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DIFFERENTIAL EXPRESSION OF 9-*O*-ACETYLATED SIALOGLYCOCONJUGATES ON LEUKEMIC BLASTS: A POTENTIAL TOOL FOR LONG-TERM MONITORING OF CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Earlier studies have demonstrated overexpression of 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) on lymphoblasts, concomitant with high titers of anti-9-*O*-AcSG antibodies in childhood acute lymphoblastic leukemia (ALL). Our aim was to evaluate the correlation between expression of different 9-*O*-AcSGs during chemotherapeutic treatment. Accordingly, expression of 9-*O*-AcSGs on lymphoblasts of ALL patients (n = 70) were longitudinally monitored for 6 years (1997–2002), using Achatinin-H, a 9-*O*-acetylated sialic acid (9-*O*-AcSA) binding lectin with preferential affinity for 9-*O*-AcSGs with terminal 9-*O*-AcSAα2$\rightarrow$6GalNAc. Western blot analysis of patients (n = 30) showed that 3 ALL-specific 9-*O*-AcSGs (90, 120 and 135 kDa) were induced at presentation; all these bands disappeared after treatment in patients (n = 22) who had disease-free survival. The 90 kDa band persisted in 8 patients who subsequently relapsed with reexpression of the 120 kDa band. FACS analysis revealed that at presentation (n = 70) 90.1 ± 5.0% cells expressed 9-*O*-AcSGs, which decreased progressively with chemotherapy, remained <5% during clinical remission and reappeared in relapse (80 ± 10%, n = 18). Early clearance of 9-*O*-AcSG$^+$ cells, during 4–8 weeks of treatment showed a good correlation with low risk of relapse. Sensitivity of detection of 9-*O*-AcSG$^+$ cells was 0.1%. Numbers of both high- and low-affinity binding sites were maximum at presentation, decreased with treatment and increased again in clinical relapse. We propose that close monitoring of 90 and 120 kDa 9-*O*-AcSGs may serve as a reliable index for long-term management of childhood ALL and merits therapeutic consideration.
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Key words: *Achatinin-H; 9-*O*-acetylated sialic acid binding lectin; acute lymphoblastic leukemia; carbohydrate; minimal residual disease; sialic acid*

ALL is a malignant transformation of lymphoblasts and represents the single most common type of cancer in the pediatric population. With the advent of modern chemotherapy for the treatment of childhood ALL, virtually all patients achieve remission and approximately 80% are cured.[1,2] With existing treatment protocols, the risk of relapse remains about 20% as patients in remission may harbor residual leukemic blasts (MRD).[1] Considering the increasing numbers of new ALL cases diagnosed worldwide,[2,3] an urgent need exists to identify ALL-specific biomarkers that are easily detectable and stably expressed the altered expression of which could be an index for reliable monitoring of ALL. Several methods for detection of MRD are currently being tested, but the technical expertise required and availability of specific probes limit their widespread clinical acceptability, especially in the developing world. Therefore, novel probes to identify unique disease-specific molecular markers are of utmost biomedical importance.

SAs, a family of 9-carbon carboxylated monosaccharides, are important constituents of the cell membrane known to influence many biologic reactions either by reacting with specific surface receptors or *via* masking of carbohydrate recognition sites.[4–7] Among the multiple variations of SA, the most frequently occurring substitutions are *O*-acetylation at positions C-7, -8 and -9 to form *N*-acetyl-7, -8 and -9-*O*-AcSAs, respectively, thus generating a family of *O*-AcSGs. However, as *O*-acetyl esters from positions C-7 and C-8 spontaneously migrate to C-9, even under physiologic conditions, *O*-acetylation at C-9 is considered the most common biologically occurring modification.

Both lectins and MAbs have been used to study sialylation patterns.[4,8–11] 9-*O*-acetyl-GD3, considered an oncofetal marker, has been identified using a lectin, *Cancer antennarius,* which recognizes SAs that are *O*-acetylated at both C-4 and C-9 and biomarkers of human melanoma.[10,12] The 9-*O*-acetylated disialosyl carbohydrate sequence of CDw60 has been reported as a marker on activated human B lymphoytes.[13] A consistent decrease of *O*-AcSA has been reported in a few diseases.[4] 9-*O*-acetylated sialoglycans are detectable at low levels on human B lymphocytes,[14] and pathologic variations have been found.[15–18] However, their detailed biologic significance, especially as potential biomarkers, remains obscure.

The preferential affinity of a lectin, Achatinin-H, toward terminal 9-*O*-AcSAα2$\rightarrow$6GalNAc was harnessed to identify the selective presence of 9-*O*-AcSGs on lymphoblasts of patients with ALL, which are minimally present on PBMCs of normal individuals.[5,19–23] Subsequently, we reported an enhanced level of anti-

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Abbreviations: 9-*O*-acetyl-GD3, 9-*O*-acetylated ganglioside; 9-*O*-AcSA, 9-*O*-acetylated sialic acid; 9-*O*-AcSG, 9-*O*-acetylated sialoglycoconjugate; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BM, bone marrow; CCG, Children's Cancer Group; CCR, continuous clinical remission; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; cyt-μ, cytoplasmic μ; DFS, disease-free survival; dsBSA, desialylated bovine serum albumin; FAB, French–American–British; FITC, fluorescein isothiocyanate; HRP, horse radish peroxidase; MAb, monoclonal antibody; MRD, minimal residual disease; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; SA, sialic acid; SIg, surface membrane Ig; TBS, TRIS-buffered saline; WBC, white blood cell.

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bodies against 9-*O*-AcSGs in these patients.[24,25] Absence of 9-*O*-AcSGs and anti-9-*O*-AcSGs in patients with other cross-reactive hematologic disorders, such as CML, AML, CLL, NHL, thalassemia and aplastic anemia, confirmed the specificity of these biomarkers.[19–25]

As modern treatment protocols utilize biologic and clinical information to tailor the intensity of therapy to the risk of relapse, using Achatinin-H as an analytic tool, the present study reports the differential expression of 9-*O*-AcSGs in different phases of treatment by *(i)* Western blotting, *(ii)* flow cytometry and *(iii)* measurement of receptor density by Scatchard analysis, to assess their correlation with disease status. Additionally, a small number of 9-*O*-AcSG$^+$ cells were detected as MRD.

MATERIAL AND METHODS

Patients

Subjects included clinically confirmed ALL patients at presentation (n = 70), referred to Vivekananda Institute of Medical Sciences (Kolkata, India) during 1997–2002. The group comprised 46 males and 24 females, the M:F ratio being 2:1; median age was 6 years (range 0.8–16) and median presenting WBC count was 12×10^9/l (range 0.4 to $1,000 \times 10^9$/l). A further 153 samples were obtained at different stages of chemotherapy. Diagnosis was established by cytologic examination of BM smears according to the FAB group recommendations,[26] belonging to L1 or L2, which were immunophenotyped using antibodies against TdT, cyt-μ, SIg, CD2, CD3, CD7, CD10, CD 13, CD19, CD20, CD33 and CD34. Among cases diagnosed as ALL, immunologic subgroups were defined as follows: common ALL (CD19$^+$, CD20$^\pm$, CD10$^+$, cyt-μ$^-$, SIg$^-$; n = 45), pre-B-ALL (CD19$^+$, CD20$^\pm$, CD10$^\pm$, cyt-μ$^+$, SIg$^-$; n = 10) and T-ALL (CD2$^\pm$, CD3$^+$, CD7$^+$; n = 15). Eighteen of these 70 patients relapsed: either BM (n = 8), CNS (n = 5), joint BM and CNS (n = 4) or testicular (n = 1). Recurrence was demonstrated after 18, 38, 42, 52, 58, 72, 74, 87, 98, 100, 116, 138, 154, 176, 199, 211, 220 and 224 weeks of diagnosis. Median follow-up was 36 months (range 1–84).

Children were entered into the UKALL X protocol[27] with addition of further drugs, *i.e.,* etoposide and cytosine, for intensification. They received vincristine, daunorubicin and prednisolone with L-asparaginase for induction. Intrathecal methotrexate was employed with or without cranial irradiation. Followed-up patients during maintenance therapy received intrathecal methotrexate, i.v. vincristine, oral methotrexate and mercaptopurine. They were broadly grouped as follows: induction of remission (phase A, 0–4 weeks), consolidation/early intensification (phase B, 4–8 weeks), period of maintenance therapy (phase C, 8 weeks–2.5 years), follow-up case (phase D, 2.5 years onward) and relapse (phase E). Controls included age-matched healthy individuals (n = 18) of both sexes. All newly diagnosed ALL patients (n = 70) were divided into risk-adjusted groups according to the CCG protocol.[28] Children 2–9 years of age with WBC count <10,000/ml, 10,000–49,999/ml and ≥50,000/ml were classified as low-, intermediate- and high-risk groups, respectively. Children 1–2 years of age with WBC count <50,000/ml were placed in the intermediate-risk group.

Venous blood (3–4 ml) or BM was collected at Vivekananda Institute of Medical Sciences and then sent to the Indian Institute of Chemical Biology, where PBMCs were separated by Ficoll-Hypaque density centrifugation. Informed consent was obtained from donors, patients and parents or guardians. The study was approved by the Institutional Human Ethical Committee per the protocol of the Indian Council of Medical Research.

Probe

The lectin Achatinin-H was affinity-purified from the hemolymph of the African giant land snail *Achatina fulica,*[29] which binds preferentially to glycoconjugates having terminal 9-*O*-AcSAα2→6GalNAc derivatives.[29–32] This was used as an analytic probe for all experiments.

Western blot analysis

PBMC membranes (30 μg/lane) from individual ALL patients at different phases of treatment were electrophoresed on SDS-PAGE (7.5%, Bio-Rad Minigel apparatus; Bio-Rad, Hercules, CA).[19,24] Proteins were transblotted on a nitrocellulose paper at 100 V for 2 hr. Membranes were blocked in TBS (0.05 M, pH 7.4) containing 10% dsBSA (TBS-dsBSA) and probed with Achatinin-H (160 μg/ml) in the presence of Ca^{2+} (0.05 M). After washing, blots were incubated at 4°C with rabbit anti-Achatinin-H (diluted 1:500), and the antigen–antibody complex was detected using HRP-conjugated goat antirabbit IgG (Cappel, Malvern, PA; 1:10,000).

Flow-cytometric analysis

Differential expression of 9-*O*-AcSGs on lymphoblasts of ALL patients during treatment was evaluated by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using FITC–Achatinin-H and anti-CD MAbs.[19,24] Briefly, cells (1×10^6/100 μl) from both T- and B-ALL patients in RPMI-1640 supplemented with 2 mM glutamine, gentamycin and 10% heat-inactivated human AB serum (medium A) were blocked with goat serum (10%) and then individually labeled with FITC–Achatinin-H or PE-conjugated anti-CD markers along with appropriate isotype controls (Pharmingen, San Diego, CA) on ice for 1 hr. Cells were washed and fixed in paraformaldehyde (1%), and binding was assessed. PBMCs were gated for lymphocytes by staining with anti-CD45 MAb, and >98.0% positive cells were present (data not shown). Binding of lectin and MAbs to lymphoblasts was evaluated by both single- and double-color flow cytometry. Analysis and calculations were performed using Cell Quest software (Becton Dickinson, San Jose, CA).

Evaluation of minimum number of 9-O-AcSG$^+$ cells by flow cytometry using FITC–Achatinin-H

To estimate the minimal number of 9-*O*-AcSG$^+$ cells detectable by Achatinin-H, its binding was examined using cells from T-(MOLT4) and B- (NALM-6) ALL cell lines, which were mixed in varying proportions with PBMCs of normal donors at ratios of 1:1, 1:10 and 1:100, keeping the total cell number constant (1×10^6). In parallel, lymphoblasts of an untreated B-ALL patient (cytomorphologic estimate of 98% blasts) were similarly analyzed.

Scatchard analysis

PBMCs from ALL patients (2×10^6/150 μl in each tube) at different phases of treatment were suspended in RPMI-1640 medium containing 20 mM HEPES buffer (pH 7.2) and 5 mg/ml dsBSA. Achatinin-H was iodinated with ^{125}I Na using the chloramine T method.[33] Cells were incubated with increasing doses of ^{125}I Achatinin-H for 90 min at 4°C in the presence or absence of a 100-fold excess of unlabeled Achatinin-H. ^{125}I Achatinin-H-bound cells were separated from free ^{125}I Achatinin-H by layering the mixture onto a 5% sucrose solution followed by centrifugation at 12,857g for 10 min at 4°C.[34] Cell-bound radioactivity in the pellet was measured by a gamma counter. The number of receptors per cell was extrapolated by the intersection of the curve with the x axis, and the K_d was obtained by dividing the number of receptors per cell by the bound/free ratio at the y axis intercept.[35,36]

Statistical analysis

Statistical analysis was performed using the GraphPad (San Diego, CA) Prism statistics software program. Results are expressed as means ± SD for individual experiments. Student's t-test and ANOVA or the Mann-Whitney test were applied depending on the normality of the sample. Reported values are 2-tailed, with $p <$ 0.05 considered statistically significant. Probabilities of survival were calculated by the Kaplan-Meier method and statistical differences between groups evaluated by log-rank tests.[37]

RESULTS

*Monitoring 9-*O*-AcSG expression in different phases of treatment by western blot analysis*

Differential expression of 9-*O*-AcSGs in ALL patients (n = 30) at different phases of treatment was demonstrated by Western blot analysis (Fig. 1*a*). The presence of 3 ALL-specific 9-*O*-AcSGs (135, 120 and 90 kDa) was established in all patients at presentation of the disease (phase A). At phase B, the 135 kDa band disappeared, and only the other 2 bands (120 and 90 kDa) were observed. Subsequently, in phase C and later in phase D, the 120 kDa band also disappeared. However, during follow-up there appeared to be 2 distinct patterns. The first pattern showed that, in a few patients (n = 8), the 90 kDa band remained in all phases of treatment, indicating persistence of a minimal number of lymphoblasts expressing 9-*O*-AcSG, possibly responsible for MRD; this was associated with clinical relapse. In all of these relapsed patients (n = 8), the 120 kDa band reappeared along with the 90 kDa band (phase E).

In contrast, the second group of patients (n = 22) consistently showed absence of all bands including the 90 kDa band at phase D1, indicating CCR, and good correlation with DFS. Serial monitoring of the 90 kDa band from this group was critical in determining distinctions at the MRD stage. Two bands, corresponding to 144 and 36 kDa, were consistently visible in normal donors. Assay sensitivity was 100%.

*Close association of DFS with lack of detectable 90 kDa 9-*O*-AcSG during clinical remission*

DFS of patients was evaluated based on the presence or absence of expression of the 90 kDa molecule during clinical remission (Fig. 1*b*). Survival curves were compared using the log-rank test. Expression of the 90 kDa molecule (n = 8) consistently showed a positive correlation with an increased probability of relapse ($p <$ 0.001). In the cohort of 90 kDa-positive patients, all 8 relapsed over a median observation time of 124 weeks (8–224 weeks), whereas no relapse in the 90 kDa-negative cohort (n = 22) was observed. According to Kaplan-Meier estimation, the probability of DFS in 90 kDa-positive patients was 0.0 at 3 years, whereas that of 90 kDa-negative patients was 100 (Fig. 1*b*). These data suggest the prognostic reliability of detecting the 90 kDa molecule during clinical remission.

*Variable expression of 9-*O*-AcSGs by FACS analysis*

Differential expression of 9-*O*-AcSGs on leukemic blasts at different phases of treatment was analyzed by flow cytometry using FITC–Achatinin-H (Fig. 2). At presentation (phase A, n = 70), leukemic blast populations (range 40–90% as morphologically estimated) were consistently positive, with Achatinin-H showing 90.1 ± 5.0% 9-*O*-AcSG$^+$ cells. Following chemotherapy, there was a progressive decrease in the percentage of 9-*O*-AcSG$^+$ cells, being 12.20 ± 5.35% in phase B (n = 60) and remaining at basal levels (<5%) in phases C (n = 34) and D (n = 30). Interestingly, in patients with frank relapse in phase C (n =11) and phase D (n = 7), the number of 9-*O*-AcSG$^+$ cells significantly increased (80 ± 10%, p = 0.0067) and showed a strong positive correlation (r = 0.94) with reemergence of the 90 and 120 kDa molecules on leukemic blasts (Fig. 1). The number of 9-*O*-AcSG$^+$ cells in normal individuals (n = 18) was only 3.0 ± 1.8% using the same dose of Achatinin-H.

*Status of 9-*O*-AcSG expression at early stage of treatment (phase B): a possible indicator for prognosis of ALL*

Based on the percentage of 9-*O*-AcSG$^+$ cells in phase B (n = 60), patients were divided into high-risk (n = 26, ≥5%) and low-risk (n = 34, <5%) groups (Fig. 2). DFS was monitored for these patients during long-term chemotherapeutic treatment. Survival curves were evaluated using the log-rank test (Fig. 3*a*). A positive correlation was present between the high-risk group (having higher numbers of 9-*O*-AcSG$^+$ cells) and increased probability of relapse. In this group, relapses (n = 18, 70%) were detected over a median observation time of 118 weeks (8–224 weeks), whereas there was no relapse in the low-risk group. According to Kaplan-Meier estimation, the probability of DFS was 30 at 3 years

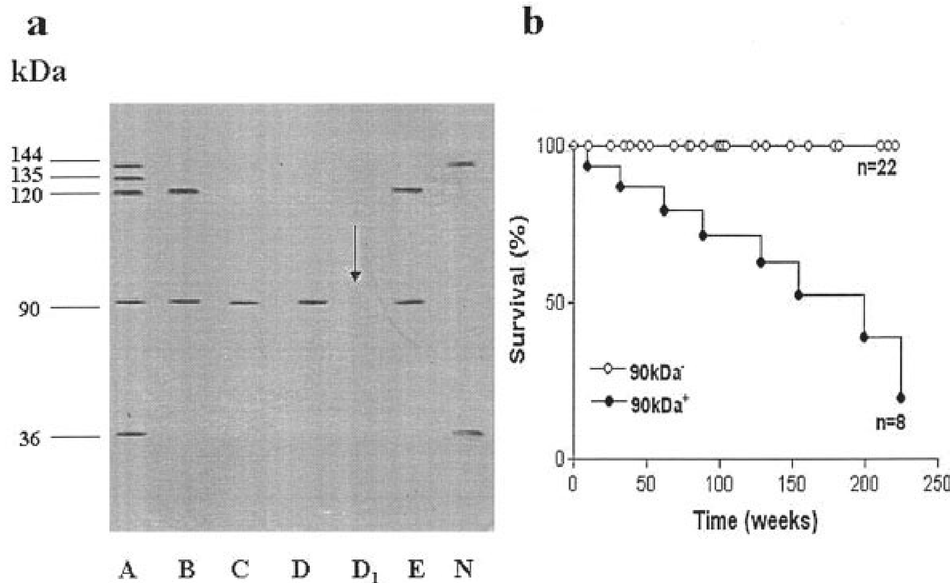

FIGURE 1 – *(a)* Representative Western blot analysis from an ALL patient at different phases of treatment. PBMC membrane fractions (30 μg) of an ALL patient at different phases of treatment and a normal donor were electrophoresed on a 7.5% SDS-PAGE gel, transferred to nitrocellulose paper and probed with Achatinin-H. Lanes A, B, C, D, E and N represent 9-*O*-AcSG profiles of patients from induction of remission, consolidation/early intensification, maintenance, follow-up and relapse as well as a normal donor, respectively. Lane D_1 represents a follow-up patient who did not relapse till the end of the period of study. *(b)* DFS curves of patients with ALL categorized according to persistence or disappearance of 90 kDa 9-*O*-AcSG at clinical remission as evidenced by Western blotting. Patients at various follow-up time points showed significantly poor DFS with persistence of 90 kDa 9-*O*-AcSG (n = 8) in contrast with good DFS in patients (n = 22) where 90 kDa 9-*O*-AcSG was absent (p = 0.002). Arrow indicates the absence of the 90 kDa 9-*O*-AcSG band at lane D1.

in the high-risk group and 100 in the low-risk group (Fig. 3*a*). The probability of DFS was comparable to the categories set by the CCG, relapses being 63% and 22% in high- and intermediate-risk patients, respectively (Fig. 3*b*). Risk of recurrence was further analyzed with reference to ALL lineage (Fig. 3*c*). There was a significant difference in outcome between the B- and T-ALL groups. In the high-risk group, 66% and 75% relapses were detected in B- and T-ALL patients, respectively. However, according to the categories set by the CCG, in high- and intermediate-risk groups, the rates of relapse were 58% and 14% in B-ALL and 80% and 50% in T-ALL patients, respectively (Fig. 3*c*). In both categories, T-ALL had a higher probability of relapse than B-ALL. Taken together, these data clearly demonstrate that early monitoring (phase B) of 9-*O*-AcSG$^+$ cells using Achatinin-H may give insight into the prognosis of ALL patients.

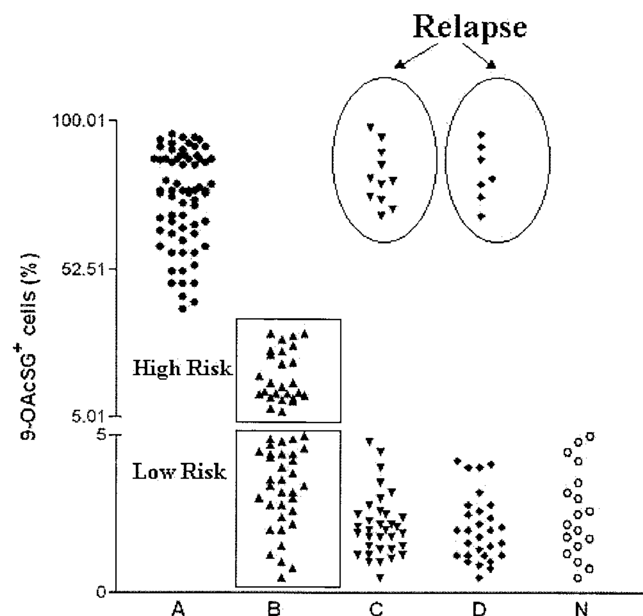

FIGURE 2 – Differential expression of 9-*O*-AcSGs in childhood ALL patients at different phases of treatment by FACS analysis using Achatinin-H as probe. Data are expressed as percentage of 9-*O*-AcSG$^+$ cells in leukemic blasts of an individual patient. Phases A, B, C, D and N represent patients at induction remission (n = 70), consolidation/early intensification (n = 60), maintenance therapy (n = 45) and follow-up (n = 37) as well as normal donors (n =18), respectively. All patients at phase B were divided into high-risk (n = 26) and low-risk (n = 34) groups corresponding to 9-*O*-AcSG$^+$ being ≥5% and <5%, respectively.

Sensitivity and quantitation of minimal number of 9-O-AcSG$^+$ cells using achatinin-h by FACS analysis

To simulate the *in vivo* scenario, the binding of Achatinin-H to a heterogeneous cell population with different ratios of normal PBMCS and MOLT4/NALM-6/PBMCS of a B-ALL patient was examined (Fig. 4). In the mixed cell populations, Achatinin-H could detect one leukemic blast in a total population of 10,[3] *i.e.,* 0.01%; therefore, this may be considered for detection of MRD. The percentage of blasts in these samples ranged 0.01–100%.

Differential expression of 9-O-AcSG: a potential marker for detection of MRD

We monitored this biomarker in 58 samples of BM, peripheral blood or both obtained from children (n = 27) with ALL in clinical remission using FITC–Achatinin-H in combination with PE antibodies against established CD markers (CD19, CD10, CD7, CD3 or CD34). Expression of 9-*O*-AcSG and other CD markers (CD19, CD10, CD3 and CD7) in 30 and 11 samples from B (n = 15) and T (n = 5) ALL patients, respectively, showed a consistently identical pattern of expression similar to that of normal PBMCs, suggesting that patients were in CCR. The remaining 17 samples, from B (n = 5) and T (n = 2) ALL, showed minimal levels of 9-*O*-AcSG, which may be indicative of MRD. These patients subsequently relapsed, accompanied by reexpression of 9-*O*-AcSGs along with high expression of CD10, CD19, CD3, CD7 or CD34. A positive correlation was observed between 9-*O*-AcSG

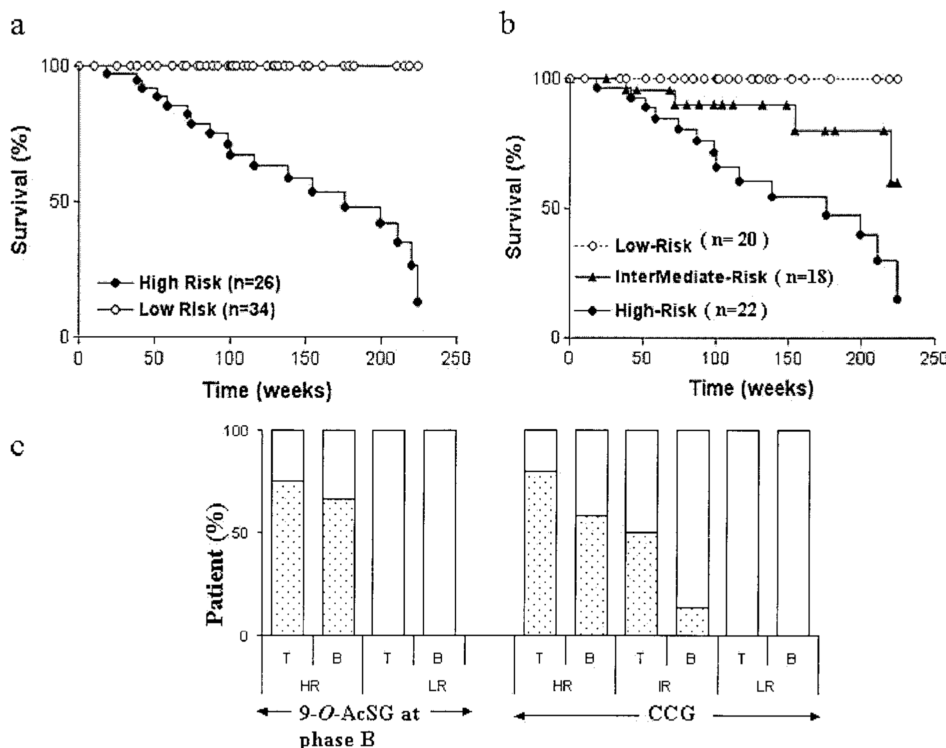

FIGURE 3 – *(a)* DFS curves of patients with ALL categorized according to 9-*O*-AcSG levels at phase B by FACS analysis. Patients at various follow-up time points showed significantly poor DFS with high levels of 9-*O*-AcSG (high-risk group, n = 26), in contrast to good DFS in the low-risk group (n = 34), where the level of 9-*O*-AcSG was <5% (p = 0.002). *(b)* DFS curves of patients with ALL categorized according to the CCG. Patients were grouped according to CCG criteria,[28] as described in Material and Methods. Significantly poor DFS in high-risk (n = 22) and intermediate-risk (n = 18) and good DFS in low-risk (n = 20, p = 0.002) groups were observed. *(c)* Comparison of ALL types according to expression of 9-*O*-AcSG at phase B and the CCG. Risk groups have been separated in B- and T-ALL patients, and percentages of relapse (stippled bars) in either lineage in individual risk groups were compared. Open bars, patients in clinical remission.

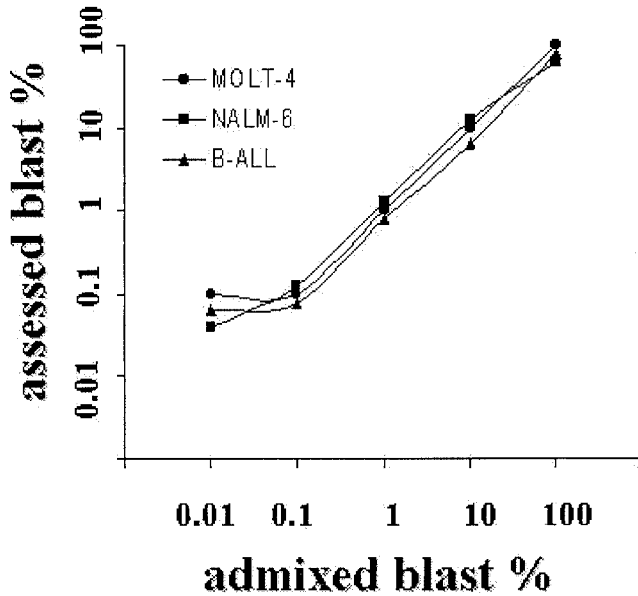

Figure 4 – Dilution experiment showing sensitivity for detection of MRD. Leukemic blasts of an untreated patient of B-ALL, MOLT-4 or NALM-6 were mixed with PBMCs of normal donors in various proportions. For each combination, 9-*O*-AcSG$^+$ cells were analyzed by FACS using FITC–Achatinin-H. The *x* axis represents the percentage of admixed blast cells. The percentage of 9-*O*-AcSG$^+$ blasts represents the mean of triplicates and is plotted on the *y* axis.

TABLE I – COMPARISON OF COMBINATION OF ACHATININ-H STAINING WITH THAT OF ESTABLISHED CD MARKER BY FACS ANALYSIS FOR MONITORING ALL PATIENTS DURING CLINICAL REMISSION

MAb/lectin combination	Frequency of application[1]
CD19–Achatinin-H	12 (60%)
CD10–Achatinin-H	9 (45%)
CD19–CD10	6 (30%)
CD19–CD34	5 (25%)
CD7–Achatinin-H	5 (100%)
CD5–Achatinin-H	3 (60%)
CD7–CD5	2 (40%)

[1]Percentages of samples with B- and T-ALL followed using PE–anti-CD MAbs or FITC–Achatinin-H, respectively.

and the established CD markers at presentation of the disease. In these samples ($n = 17$), during clinical remission, a direct comparison between the presence of these CD markers in combination with 9-*O*-AcSG is provided in Table I, indicating the usefulness of lectin staining for ALL monitoring. Representative profiles of patients are shown in Figure 5, in which 78% of cells from a T-ALL patient were CD7$^+$9-*O*-AcSG$^+$ at diagnosis. During remission, the number of these cells decreased to 4.2%. Similarly, 80% of cells from a B-ALL patient at presentation were CD10$^+$9-*O*-AcSG$^+$. Following chemotherapy, only 0.42% of these cells were detected in clinical remission (Fig. 5). Minimal levels of 9-*O*-AcSGs were detected on cells obtained from both BM ($n = 11$) and peripheral blood ($n = 6$) from patients irrespective of lineage. In general, significant increases of the CD10$^+$9-*O*-AcSG$^+$ (70 ± 12%) and CD7$^+$9-*O*-AcSG$^+$ (90 ± 8%) populations in B- and T-ALL patients correlated well ($r = 0.94$) with the reappearance of the 90 and 120 kDa molecules (Fig. 1) on leukemic blasts leading to clinical relapse.

Monitoring differential expression of 9-O-AcSGs by scatchard analysis

The observation that Achatinin-H binds to leukemic blasts of ALL patients prompted us to study the nature of their binding by Scatchard analysis. The binding of ^{125}I Achatinin-H was stronger at 4°C than at 25°C and needed approximately 60 min to achieve saturation under the experimental conditions used. Unlabeled Achatinin-H competed with the binding of ^{125}I Achatinin-H, and 100-fold excess completely abolished the binding. Analysis of binding curves revealed that the binding was biphasic at presentation and relapse, indicating the existence of at least 2 types of binding site with about a 4-fold differences in affinity (Fig. 6, Table II). A representative Scatchard plot from a patient at presentation is shown in Figure 6. Striking differences were observed in the number of binding sites. At presentation ($n = 5$), large numbers of both high-affinity (62,000/cell, low K_d) and low-affinity (197,000/cell, high K_d) binding sites were observed. Following treatment, the high-affinity sites disappeared and the low-affinity sites reduced by 66-fold. Interestingly, at relapse high-affinity sites reappeared (11,200/cell) and low-affinity sites increased (27,500/cell).

DISCUSSION

Interest in the use of biomarkers to evaluate future disease risk has been greatly enhanced since they are observable end points in disease. Assessment of the *O*-acetylated sialoglycan profile in ALL is a relatively new domain in leukemia sialobiology, where the increased presence of 9-*O*-AcSA have been established as an important disease-specific determinant.[5,19–25] Minimal expression in normal healthy donors and in cross-reactive hematologic diseases substantially proves that 9-*O*-AcSGs are novel biomarkers unique in childhood ALL. The major achievements of the current investigation includes the demonstration of *(i)* the involvement of a unique 120 kDa molecule as an important indicator for predicting relapse in ALL irrespective of lineage, *(ii)* good correlation between absence of the 90 kDa molecule during clinical remission with DFS and *(iii)* a positive association between early (phase B) clearance of 9-*O*-AcSG$^+$ cells with reduced incidence of relapse.

The selective binding affinity of Achatinin-H allowed for the successful detection of membrane-bound 9-*O*-AcSGs (Figs. 1, 2, 4–6) corresponding to 144, 135, 120, 90 and 36 kDa, which were consistently observed at disease presentation (Fig. 1). Of these, 2 9-*O*-AcSGs, corresponding to 144 and 36 kDa, were constitutively present on normal PBMCs[5,19,24,25] and cannot therefore be designated as ALL-specific. However, these 2 9-*O*-AcSGs consistently disappeared with initiation of chemotherapy and remained undetectable 1 year after treatment or in relapse (Fig. 1); the cause of this loss is presently not clear. Although all 5 bands possess a similar glycotope, namely, 9-*O*-AcSAα2→6GalNAc, the molecular properties of the individual sialoglycoproteins vary from each other. Fluorimetric quantitation revealed low amounts of 9-*O*-AcSA on normal PBMCs, indicating that the 36 and 144 kDa bands are less 9-*O*-acetylated than the ALL-specific 9-*O*-AcSG.[5,19] Earlier reports also suggested that both the 36 and 144 kDa forms are less immunogenic, as evidenced by reduced complement-mediated cell lysis.[24,25]

Of the 3 ALL-specific molecules corresponding to 135, 120 and 90 kDa, only the 90 kDa band persisted during clinical remission in patients who relapsed ($n = 8$), suggesting its potential application for monitoring of MRD (Fig. 1*b*). The absence of the 90 kDa molecule in phase D correlated (100%) with DFS, reinforcing the prognostic value of this molecule.

The disappearance of the 120 kDa band as early as 8 weeks of treatment and its continued absence during clinical remission suggest its utility in evaluating disease status. Early clearance of leukemic cells is reported to have a favorable prognostic relevance;[38] therefore, close monitoring of the 120 kDa molecule using Achatinin-H/anti-120 kDa MAb by FACS analysis is expected to be highly beneficial in the prediction of relapse. Reinduction of the 120 kDa band in phase E clearly indicates its potential for prediction of relapse during maintenance therapy. We therefore propose that critical examination of these 2 newly in-

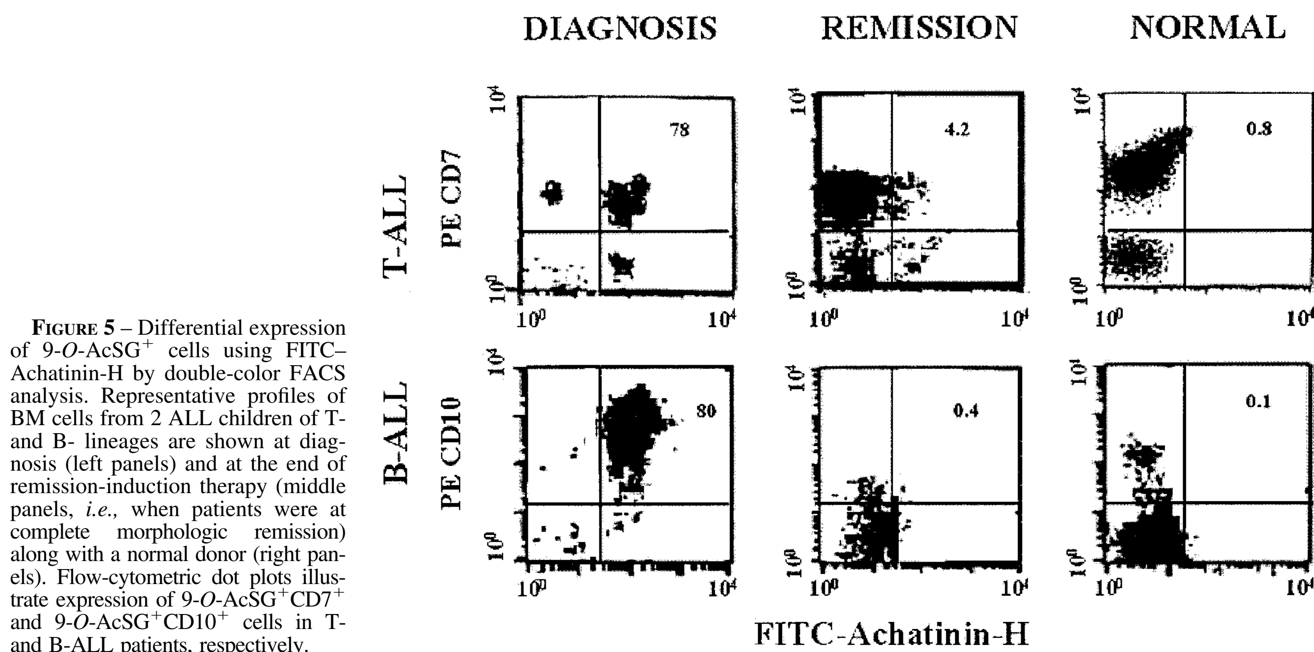

Figure 5 – Differential expression of 9-*O*-AcSG^{+} cells using FITC–Achatinin-H by double-color FACS analysis. Representative profiles of BM cells from 2 ALL children of T- and B- lineages are shown at diagnosis (left panels) and at the end of remission-induction therapy (middle panels, *i.e.,* when patients were at complete morphologic remission) along with a normal donor (right panels). Flow-cytometric dot plots illustrate expression of 9-*O*-AcSG^{+}CD7^{+} and 9-*O*-AcSG^{+}CD10^{+} cells in T- and B-ALL patients, respectively.

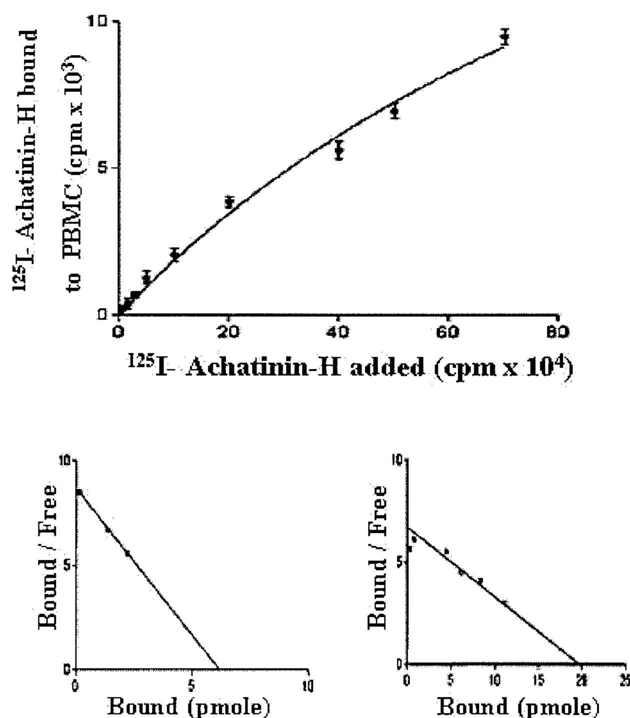

Figure 6 – Scatchard analysis of 9-*O*-AcSG receptors present on mononuclear cells from ALL patients. Cells (2×10^{6}/tube) suspended in RPMI-1640 medium were incubated for 90 min at 4°C with varying doses of ^{125}I Achatinin-H. Specificity of binding was determined in parallel competition experiments using a 100-fold excess of unlabeled Achatinin-H. To determine specific binding, the nonspecific binding (obtained from 100-fold excess of unlabeled Achatinin-H in the presence of the same amount of ^{125}I Achatinin-H used in binding to PBMCs) was subtracted from the total binding of ^{125}I Achatinin-H. K_d values and numbers of Achatinin-H binding sites per cell were derived from Scatchard analysis. Data are means of 3 independent experiments from a patient at presentation.

duced antigens may provide an efficient tool for universal monitoring of MRD, potentially applicable for designing patient-specific chemotherapy of childhood ALL.

Analytic methods for quantification of these alkali-labile *O*-AcSGs are prone to error,[39] and substantial loss of this *O*-acetylation during membrane preparation was reported earlier.[18] Therefore, accurate determination of these saponifiable groups, in the proper sterical context, on the intact cell surface is feasible only by noninvasive approaches, such as FACS analysis with lectins (Fig. 2) or use of antibodies as probes.[19,24,25]

FACS analysis showed a higher degree of Achatinin-H binding at presentation compared to patients in clinical remission (Fig. 2). This corroborated with enhanced expression of the 135, 120 and 90 kDa molecules before treatment, whereas minimal binding of Achatinin-H during remission was possibly due to the persistent 90 kDa molecules. The sharp increase in 9-*O*-AcSG^{+} cells with clinical relapse was in agreement with the reappearance of the 120 kDa band in Western blots. Patients belonging to the high-risk group in phase B exhibited poorer DFS than those in the low-risk group (Fig. 3*a*). Patients in the low-risk group did not relapse even after 3 years (Fig. 3*a*). Hence, our results suggest that measurement of 9-*O*-AcSG^{+} cells at phase B can be utilized for prognostic evaluation.

Single-color FACS analysis using Achatinin-H was corroborated by double-color analysis (Fig. 5), wherein the high expression of 9-*O*-AcSG concomitant with expression of CD10^{+}, CD19^{+}, CD3^{+}, CD7^{+} or CD34^{+} was used as a measure of circulating lymphoblasts of B- and T- ALL. However, minimal presence ($<5\%$) of such cells at clinical remission may be considered MRD as supported by clinical parameters (Fig. 5).

Identification of MRD by their morphologic features is subjective and lacks sensitivity mainly due to the limited number of specific CD markers used for routine immunophenotyping. So far, progress in the identification of new leukemia-specific markers has relied on testing the expression of known CD markers, *e.g.,* Tdt, CD66, CD58, CD45, CD34, CD19, CD20, CD22, CD10, CD3, CD5, *etc.,* which is largely based on trial and error and is slow.[3,40–42] As experienced in BIOMED Concerted Action on MRD, using different triple combinations of antibodies (CD10–CD20–CD19, TdT–CD10–CD19, TdT–CD5–CytCD3), sensitivity of 10^{-4} in 82% and 10^{-5} in 12% of the samples was reported.[42] Detection of MRD with a sensitivity of 0.01–1.13% has been

TABLE II – SCATCHARD ANALYSIS OF THE BINDING OF ACHATININ-H TO PBMCs OF ALL PATIENTS AT DIFFERENT PHASES OF TREATMENT[1]

Phases of treatment	High-affinity binding site		Low-affinity binding site	
	Binding sites/cell	K_d (pM)	Binding sites/cell	K_d (pM)
Presentation	62,000 ± 344	0.77	197,000 ± 676	2.98
Clinical remission	—	—	3,000 ± 68	2.60
Relapse	11,200 ± 211	1.38	27,500 ± 324	3.98
Normal donors	—	—	4,500 ± 66	4.89

[1]PBMCs (2×10^6) from ALL patients at different phases of treatment and from normal donors were incubated with ^{125}I Achatinin-H at 4°C. A 100-fold excess of unlabeled Achatinin-H was used to evaluate the specific nature of binding. Bound and unbound Achatinin-H were separated, and bound radioactivity was measured as described in Material and Methods. —, Not found.

observed using anti-CD58 antibody.[41] Achatinin-H is capable of detecting 0.1–1% leukemic cells by identifying 9-*O*-AcSG$^+$ cells as a measure of MRD (Fig. 4). This assay gave a sensitivity of 10^{-3} in 60% of total samples from both B- and T-ALL patients. However, in 29% of the total cases, sensitivity was 10^{-4} and maximum sensitivity, under these circumstances, was 10^{-5} in 11% of the total cases. It may be possible to further increase the level of sensitivity, in most patients, using Achatinin-H along with different triple combinations of anti-CD antibodies.

Use of peripheral blood to detect MRD is debatable[42] as 2 recent studies[43,44] have shown that paired MRD values in BM and peripheral blood are highly concordant in T-ALL. Conversely, large differences frequently occur in B-ALL, where levels are much higher in BM than in peripheral blood. Thus, there are different amounts of ALL cells of different lineages in blood after treatment as measured by known CD markers. However, Achatinin-H is able to consistently detect 9-*O*-AcSGs, as an ALL-specific marker, in both peripheral blood and BM irrespective of lineage (Figs. 2, 5).[24] However, larger trials are needed to establish whether peripheral blood investigation can substitute for BM analysis. Achatinin-H along with antibodies against the 90 and 120 kDa bands can serve as new armaments in ALL research.

Differential expression of 9-*O*-AcSGs was reflected in the Scatchard analysis (Table II). It was consistently observed that there were large numbers of both high- and low-affinity receptors at disease presentation, very low levels of low-affinity binding sites during clinical remission and increased levels of both during relapse. Therefore, the 120 and 90 kDa molecules may be associated with the high- and low-affinity receptors, respectively; however, the variations of K_d in different phases of treatment are difficult to explain in terms of discrete molecular entities.

O-Acetylation of sialoglycoconjugates depends primarily on *O*-acetyltransferases and possibly on sialyltransferases. Homeostasis of these enzymes, responsible for the resultant expression of *O*-acetyl derivatives, may become perturbed during medication, giving rise to differential effects on different 9-*O*-AcSGs and accounting for slight variations in K_d values. In future, it will be important to determine the role of fine-tuning these enzymes in disease conditions. However, measurement of *O*-acetyltransferase activity is extremely difficult,[45] though the substrate specificity and subcellular localization of this enzyme in normal human colonic mucosa have been demonstrated.[46]

Modification of cell surface sialyl residues can be correlated to a differentiation marker in developmental processes and protection against sialidases.[4,5,47] However, the relationship between 9-*O*-acetylation of SAs during neoplastic transformation and immune system abnormalities remains poorly understood. 9-*O*-acetylation is known to abrogate the normal functions of SAs in preventing the activation of alternate complement pathways due to involvement of the exocyclic side chain of SA. 9-*O*-AcSGs on erythrocytes of patients suffering from visceral leishmaniasis are potent complement activators that cause enhanced hemolysis.[48] Interestingly, derivatives of the exocyclic side chain of SA are also important determinants of Achatinin-H binding.[29] The ligand of human B-cell adhesion molecule (CD22) is a ubiquitously present glycotope with α2-6 linked SA, and it has been postulated that 9-*O*-AcSGs play a regulatory role, preventing undesirable CD22-mediated interactions with inappropriate targets through this moiety.[49] Hence, the increased expression of 9-*O*-AcSGs on lymphoblasts hinders the cascade of CD22-mediated cellular interactions by masking their binding sites, which is also the epitope of Achatinin-H,[29,30,32] thereby influencing cell adhesion-dependent immune responses. Therefore, it is reasonable to hypothesize that the increased *O*-acetylation may well be an effective strategy adopted by leukemic blasts to circumvent immune surveillance.

In summary, our results indicate that the 90 and 120 kDa molecules are 2 novel lymphoblastoid antigens, which would allow the design of disease-specific primers for detection of MRD and may help in the development of new therapeutic strategies. The availability of MAbs against these molecules is expected to be useful for immunophenotyping and drug targeting in ALL.

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