidase activity and expression in ALL-cell lines. (*a*) The activity of sialidase in the membrane fraction from B-(REH, ALLPO), T-(MOLT4) ALL-cell lines and normal PBMC (N) was measured by fluorimetric method taking MU-Neu5Ac as substrate, as described in "Material and methods." Additionally, sialidase activity was measured by radiometric method taking [^{3}H]GD1a as substrate, as described in "Material and methods." Significance: ***$p < 0.001$. (*b*) The genetic expression of Neu3 was measured by quantitative real-time PCR using respective cDNA from normal PBMC (N, $n = 10$), T- and B-ALL-cell lines. The genetic expression data shown were normalized against the genetic expression of β-actin. Significance: ***$p < 0.001$. (*c*) Neu3 mRNA level in a representative ALL-cell lines (ALL) and a representative healthy normal PBMC (N) was evaluated by semi-quantitative RT-PCR. The expression of β-actin was also measured as an internal control for normalization Neu3 expression.

annexin-V and propidium iodide as per manufacturer's instructions. Cells were then incubated for 30 min in dark at 20–25°C and analyzed as described earlier.

Cell cycle analysis

The cell cycle status of Neu3-transfected cells was analyzed using Cycle TEST PLUS kit (BD Pharmigen) according to the manufacturer's instruction and the percentage of cells in different phases of cell cycle was determined.

Data analysis

Data analysis was performed using the Graph-Pad Prism statistics software program (Graph-Pad Software, San Diego, CA). Results are expressed as mean ± SD for individual sets of experiments. Each experiment was performed 4 to 5 times, and the results are representative of each set of experiment. Statistical analysis of data was performed using two-tail student *t*-test.

Error bars represent standard error of mean (SEM). *** represents the $p < 0.001$ considered statistically significant. The correlation coefficient was determined between individual Neu3 activities and *Neu3* mRNA level of patients and percentage of 9-*O*-AcGD3$^+$ cells present in lymphoblasts along with normal PBMC.

Results

Sialidase expression in primary cells from ALL patients

Lymphoblasts were immunophenotyped as B-(CD19$^+$ and CD10$^+$) and T-(CD3$^+$ and CD7$^+$)-ALL. A high number of Neu5,9Ac$_2$-GPs$^+$ (>80% *vs.* 5–8% normal PBMC) cells were revealed. They were also typified by an elevated content of 9-*O*-AcGD3 (+81%) in comparison to healthy PBMC.[19] Enhanced levels of 9-*O*-AcGD3, irrespective of their lineages, were always higher than disialoganglioside GD3 in the lymphoblasts compared to normal cells.[19] Thus, it may be envisaged that the *O*-acetylation machinery is upregulated in ALL.[11,12]

We assayed sialidase activity in lysosomes, cytosol and membrane fractions of lymphoblasts from T- and B-ALL patients. A marked decrease of sialidase activity associated to

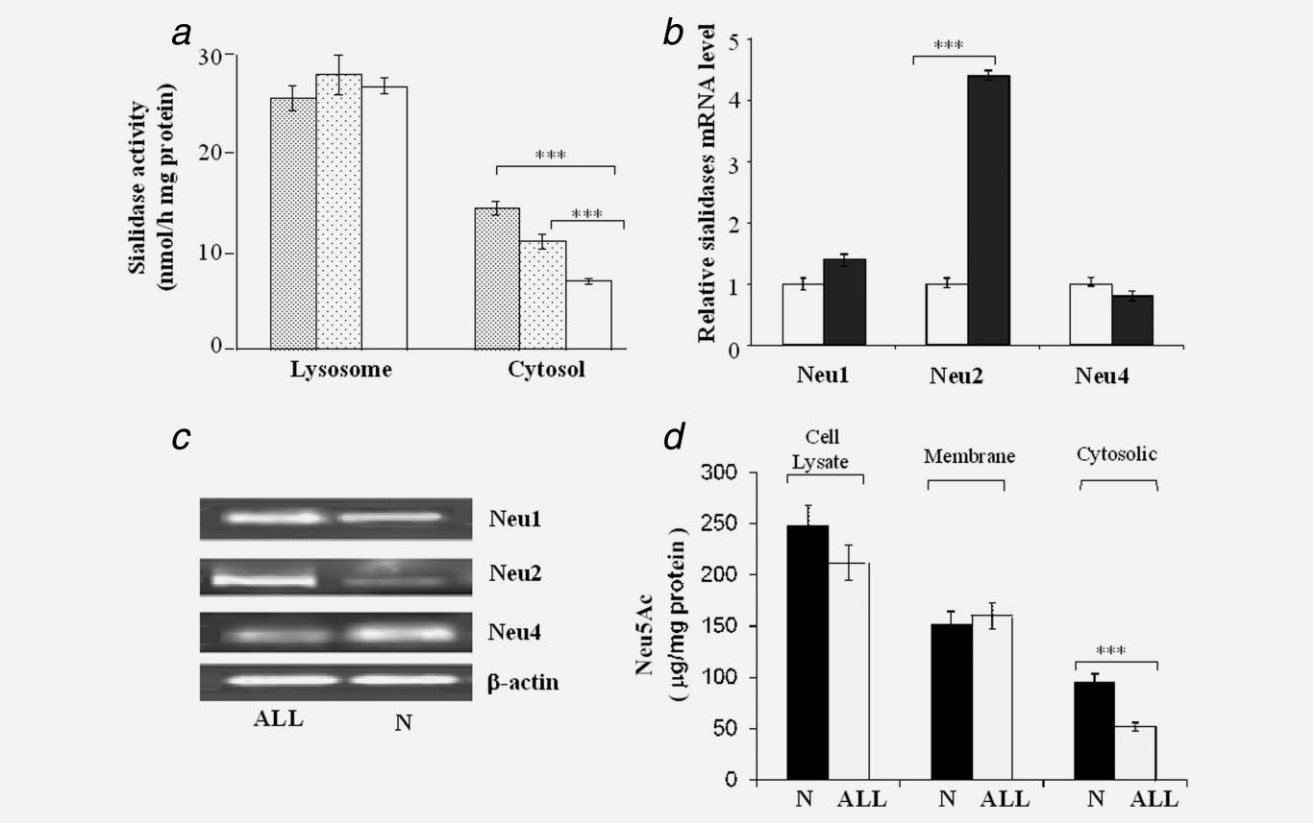


Figure 3. Sialidase (Neu1, Neu2 and Neu4) activity and expression and sialic acid content in ALL-cell lines. (*a*) Sialidase activity in the lysosomal and cytosolic fractions from B-ALL (▨), T-ALL (▨) cell lines and normal PBMC (□) was measured by fluorimetric method taking MU-Neu5Ac as substrate, as described in "Material and methods." Significance: ***$p < 0.001$. (*b*) Real-time PCR analysis of Neu1, Neu2 and Neu4 was performed using respective cDNA from ALL-cell lines (■), and normal PBMC (□, N, $n = 5$). The genetic expression data shown were normalized against the expression of β-actin. Significance: ***$p < 0.001$. (*c*) Genetic expression of Neu1, Neu2 and Neu4 was evaluated by semi-quantitive RT-PCR and using respective cDNA from a representative ALL-cell line and a representative normal PBMC (N). The expression of β actin was also measured as an internal control for normalization of the other genes. Significance: ***$p < 0.001$. (*d*) Quantification of sialic acid (Neu5Ac) by fluorimetric estimation using acetylacetone method. Lysate (15 μg), membrane and cytosolic protein (25 μg) from normal (■) and ALL cells (□) were used for quantification as described in "Material and methods." Significance: ***$p < 0.001$.

the plasma membrane (−51 to 53%) toward 4-MUNeu5Ac in lymphoblasts in comparison to healthy PBMC was observed. In contrast, cytosolic sialidase activity showed an increase (+49.5 to 60.4%) and lysosomal sialidase activity remain unchanged in lymphoblasts (Fig. 1*a*). We further confirmed the decrease of membrane sialidase activity (almost −29.9 to 30.6%) in lymphoblasts using ganglioside [^{3}H]GD1a, a preferred substrate for sialidase Neu3 (Fig. 1*b*). No significant difference in sialidase activity was observed in membrane fractions between PB and BM lymphoblasts of these patients (Fig. 1*b*).

Then, we analyzed the status of sialidase Neu3 on healthy and patient's cells by flow cytometry and confirmed that the decrease of membrane sialidase associated activity was related to a decrease of the sialidase Neu3. In fact, mean fluorescence intensity, due to the binding of anti-Neu3 antibody, was 16-fold lower in lymphoblasts compared with normal PBMC (Fig. 1*c*). In addition, Neu3 protein was 30% lower in patients compared with normal as confirmed by Western blot (Fig. 1*d*). Accordingly, a 40% reduction in *Neu3* gene expression was observed in patients by real-time PCR (Fig. 1*e*).

Sialidase expression in ALL-cell lines

As we revealed a reduced expression of Neu3 sialidase in primary lymphoblasts, we further checked our finding in several ALL-cell lines. A significant decrease in sialidase activity (−51.06 to 52.3%) was also found in membrane fractions of cell lines, compared with normal PBMC, toward 4-MUNeu5Ac (Fig. 2*a*). The employment of ganglioside GD1a confirmed this trend (−25.6 to 26.6%) (Fig. 2*a*). Concordantly, a decrease in *Neu3* gene expression (∼30%) was observed in these cell lines (Figs. 2*b* and 2*c*), in comparison to healthy PBMC.

Instead, no significant differences in sialidase activity in lysosomal fractions between healthy PBMC and ALL-cell lines were observed; whereas, in cytosolic fractions, sialidase

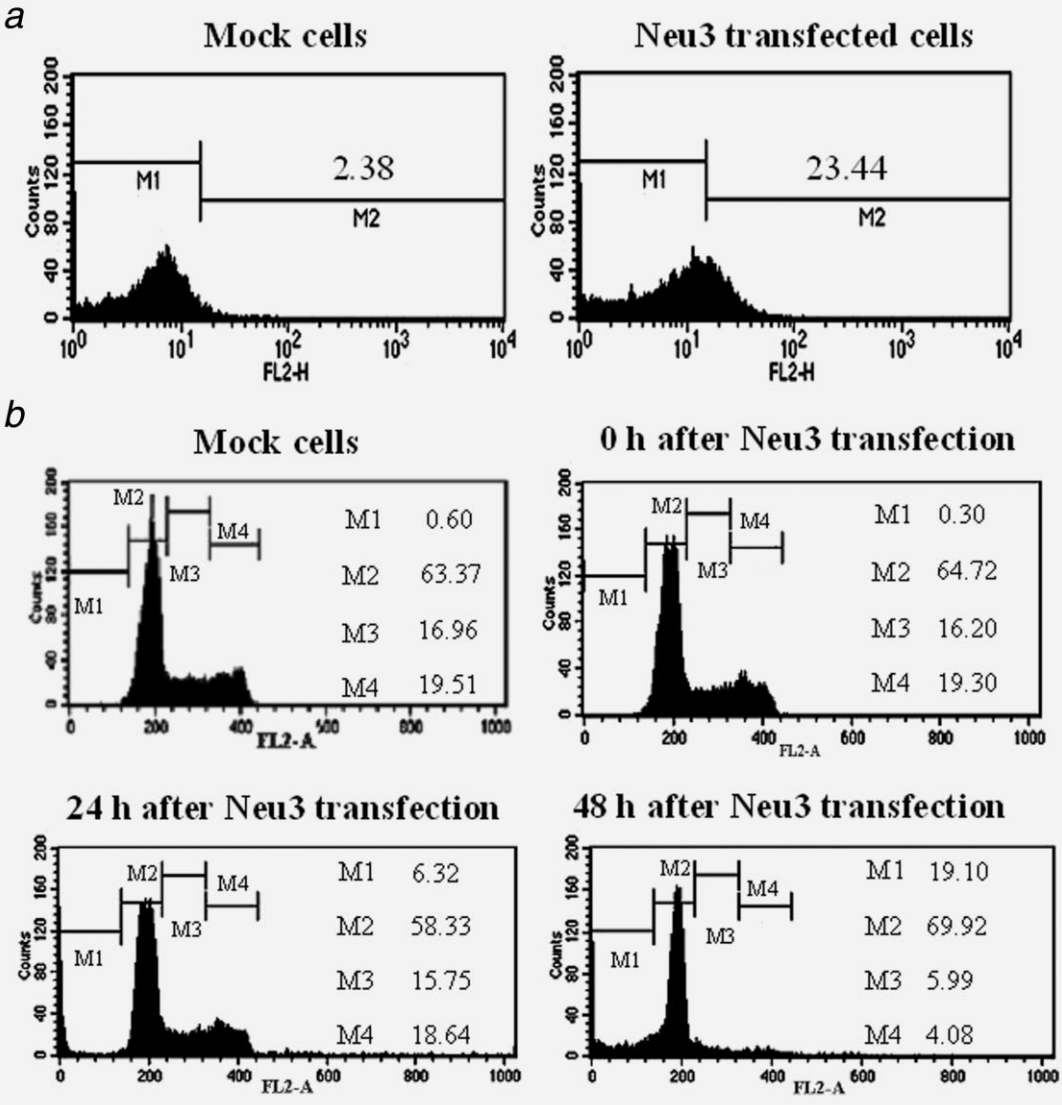


Figure 4. Transfection of *Neu3* in MOLT-4 cells and cell cycle analysis. (*a*) MOLT-4 cells were transfected with *Neu3* cDNA, as described in "Material and methods." The transfection efficiency was measured by flow cytometry, determining the expression of Neu3 protein by using PE conjugated anti-Neu3 antibody. (*b*) Mock-transfected and Neu3 transfected MOLT-4 cells were analyzed for cell cycle at different times after transfection. The cells were analyzed through FACS analysis after propidium iodide staining. Gates were set to assess % of cells in Sub G0–G1 (<2n DNA, M1), G1 (2n DNA, M2), S (>2n DNA, M3) and G2+M (4n DNA, M4). Bars denote the boundaries of cell cycle phases.

activity was doubled (Fig. 3*a*). Then, the mRNA expression of sialidases (Neu1, Neu2 and Neu4) revealed a significant increase (4.4-fold) in Neu2 gene expression (Fig. 3*b*) in cell lines compared with normal PBMC. No such difference was observed in Neu1 expression while Neu4 expression decreased slightly in ALL cells (Figs. 3*b* and 3*c*).

Comparison of kinetic constants of Neu2 and Neu3 between ALL-cell lines and normal PBMC

The reduced expression of Neu3 protein resulted in a decreased enzymatic activity in ALL-cell lines compared with normal. In fact, the V_{max} of membrane sialidase activity was 30.87 ± 2.09 and 58.7 ± 3.73 nmole/hr × mg protein in the membrane fraction of ALL-cell lines and normal PBMC, respectively, using MU-Neu5Ac as substrate. Accordingly, hydrolysis of GD1a by membrane-associated sialidase was much lower in all types of ALL cells. In fact, the V_{max} value was 1.3-fold less in ALL-cell lines (1.92 μmole/hr × mg protein) compared with normal (2.5 μmole/hr × mg protein). In contrast, higher sialidase activity in cytosolic fraction of ALL cells was observed, V_{max} being 15.1 ± 1.05 nmole/hr × mg protein *vs.* 7.32 ± 1.67 nmole/hr × mg protein/min, respectively, using MU-Neu5Ac as a substrate. K_M values of membrane and cytosolic sialidases remained unchanged both in

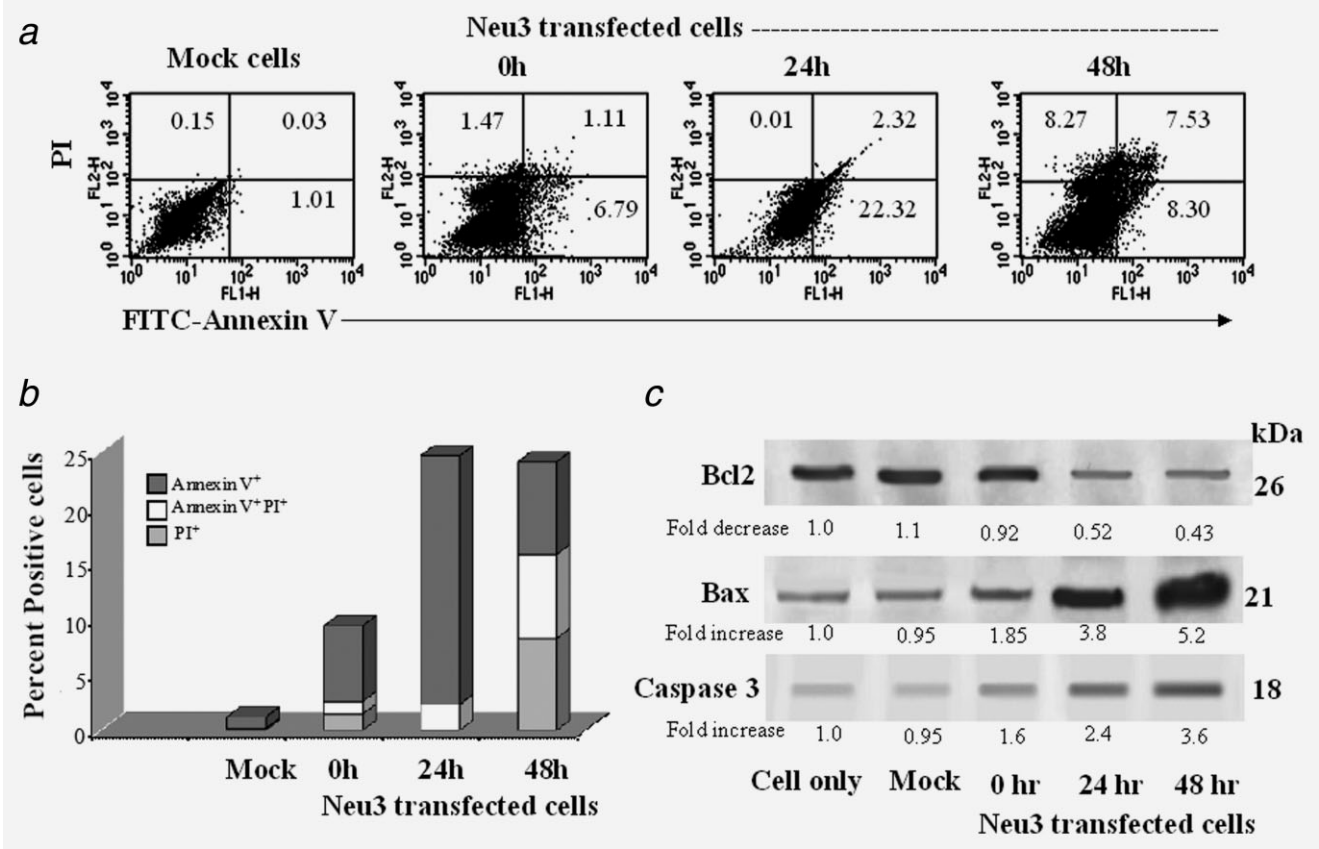


Figure 5. Analysis of apoptosis in transfected MOLT-4 cells: externalization of phosphatidylserine and apoptosis regulating molecules analysis. (*a*) Transfected MOLT-4 cells were stained with FITC-annexin-V and propidium iodide (PI). The percentage of apoptosis (annexinV/PI) in *Neu3* transfected and mock-transfected cells have been shown in dot plot at different times after transfection. (*b*) The percentage of annexin-V^+, annexin-V^+PI^+ and only PI^+ in Mock-transfected and *Neu3* transfected cells at different time after transfection was plotted. (*c*) Expression of Bcl-2, Bax and active caspase3 in the cell lysate was determined by Western blot analysis using anti-Bax (1:100), anti-Bcl-2 (1:100) and anti-active-caspase3 (1:100) antibodies. Fold decrease /increase was determined when compared with cell only.

normal and ALL, in the range of $3–4 \times 10^{-4}$ M (data not shown).

Total sialic acid content of ALL-cell lines and normal PBMC

A slight decrease of total sialic acid (−15%) was revealed in ALL-cell lines in comparison to normal PBMC. This may be due to the fact that more than 42% of sialic acids are *O*-acetylated in the lymphoblasts of ALL in comparison to normal PBMC.[21] No significant differences in membrane sialic acid content were observed, being 152 ± 6 and 160 ± 8 μg/mg protein in the membrane fraction of normal and ALL cells. In contrast, the cytosolic fraction showed a decrease by 46% of sialic acids in ALL cells compared with normal, being 52 ± 4 and 96 ± 5 μg/mg protein, respectively (Fig. 3*d*).

Induction of apoptosis in Neu3 transfected MOLT-4 cells

To understand the effects of the reduced expression of Neu3 in the pathogenesis of ALL, we introduced the *Neu3* gene into a representative T-ALL-cell line (MOLT-4). After transient transfection, a 9.8-fold increase in Neu3 protein level was observed (Fig. 4*a*), in comparison to mock cells. Neu3 activity toward GD1a increased by 2.5-fold in transfected cells in comparison to mock cells (data not shown).

Transfected cells were further analyzed for cell cycle (Fig. 4*b*). Time 0 hr was considered the time when transfection mixture was removed. First, we assessed a marked reduction of the proliferation rate, explained by the slowing down of the progression from the G1 phase to the S phase after 48 hr of transfection. Moreover, importantly, the population in the sub G0–G1 phase (apoptotic cells) was significantly increased in *Neu3*-transfected cells compared with mock cells, after 24 hr of transfection (6.32%) and above all, after 48 hr of transfection reaching 19.10% of the cells. Therefore, the induction of apoptosis was confirmed as annexin-V positivity increased to 6.79%, after 0 hr and to 22.32% after 24 hr of *Neu3*-transfection (mock cells: 1.01%) (Figs. 5*a* and 5*b*). In contrast, as expected the percentage of annexin-V^+/PI^+ increased gradually to 7.53% after 48 hr of *Neu3*-transfection (mock cells: 0.03%). These results indicate that Neu3 overexpression induced an increased susceptibility of MOLT-4 cells toward apoptosis.

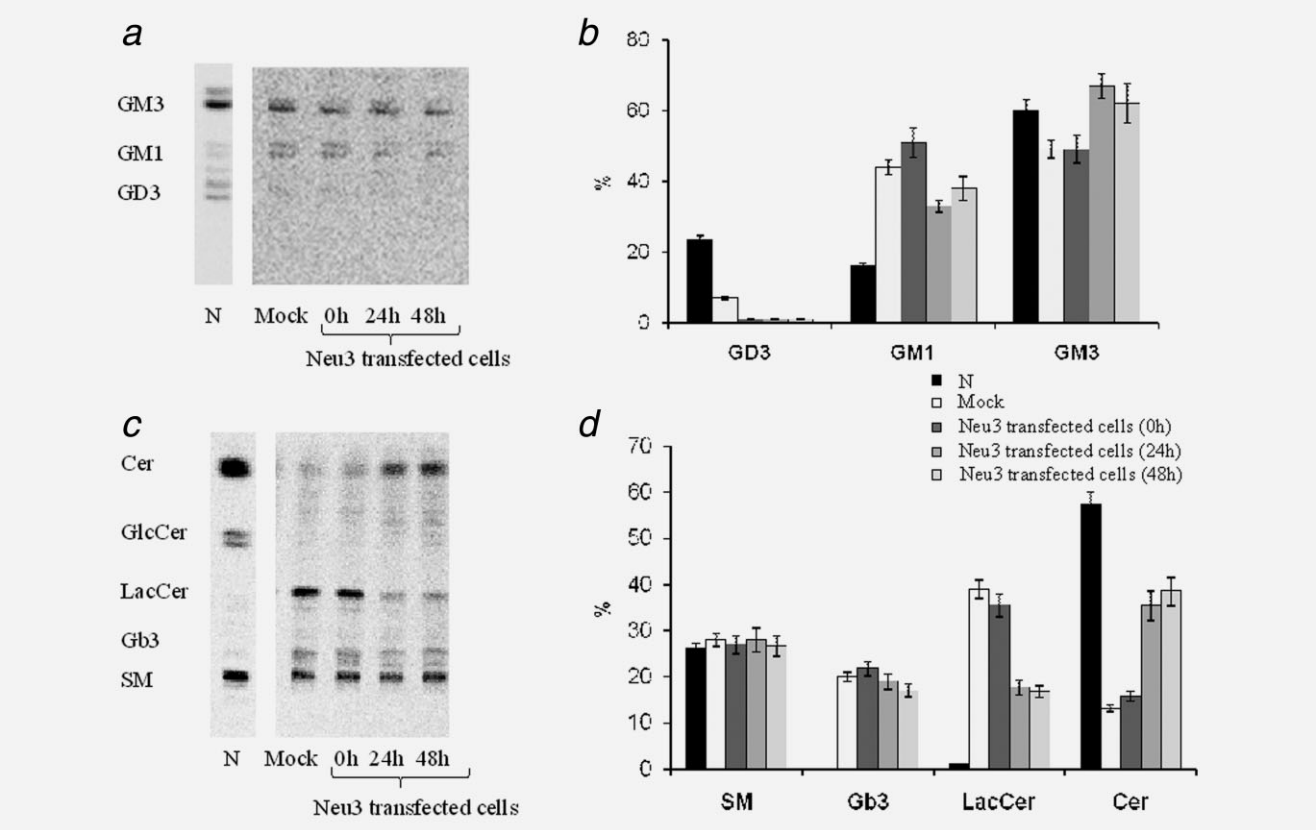


Figure 6. Sphingolipid profile of normal PBMC and transfected MOLT-4 cells. (*a*) HPTLC separation of gangliosides. Doublets are due to the heterogeneity of the ceramide moiety. Solvent system: chloroform/methanol/0.2% aqueous $CaCl_2$ 60:40:9 (v/v). Image acquired by radiochromatoscanning (Beta Imager 2000). N: normal PBMC. (*b*) Ganglioside content of normal PBMC (N) and Mock-transfected and *Neu3* transfected MOLT-4 cells at different times of transfection. (*c*) HPTLC separation of neutral glycolipids. Doublets are due to the heterogeneity of the ceramide moiety. Solvent system: chloroform/methanol/H2O 55:20:3 (v/v). Image acquired by radiochromatoscnning (Beta Imager 2000). N: normal PBMC. (*d*) Neutral glycolipid content of normal PBMC and Mock-transfected and Neu3 transfected MOLT-4 cells at different times of transfection.

Expression of apoptosis related molecule in Neu3 transfected MOLT-4 cells

To verify the role of Neu3 in apoptosis induction, we evaluated the expression of a few apoptosis governing molecules, Bcl-2, Bax and caspase 3, in *Neu3*-transfected cells. After 24 and 48 hr of transfection, Western blot analysis showed a sharp increase in Bax expression while Bcl-2 level decreased, and then also Bcl-2/Bax ratio decreased (Fig. 5*c*). Possibly, these events drove MOLT-4 cells toward death. Consistent with the results of Bcl-2/Bax above, the cleavage level of procaspase-3 was found to be higher in *Neu3*-transfected cells after 24 and 48 hr (Fig. 5*c*).

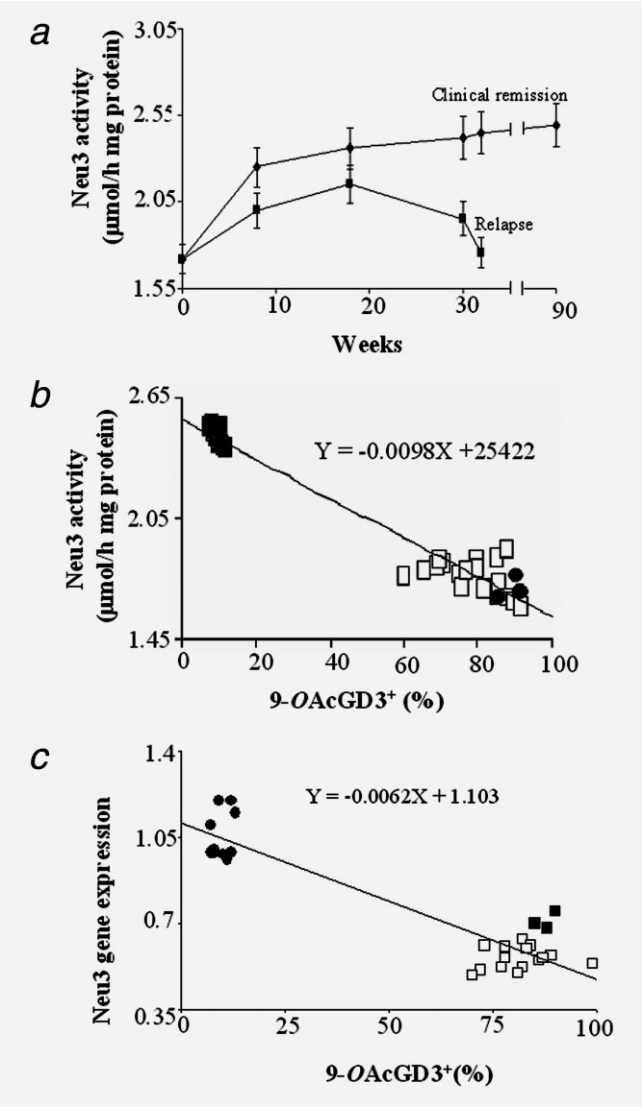
Sphingolipid pattern of normal PBMC and transfected MOLT-4

To identify a molecular connection between Neu3 overexpression in MOLT-4 and the events illustrated earlier, we examined the modifications that occurred in sialoglycosphingolipids. The sphingolipid pattern was explored through a metabolic labeling with [3-^{3}H]sphingosine, after a 2 hr pulse and a 24 hr chase. Ganglioside pattern of normal PBMC and mock-transfected MOLT-4 was mainly composed by GM3, GM1, and GD3 (Figs. 6*a* and 6*b*). After *Neu3* transfection, the content of GD3 and GM1 significantly diminished while GM3, which constitutes the major ganglioside present in MOLT-4 cells, did not change or slightly increased (Fig. 6*b*), in comparison to mock cells.

Neutral glycolipids expressed by mock-transfected MOLT-4 were very different in comparison to that expressed by normal PBMC (Fig. 6*c*). In particular, healthy cells showed a significant content of ceramide and the lack of lactosylceramide; instead, mock-transfected MOLT-4 was characterized by a marked prevalence of lactosylceramide over ceramide. Nevertheless, Neu3 overexpression gave rise to significant changes in these cells; lactosylceramide decreased by 55% immediately after 18 hr of transfection, and ceramide increased by 63% after 24 hr, and by 66% after 48 hr of transfection (Fig. 6*d*), in comparison to mock cells.

Status of Neu3 with disease progression

Ascertained the reduced expression of Neu3 in lymphoblasts and its connection with apoptosis resistance, we decided to examine the status of Neu3 activity of lymphoblasts during a longitudinal follow-up of 10 patients. These patients were monitored at diagnoses, clinical remission and relapse. In 6 patients, there was a progressive increase in Neu3 activity being 27% higher between 2 and 40th weeks in clinical remission when compared with presentation of the disease (Fig. 7*a*). In contrast, 4 patients showed decreased Neu3 activity at the diagnosis, which progressively increased upto the 18th week and subsequently, decreased again on 30th week (Fig. 7*a*). These patients showed clinical symptoms for relapse at the 32nd week that also reflected on Neu3 activity. Taken together, these results suggested that decrease of Neu3 activity is strictly associated to ALL and could reasonably predicted an eventual clinical relapse.



Interestingly, we found a significant correlation between the Neu3 activity and increased expression of ALL-associated 9-*O*-AcGD3[19] both in patients and cell lines. In particular, a negative correlation between Neu3 activity and the expression of 9-*O*-AcGD3 was observed ($r^2 = 0.95$, Fig. 7*b*). Similarly, a negative correlation was also observed between *Neu3* gene expression and cell surface expression of 9-*O*-AcGD3 ($r^2 = 0.84$, Fig. 7*c*).

Discussion

Cancer cells frequently alter the regulation of glycosylation processes leading to the appearance of characteristic glycosylated molecules. In particular, ALL lymphoblasts show the peculiarity to attach *O*-acetyl group on some sialoglycoproteins[12,21–23] and sialoglycosphingolipids[19] possibly to impair their catabolism by desialylation, their recognition and acquire an advantage in malignancy.[15–20]

Here, we demonstrated that lymphoblasts exploit an additional tool to preserve a particular configuration of glycosphingolipids that help cells to survive. In fact, glycosphingolipids are pivotal components of the plasma membrane where they can play important roles in modulating signal transduction processes.[25,43,44] An anomalous expression of some glycosphingolipids is reported as a typical feature in cancer cells[19,25,44] and this event significantly contributes to alter cell homeostasis and strengthens the malignant behavior, enhancing proliferation, resistance to apoptosis and invasiveness.[45]

A key enzyme for the extralysosomal catabolism of sphingolipids, through the desialylation of gangliosides, is

Figure 7. Neu3 activity at different phases of therapeutic treatment. (*a*) Plot of Neu3 activity determined by using [^{3}H]GD1a as substrate *vs.* duration of treatment (weeks). The children ($n = 10$) were longitudinally monitored using membrane fraction (20 µg) of lymphoblasts from BM. The change of Neu3 activity was monitored for 90 weeks during treatment. Four patients showed relapse whereas 6 were in clinical remission. (*b*) Correlation of Neu3 activity with the percentage of 9-*O*-AcGD3$^+$ cells. The Neu3 activity was measured in clinically confirmed ALL patients ($n = 15$) at diagnosis, using [^{3}H]GD1a as substrate, as described in "Material and methods." The percentage of 9-*O*-AcGD3$^+$ cells was determined by FACS analysis using monoclonal anti 9-*O*-ACGD3 antibody as described earlier.[19] Correlation plots for ALL patients (□), ALL-cell lines (●) and normal individuals (■, $n = 10$) are shown. The straight line represents regression analysis. (*c*) Correlation of *Neu3* gene expression with the percentage of 9-*O*-AcGD3$^+$ cells. The *Neu3* gene expression of clinically and immunophenotypically confirmed ALL patients ($n = 15$) was measured by real-time PCR, as described in "Material and methods." The percentage of 9-*O*-AcGD3$^+$ cells was determined as described in Figure 7*b*. Similar correlation plots were obtained for ALL patients (□), ALL-cell lines (■) and normal individuals (●, $n = 10$). The straight line represents regression analysis.

undoubtedly the sialidase Neu3.[33] Neu3 is localized in lipid rafts, strictly associated to gangliosides and signal molecules and is involved in trans-membrane signaling.[46,47] Several reports demonstrated the importance of Neu3 in carcinogenesis and evidenced its marked upregulation in some tumors such as carcinomas and CML.[32,34,35,48] In this study, we demonstrated that ALL constitutes an exception in this view. In fact, we analyzed the expression level of sialidases in lysosomal, cytosolic and membrane fractions of lymphoblasts of cell lines and primary cells of these patients. A reduced Neu3 expression in terms of mRNA content and enzymatic activity toward an artificial substrate and gangliosides was detected in lymphoblasts compared with normal PBMC. This result was confirmed using several ALL-cell lines. In addition to this event, a higher expression of the cytosolic sialidase Neu2 characterized ALL cells. This event seems to be related to a decrease of the intracellular sialic acid content in these lymphoblasts in comparison to healthy PBMC, but its connection with the disease is still obscure and will be object of our future investigation.

No significant difference of Neu1 and Neu4 expression between normal PBMC, lymphoblasts and cell lines was detected, possibly indicating their minimal or negligible involvement in the disease. Prompted by these results and aimed to explain the significance of Neu3 downregulation in ALL, we overexpressed Neu3 in a representative ALL-cell line, MOLT-4. First, Neu3 overexpression significantly reduced the proliferation rate, impairing the progression through the cell cycle. Second but more importantly, this event pushed MOLT-4 cells toward apoptosis as revealed by the increase of the population in the sub G0–G1 phase in the cell cycle and by the annexin-V positivity. In effect, Neu3 overexpression radically altered the main system, which controls apoptosis, increasing the expression of the proapoptotic protein Bax and decreasing the expression of the anti-apoptotic protein Bcl-2. Correspondently, caspase 3 resulted to be activated. These results are linked to extensive modifications induced by Neu3 overexpression in the sphingolipid composition of MOLT-4. Particularly, ceramide was significantly decreased in lymphoblasts compared with healthy normal cells while lactosylceramide was abundantly present in lymphoblasts, which was completely absent in healthy cells. These phenomena possibly indicate towards a mechanism of resistance to apoptosis of lymphoblasts.

To demonstrate the existence of a catabolic process, GD3 disappeared but surprisingly, the cellular content of GM3 slightly modified. This last event could be explained hypothesizing that other enzymes involved in sphingolipid metabolism change their activity, in parallel to Neu3 overexpression; this occurrence was already established in other cells.[34,35,49] Nevertheless, ceramide has been shown to participate in the activation of the apoptotic process,[50] this supports a relation between the increase of ceramide subsequent to Neu3 overexpression and the death by apoptosis. These data contrast with other studies performed overexpressing Neu3 in colon adenocarcinoma; in fact, in these cells the high activity of Neu3 led to an increase of lactosylceramide, not ceramide, and therefore, enhanced resistance to apoptosis.[35] These results could be related to a complex regulation of the overall metabolism of sphingolipids and to a different activation of other enzymes, which are involved in these processes in association to Neu3, in ALL in comparison to other tumors. This situation is fairly similar to the results obtained after Neu3 overexpression in fibroblasts.[49] Therefore, we may conclude that by downregulating Neu3 expression, ALL cells evolved the way to survive.

In addition, when we checked Neu3 activity in relation to disease progression, clinical remission after chemotherapy and relapse, we found that it effectively changed as the patient healed or relapsed. In particular, we found that Neu3 activity increased in clinical remission after chemotherapy and decreased again in patients that relapsed. These results demonstrated that the reduction of Neu3 is closely linked to ALL. Thus, Neu3 could represent itself another biomarker in leukemia. Interestingly, we observed that Neu3 activity varied, along with the percent of 9-*O*AcGD3 positive cells; in particularly when Neu3 activity increased, 9-*O*AcGD3 decreased. This event could be related to a decrease of GD3, induced by Neu3, as demonstrated by overexpression results. In conclusion, downregulation of Neu3 is an effective strategy adopted by leukemic cells to escape the increase of ceramide and thereby enhance their survival; this feature could be followed to assess the efficacy of therapeutic protocols in ALL patients.

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