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Antibodies against 9-*O*-acetylated sialoglycans: a potent marker to monitor clinical status in childhood acute lymphoblastic leukemia

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Abstract

Background: Although childhood acute lymphoblastic leukemia (ALL) is highly responsive to chemotherapy, reliable techniques are needed to determine treatment outcome and predict impending relapse. In ALL, the cell surface over expression of 9-*O*-acetylated sialoglycans (9-*O*AcSGs) on lymphoblasts and concomitant high antibody titers in patients' sera was reported.

Objectives: The present study was aimed to evaluate whether anti-9-*O*AcSG titers can be harnessed to monitor the clinical outcome of ALL.

Design and methods: Anti-9-*O*AcSGs were analyzed by ELISA in children receiving either UK ALL X ($n = 69$, Group I) in India or UK ALL 97 ($n = 47$, Group II) in UK along with age-matched normal healthy controls at different time points over a period of >2 years. An attempt was also made to investigate subclass distribution of disease-specific IgG. Moreover, 17 patients having a higher sample size were longitudinally monitored.

Results: Antibody levels were raised at disease presentation, decreased with remission induction, and importantly, reappeared with clinical relapse. Sera from patients with other hematological disorders and normal controls showed negligible levels of circulating anti-9-*O*AcSGs. In patients of both Groups I and II, the assay showed high sensitivity (98.92% and 96.77%) and specificity (92.1% and 95.91%), respectively. IgG subclass analyses during different phases of treatment revealed that 9-*O*AcSG-specific IgG_1 could serve as a better prognostic marker in ALL.

Conclusions: This study demonstrated the potential of this disease-specific antibody as an alternate marker in diagnosis and long-term assessment of ALL patients, suggesting its application in detection and prediction of impending relapse. Therefore, the expression of anti-9-*O*AcSGs, irrespective of their treatment protocol, may serve as an economical yet effective index for monitoring of childhood ALL.
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Keywords: Antibodies against 9-*O*-acetylated sialic acids; Acute lymphoblastic leukemia (ALL); Bovine submaxillary mucin; *O*-acetylated sialic acids; Achatinin-H

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant transformation of lymphoblasts and represents the single commonest type of cancer in the pediatric population. With existing treatment protocols, the risk of relapse remains about 20% as patients in remission may harbor residual leukemic blasts [1]. Although leukemic cells of patients in remission cannot be identified by routine cytomorphology, they persist in some cases and can recur unless further chemotherapy is given. The mandatory technical expertise required for detection of minimal residual disease (MRD) limits its widespread clinical acceptability, and an urgent need exists to identify biochemical markers whose altered expression could be exploited for evaluating individual chemotherapeutic response and predicting impending relapse [2].

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Sialic acids, a family of 9-carbon carboxylated monosaccharides, are important constituents of lymphocyte cell membranes and play a significant role in the mediation of many biological phenomena involving cell–cell interactions either by reacting with specific surface receptors or via masking of carbohydrate recognition sites [3,4]. Amongst the diverse multitude of variations of the parent sugar Neu5Ac, the most frequently occurring modification is *O*-acetylation at positions C-7, 8, and 9 to form *N*-acetyl-7,8,9-*O*-acetyl neuraminic acid, respectively, thus generating a family of *O*-acetylated sialoglycoconjugates or *O*-AcSGs [5]. However, as *O*-acetyl esters from C-7 and C-8 positions spontaneously migrate to C-9 position, even under physiological conditions, *O*-acetylation at C-9 position is considered as the commonest biologically occurring modification [6].

Although the presence of antibodies against *O*-acetylated sialic acids in normal human sera has long been identified [7], little progress has been made in either identifying the presence of such an antibody fraction in disease conditions or in assigning it a biological role. In previous studies, we have shown that the selective presence of 9-*O*-acetylated sialoglycans (9-*O*AcSGs) on peripheral blood mononuclear cells of patients with ALL can serve as a surrogate marker for impending relapse [3,8–10]. Furthermore, immunogenicity of 9-*O*AcSGs was demonstrated by increased levels of IgM, IgG_1, and IgG_2 subtypes [11,12]. In the present investigation, we undertook a prospective study to correlate the disease status of childhood ALL patients with antibody levels directed against 9-*O*AcSGs.

Materials and methods

Serum samples from childhood ALL patients and controls

The study included sera from children with acute lymphoblastic leukemia (ALL) received at Vivekananda Institute of Medical Sciences, Kolkata (India; $n = 69$, Group I), and Cancer Research Unit, University of Newcastle upon Tyne Medical School (UK; $n = 47$, Group II). Diagnosis of leukemia was performed by standard cytological and histochemical examination of bone marrow and blood smears according to French–American–British (FAB) classification [13] and by immunophenotyping using a panel of monoclonal antibodies that included CD 2, 3, 4, 7, 8, 10, 19, 20, 36, 45, 13, 33, 34, HLA-DR, and surface immunoglobulin (SIg). Patients with L3 (according to French–American–British criteria) morphology and/or B-ALL (SIg positive) were excluded from the study. Age of the children ranged from 2.5 to 11 years with a male/female ratio of 2:1. Patients of Group I received therapy according to UK ALL X, whereas the UK group (i.e., Group II) received UK ALL 97 [14].

The patients were grouped according to the stages of remission, that is, induction of remission (phase A, 0–4 weeks), consolidation/early intensification (phase B, 4–8 weeks after starting chemotherapy), period of maintenance therapy (phase C, 8–104 weeks), follow-up (phase D, >2 years), and patients who relapsed (phase E). Patients of Group I were given multiple drugs as per UK ALL X protocols with addition of further drugs, that is, etoposide and cytosar, for intensification. They received vincristine, daunorubicin, and prednisolone with L-asparaginase for induction. Intrathecal methotrexate was employed with or without cranial irradiation. Patients who were followed-up during maintenance therapy received intrathecal methotrexate, intravenous vincristine, oral methotrexate, and mercaptopurine. Patients of Group II were randomized to receive thiopurine drugs, 6-mercaptopurine (6-MP), or 6-thioguanine (6-TG) during continuing therapy.

At each consecutively numbered visit, clinical information and blood samples were obtained, and sera were stored at $-70°C$. The study included a total of 131 and 111 samples in Groups I and II, respectively, where 69 and 47 patients were recruited with new-onset ALL (phase A). Subsequently, Groups I and II had 24 and 15 samples, respectively, in phase B, whereas 21 and 41 in phase C. In phase D, 17 and 8 samples were assessed. This discrepancy in sample number at different time points of the study was due to logistic constraints in acquiring the samples, and in some cases, due to death of patients. Controls were normal healthy donors ($n = 70$, Group I and $n = 58$, Group II). To assess the assay specificity, sera from patients with other hematological disorders were analyzed viz., chronic myelogenous leukemia (CML, $n = 16$, age range >40 years), chronic lymphocytic leukemia (CLL, $n = 14$, >60 years), acute myelogenous leukemia (AML, $n = 18$, 18–67 years), aplastic anemia ($n = 6$, 12.4–78 years), thallassemia ($n = 8$, 2–14.1 years), and non-Hodgkin's lymphoma (NHL, $n = 18$, 4.6–58 years) along with adult acute lymphoblastic leukemia ($n = 10$, 33–67 years). Clinical remission was defined as <5% blasts in the bone marrow and absence of leukemic cells in peripheral blood. Informed consent was taken from parents and patients. The Institutional Human Ethical Clearance Committee approved the study.

Preparation of bovine submaxillary mucin and its derivatives

Bovine submaxillary mucin (BSM) was prepared according to the method of Murphy and Gottschalk [15]. Briefly, tissues were homogenized and extracted with water by centrifugation at $10000 \times g$ for 15 min at 4°C. The supernatant was collected, pH adjusted to 4.5, and the resulting precipitate removed by centrifugation at $5000 \times g$ for 20 min. The supernatant was neutralized (pH 6.0) and dialyzed against water. Barium acetate was slowly added to the dialysate to make it 0.1 M. Precooled methanol was then slowly added to give an alcohol concentration of 64% (v/v) and incubated overnight at 4°C. The precipitate formed was retrieved by centrifugation, dissolved in 0.1 M EDTA,

dialyzed extensively against water, and stored at −20°C until use. Protein content was measured by the method of Lowry et al. [16] using bovine serum albumin (BSA) as the standard. The percentage of 9-*O*AcSA derivatives present in BSM was quantified fluorimetrically [17] and by fluorimetic HPLC [18]. BSM was de-sialylated by incubation with 10% H_2SO_4 (0.05 M) for 1 h at 80°C. The *O*-acetyl group of sialic acid of BSM was removed by incubating it with NaOH (0.01 M) for 1 h at 4°C.

Evaluation of anti-9-OAcSG levels

Microtiter plates were coated with purified BSM (10 μg/ml, 100 μl/well) in 0.02 M phosphate buffer, pH 7.4, overnight at 4°C. BSM was used based on the available knowledge that it contains a high proportion of 9-*O*AcSGs linked with subterminal GalNAc of the underlying oligosaccharide chain [19]. Following blocking of nonspecific binding sites with PBS containing 2% BSA, binding of sera (diluted 1:10) was colorimetrically measured on an ELISA reader (OD_{405} nm) using horseradish peroxidase (HRP) conjugated Protein-A (diluted 1:15 000) and azino-bis thio-sulfonic acid (ABTS) as the substrate [11].

Quantification of anti-9-OAcSG

Preparation of affinity matrix using purified BSM and asialo-BSM

Activation of Sepharose 4B (Pharmacia) was done by the method of Kohn and Wilchek [20] using cyanogen bromide. Activated beads were allowed to couple with both BSM and asialo-BSM (5 mg/ml gel) separately in 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 7.4.

Purification of anti-9-OAcSG

Human sera (6.0 ml) from an ALL patient, at presentation, was used to purify an anti-9-*O*AcSG fraction with preferential affinity for 9-*O*AcSG derivatives using the method of Siebert et al. [21] and modified by Pal et al. [11]. Briefly, serum was subjected to a 33% ammonium sulfate fractionation, passed over an asialo-BSM-Sepharose 4B column to remove any galactose binding fractions, and the run through was loaded onto a BSM-Sepharose 4B column. The specific protein was eluted with 0.1 M ammonium hydroxide, pH 11.0, followed by immediate neutralization with 0.2 M sodium dihydrogen phosphate, pH 4.0, and extensively dialyzed against Tris-buffered saline (TBS). To determine the proportion of immunoglobulin present in this fraction, it was passed through Protein A Sepharose 4B, and the protein content of the bound fraction was measured by the method of Lowry [16].

Generation of standard curve using purified anti-9-OAcSGs

Affinity-purified anti-9-*O*AcSGs (0–200 μg/ml, 100 μl/well) [11,12] were added to BSM-coated microtiter plates (10 μg/ml, 100 μl/well) and incubated overnight at 4°C. The wells were washed with PBS–Tween 20 and binding measured using HRP-conjugated Protein A (diluted 1: 10 000, 100 μl/well) at 405 nm. The standard curve thus generated was applied for quantitation of total anti-9-*O*AcSGs present in sera of ALL patients at various stages of the disease.

Immunoglobulin subclass distribution of anti-9-OAcSGs

Serum IgG subclass levels were determined by incubating sera (diluted 1:10, 100 μl/well) overnight at 4°C with BSM (10 μg/ml, 100 μl/well) as the coating antigen; the wells were washed with PBS–Tween 20 and then incubated overnight at 4°C with mouse anti-human IgG_1, IgG_2, IgG_3, and IgG_4 (diluted 1:3000, 100 μl/well). After three washes with PBS–Tween 20, binding was measured using anti-mouse-HRP-linked IgG (diluted 1:10 000) and detected as above. In all experiments, positive and negative human sera, previously tested by antibody screening assays, were included as controls. Controls for nonspecific binding included binding of sera from wells that had no BSM and wells where test sera were omitted.

The results have been reported as absolute absorbances or as antibody-binding index of sera diluted 1:10 and calculated according to the following equation [22].

$$\text{Binding index} = \frac{OD_{405}(\text{serum sample at 1:10 dilution}) - OD_{405}\ (\text{background})}{OD_{405}(\text{reference normal serum sample at 1:10 dilution}) - OD_{405}\ (\text{background})}$$

The reference normal serum for each assay was the median of sera from 50 normal healthy individuals.

Statistical analysis

Statistical analyses were performed using the Graph-Pad Prism statistics software (Graph-Pad Software, San Diego, CA). Student's unpaired and paired *t* tests were used. Reported values are two tailed and *P* values less than 0.05 were considered as statistically significant. ROC analysis was used to assess the cut-off value of the assay to evaluate the two patient groups. The Spearman's correlation test was used for the comparison of independent variables and the data were accepted when probability was greater than 95% confidence level. Group statistics were performed using one-way analysis of variance with post-test for linear trend.

Results

Standardization of BSM-ELISA

To ensure measurement of total 9-*O*AcSG-specific immunoglobulins, comprising IgG of mainly IgG_1 and IgG_2 subtype [11] along with IgM [12], we used HRP conjugated Protein A as the detecting reagent. Once the

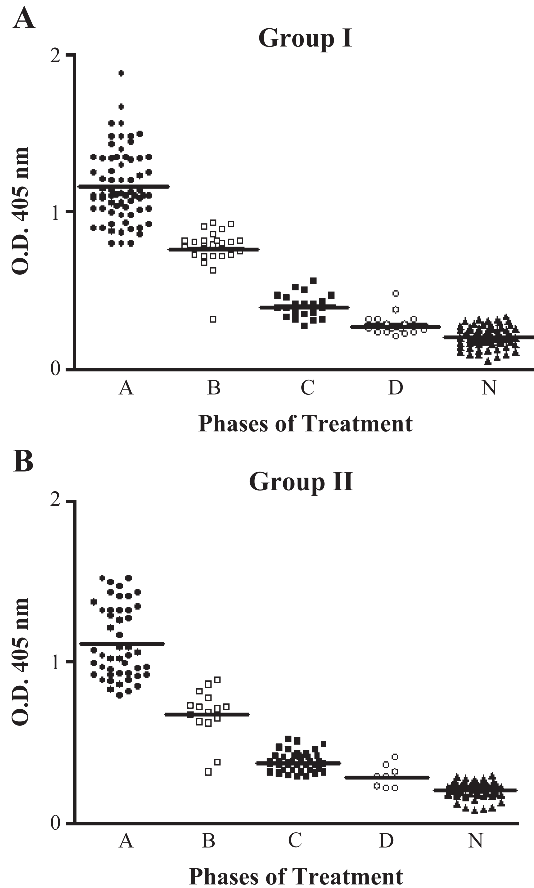

Fig. 1. ELISA-based measurement of antibodies against 9-*O*-acetylated sialoglycans in childhood ALL patients at different phases of treatment. (A) Group I (India) received UK ALL X. (B) Group II (UK) received UK ALL 97. Antibody levels against 9-*O*AcSGs were measured in ALL patients from different stages of treatment, that is, induction of remission (phase A, 0–4 weeks), consolidation/early intensification (phase B, 4–8 weeks), period of maintenance therapy (phase C, 8–104 weeks), follow-up (phase D, >2 years), and normal individuals (N) as described in Materials and methods.

assay conditions were established, we evaluated levels of anti-9-*O*AcSGs in sera of 128 age-matched normal healthy individuals whose mean OD_{405} nm ± SD was 0.21 ± 0.06. To minimize the false positivity, the cut-off value of the assay was selected as 0.4 based on the mean OD_{405} nm + 3 SD obtained from normal controls, and this was found to be significant using receiver operating characteristic curve (ROC).

Variable antibody levels against 9-OAcSGs in ALL patients at different stages of treatment

Irrespective of the chemotherapy protocol, antibodies against 9-*O*AcSGs were consistently increased at presentation (phase A), mean OD_{405} nm ± SD in Groups I (n = 69) and II (n = 47) being 1.16 ± 0.22 and 1.11 ± 0.22, respectively. In contrast, the mean OD_{405} nm ± SD in normal individuals in Groups I and II was significantly lower being 0.2 ± 0.06 and 0.21 ± 0.05, respectively (N, n = 70 and 58, P < 0.0001) (Figs. 1A, B). Absorbances of sera from patients with other hematological disorders showed negligible levels of circulating anti-9-*O*AcSGs, for example, CML (0.189 ± 0.047, n = 16), CLL (0.22 ± 0.084, n = 14), AML (0.245 ± 0.054, n = 18), aplastic anemia (0.204 ± 0.038, n = 6), thallassemia (0.241 ± 0.045, n = 8), NHL (0.198 ± 0.066, n = 18), and adult ALL (0.30 ± 0.03, n = 10).

Following chemotherapy, anti-9-*O*AcSG titers progressively decreased in patients of Groups I and II, the mean OD_{405} nm ± SD in phase B being 0.77 ± 0.12 and 0.68 ± 0.16; in phase C, 0.4 ± 0.08 and 0.38 ± 0.06; and in phase D, 0.28 ± 0.07 and 0.29 ± 0.06, respectively (Figs. 1A, B).

Quantification of circulating disease-specific antibody

Quantification of circulating anti-9-*O*AcSGs was performed using a standard curve generated by measuring affinity purified anti-9-*O*AcSGs ranging from 0 to 200 μg/ml using the BSM-ELISA. Linearity and imprecision of the assay was analyzed using two serum samples in five serial dilutions with triplicate determinations per run and two runs per day for 20 days. The intra-assay and inter-assay imprecision (CV) were 0.91–6.52% and 1.2–4.37%, respectively.

We successfully measured the absolute level of anti-9-*O*AcSGs at different time points of disease as shown in Fig. 2. The data were subjected to one-way analysis of variance with post-test for linear trend and confirmed a significant decrease of antibody titers with progress in treatment in patients of both groups (P < 0.0001).

Accuracy of the assay was confirmed in patients of Groups I and II by calculating the assay sensitivity

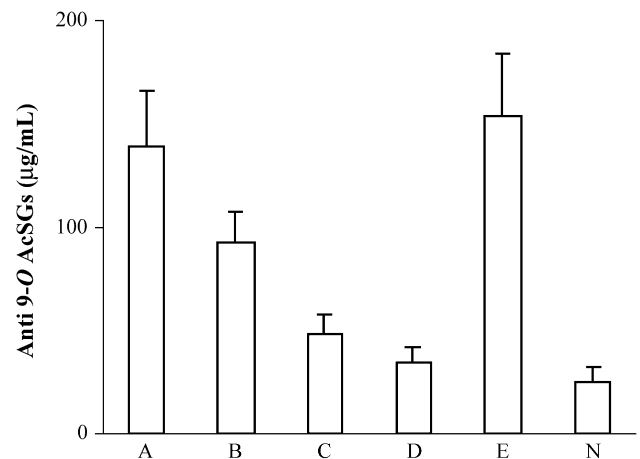

Fig. 2. Quantification of anti-9-*O*AcSGs. The concentration of circulating anti-9-*O*AcSGs in group I patients was measured as described in Materials and methods. Data is expressed as mean ± SD in μg/ml. A: phase A (n = 69), B: phase B (n = 24), C: phase C (n = 21), D: phase D (n = 17), E: relapse (n = 8), and N: normal individuals (n = 70).

(98.92% and 96.77%), specificity (92.1% and 95.91%), efficiency (96.94% and 96.39%), positive predictive value (PPV, 96.84% and 96.77%), and negative predictive value (NPV, 97.22% and 95.91%), respectively.

Subclass distribution (IgG_1 and IgG_2) of anti-9-OAcSGs in ALL patients in different phases of treatment

Since we have reported elevated levels of both 9-*O*AcSG-specific IgG_1 and IgG_2 in children suffering from ALL at presentation [11], we wanted to assess the IgG subclass status in different phases of treatment along with total 9-*O*AcSG-specific IgG. Our results reflected high titers of both IgG_1 and IgG_2 at presentation (phase A, Fig. 3A) that progressively declined with chemotherapy (phase B–D)

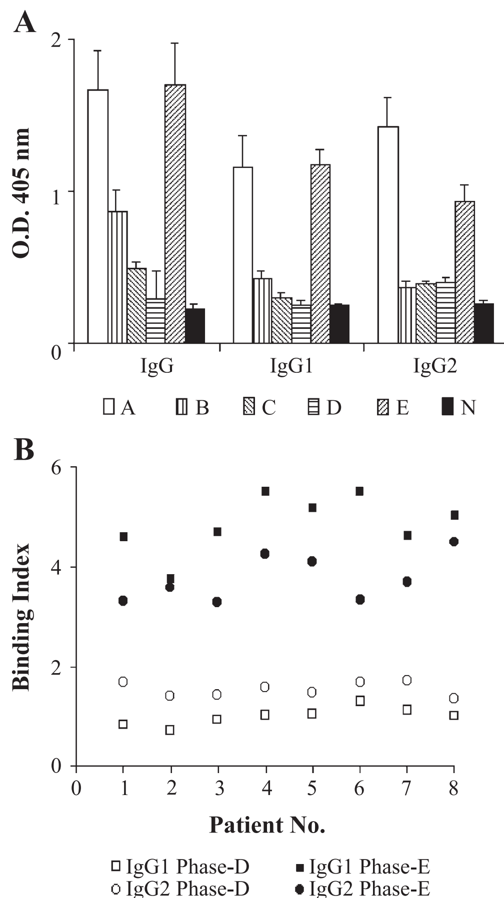

Fig. 3. IgG subclass distribution of anti-9-*O*AcSGs in ALL patients in different phases of treatment. (A) Distribution of anti-9-*O*AcSA antibodies (IgG, IgG_1, and IgG_2) in patients with childhood ALL at different phases of treatment (A, B, C, D, and E) along with normal donors. Sera were diluted 1:10 and antibody levels measured as described in Materials and methods. Data are expressed as mean ± SD of optical density at 405 nm. (B) Comparative distribution of anti-9-*O*AcSA IgG_1 and IgG_2 antibodies in eight patients of ALL at phases D and E. Sera were diluted 1:10 and antibody levels were measured as described in Materials and methods. Results are expressed as the binding index as indicated in Materials and methods.

and increased only at relapse (phase E, Fig. 3A). Concerning levels of 9-*O*AcSG-specific IgG_1 at clinical remission, antibody levels decreased to titers observed in normal donors (N, Fig. 3A). However, the same was not true for 9-*O*AcSG-specific IgG_2; although levels decreased following induction remission, they remained marginally higher than normal donors. However, in individuals that relapsed, titers of both IgG_1 and IgG_2 dramatically increased (phase E, Fig. 3A).

Titers of 9-OAcSG-specific IgG_1 may be more powerful than IgG_2 for prediction of relapse

With regard to whether 9-*O*AcSG-specific IgG_1 or IgG_2 is the better prognostic marker, the binding index of IgG_1 and IgG_2 was examined in eight patients who relapsed in phases D and E. As shown in Fig. 3B, the difference in binding index of IgG_1 levels at phases D and E was 0.93 ± 0.25. However, differences in binding index of IgG_2 levels at phases D and E was lower, being 0.54 ± 0.32. Taken together, monitoring of antibody titers of 9-*O*AcSG-specific IgG_1 appears to be more powerful than IgG_2 for prediction of relapse.

Longitudinal prospective study of 17 ALL patients showed antibody titers against 9-OAcSGs that correlate well with clinical remission or relapse

Although 116 patients were enrolled in this study, we have longitudinally monitored 17 individuals for antibodies against 9-*O*AcSGs based on the availability of a comparatively larger number of samples in these patients than the rest of the subjects. There appeared to be two distinct patterns that emerged, eight patients showed relapse whereas nine patients were in continual clinical remission (CCR) (Table 1).

The first pattern showed elevations in antibody titers within a reasonable time period before relapse (patients no. 1–8), for example, patient no. 1 was monitored for 211 weeks; at presentation, that is, phase A, high antibody titers were obtained (OD_{405} being 1.71) and levels progressively declined up to the 89th week (OD_{405} being 0.22). Subsequently, the titers increased at week 112 (OD_{405} being 0.66) and increased further (OD_{405} being 1.62) and were associated with clinical relapse at the 129th week. It is clearly evident that the antibody titers increased 17 weeks before clinical relapse and thus may be considered as MRD. In all these eight patients, a gradual increase in antibody titer was noticed 14–31 weeks before relapse indicating disease resurgence (MRD) that eventually culminated in clinical relapse. This clearly indicates a strong secondary immune response against cancer antigen at the onset of MRD although there was no clinical evidence of the disease persistence (<5% blast cells). It also suggests the high immunogenicity of the antigen even when minimally present.

Table 1
A longitudinal prospective study of 17 childhood ALL patients

Patient no., sex; age	Weeks of treatment (OD_{405} nm) [blast %]													
1, M; 4	0 (1.71) [86]	7 (0.80) [<5]	10 (0.48) [<5]	61 (0.62) [<5]	77 (0.42) [<5]	83 (0.10) [<5]	89 (0.22) [<5]	112 (0.66) [<5]	123 (0.81) [12]	129* (1.62) [46.2]	165 (0.28) [<5]	174 (0.48) [<5]	194 (0.34) [<5]	211 (0.12) [<5]
2, M; 5.5	0 (1.42) [88]	8 (0.64) [<5]	15 (0.37) [<5]	27 (0.43) [<5]	30 (0.25) [<5]	43 (0.12) [<5]	57 (0.32) [<5]	61 (0.06) [<5]	113 (0.53) [<5]	118 (0.72) [10]	127* (1.03) [62]	136 (0.24) [<5]	189 (0.31) [<5]	
3, M; 4	0 (2.50) [90]	25 (1.04) [12]	70 (0.43) [<5]	126 (0.35) [<5]	166 (0.31) [<5]	192 (0.62) [<5]	208* (1.22) [8]	209* (1.36) [41]	224 (0.52) [18]	252 (0.32) [<5]	278 (0.28) [<5]	281 (0.24) [<5]		
4, F; 2.5	0 (1.80) [85]	8 (0.64) [<5]	18 (0.26) [<5]	30 (0.79) [<5]	44* (1.11) [32]	68 (0.41) [<5]	102 (0.41) [<5]	128 (0.47) [<5]	132 (0.36) [<5]	153 (0.39) [<5]	169 (0.24) [<5]	181 (0.32) [<5]		
5, M; 3	0 (2.46) [79]	9 (0.23) [<5]	24 (0.66) [<5]	56* (1.39) [62]	78 (0.51) [<5]	92 (0.26) [<5]								
6, M; 4	0 (1.98) [88]	21 (0.82) [<5]	53 (0.72) [<5]	105 (0.61) [<5]	161 (0.49) [<5]	183 (0.63) [<5]	197 (0.81) [<5]	205* (1.42) [23.4]						
7, M; 3.5	0 (1.58) [76]	25 (0.71) [<5]	40 (0.32) [<5]	58 (0.28) [<5]	64 (0.34) [<5]	78 (0.51) [<5]	92 (0.86) [<5]	106* (1.53) [45]	157 (0.47) [<5]	181 (0.36) [<5]				
8, M; 2	0 (1.54) [62]	12 (0.62) [<5]	18 (0.38) [<5]	36 (0.58) [<5]	51 (0.73) [<5]	60* (0.92) [10]	88* (1.54) [44]	112 (0.41) [<5]	158 (0.29) [<5]					
9, M; 4.5	0 (1.42) [80]	24 (0.82) [<5]	62 (0.54) [<5]	119 (0.44) [<5]	151 (0.29) [<5]	160 (0.22) [<5]	167 (0.29) [<5]	172 (0.07) [<5]						
10, F; 3	0 (1.86) [92]	8 (0.65) [<5]	24 (0.62) [<5]	48 (0.45) [<5]	144 (0.47) [<5]	172 (0.47) [<5]	198 (0.48) [<5]	218 (0.32) [<5]	244 (0.27) [<5]	271 (0.39) [<5]				
11, F; 4	0 (1.34) [78]	18 (0.80) [<5]	45 (0.23) [<5]	67 (0.15) [<5]	71 (0.28) [<5]	97 (0.38) [<5]	122 (0.21) [<5]							
12, F; 3	0 (1.56) [71]	24 (0.74) [<5]	42 (0.43) [<5]	144 (0.47) [<5]	172 (0.47) [<5]	198 (0.48) [<5]								
13, M; 2	0 (1.56) [76]	4 (0.34) [<5]	9 (0.28) [<5]	13 (0.19) [<5]	27 (0.20) [<5]	67 (0.24) [<5]	82 (0.33) [<5]	107 (0.24) [<5]						
14, M; 2.5	0 (1.72) [82]	24 (0.64) [<5]	64 (0.42) [<5]	144 (0.47) [<5]	172 (0.36) [<5]	193 (0.41) [<5]								
15, F; 3.5	0 (2.42) [66]	6 (1.60) [<5]	25 (0.74) [<5]	76 (0.47) [<5]	101 (0.52) [<5]									
16, F; 3	0 (1.14) [66]	8 (0.94) [<5]	24 (0.33) [<5]	42 (0.39) [<5]	71 (0.41) [<5]	83 (0.36) [<5]	103 (0.38) [<5]	107 (0.29) [<5]						
17, M; 2.5	0 (1.28) [74]	24 (0.23) [<5]	58 (0.28) [<5]	63 (0.33) [<5]	98 (0.41) [<5]	116 (0.38) [<5]								

Normal individuals show mean OD_{405} nm = 0.21 ± 0.06.
* Relapse.

In contrast, the second group of patients (no. 9–17) showed a relatively horizontal antibody pattern indicating that CCR as low antibody levels was maintained throughout follow-up. Serial measurement of antibody titers from this group of patients is critical in determining distinctions at the MRD stage.

Discussion

There is a growing evidence that some physiological and pathological processes ranging from cell–cell adhesion, signaling, differentiation, and metastasis may be attributed to the appearance of *O*AcSGs [3,4,8,23]. Antibodies against

these disease-specific biomarkers are likely to play a pivotal role in diagnosis and prognosis of the disease [11,12].

A striking feature of childhood ALL is the high expression of a unique disease-specific antigen having 9-*O*AcSA α2,6GalNAc as its terminal carbohydrate epitope [8]. Their enhanced immunogenicity was reflected in an increased presence of 9-*O*AcSA directed IgG_1 and IgG_2 isotypes [11] along with IgM [12]. In this study, we have capitalized the enhanced presence of both IgG and IgM to develop a serodiagnostic assay for measurement of total 9-*O*AcSGs-specific immunoglobulin using Protein A as the detecting reagent due to its strong affinity for Fc regions of both IgG and IgM. Based on the preexisting knowledge that this disease-specific antibody possess strong affinity toward 9-*O*AcSA α2,6GalNAc, BSM was selected as the capture antigen, known to be a rich source of naturally occurring *O*-acetylated sialoglycoproteins present either as mono-8(9)-*O*-AcSA and/or higher *O*-AcSA (di- and tri-*O*-AcSA) derivatives [19].The presence and absence of antibody reactivity toward BSM and its de-*O*AcSA derivative, respectively, clearly indicated that the purified antibody is highly specific in its antigen recognition.

Based on the evaluation of anti-9-*O*AcSGs in sera of age-matched normal healthy individuals, a cut-off value of 0.4 absorbance at 405 nm was selected to minimize the false positivity. A fivefold increase in the OD_{405} nm of ALL patients from both Groups I and II at disease presentation as compared to normal individuals clearly reflected the diagnostic potential of BSM-ELISA irrespective of the chemotherapy protocol (Figs. 1A, B). Quantification of circulating anti-9-*O*AcSGs showed a gradual decline in antibody titers that coincided with disease regression (Fig. 2). The assay specificity has been confirmed using sera from patients with other hematological disorders like CML, CLL, AML, aplastic anemia, thallassemia, NHL, and adult ALL wherein negligible levels of circulating anti-9-*O*AcSGs were observed. Although it was not feasible to age match some of these patients with childhood ALL patients, the absence of cross reactivity with other hematological disorders makes this a novel serological approach for diagnosis of ALL.

The relationship between antibody isotype and clinical manifestations has been reported in several diseases. It is known that patients with squamous cell carcinoma of the head and neck exhibited consistent patterns of IgG subclasses, comprising of a decrease in IgG_1 and an increase in IgG_2 relative to total IgG that was helpful for their post-therapeutic monitoring [24]. A different scenario was observed concerning autoantibodies in patients with paraneoplastic neurological syndromes that are predominantly IgG_1 and IgG_3 isotypes [25] and hairy cell leukemia where the surface IgG_3 subclass is preferentially expressed [26]. In patients with lung cancer, the influence of different histological sites of cancer origin on the IgG subclass distribution has been reported in the context of adenocarcinoma, squamous cell, and small cell lung carcinoma [27]. Anti-polysaccharide responses have been identified to have a restricted heterogeneity concerning the IgG subclasses produced, being predominantly IgM and IgG_2 in humans [28]. Several reports indicating irregular biosynthesis of IgG subclasses at the B-cell level [29], influence of tumor antigen, adhesion molecules, and defect in T-cell interaction [26] have been attributed to the mechanism of antibody class and subclass switching in cancers; however, the effect of this interesting phenomenon is only partially explored.

To determine the prognostic significance of IgG subclasses in detection of relapse in ALL, we evaluated isotypes and subclasses of immunoglobulins in different phases of treatment by the BSM-ELISA that would permit the identification of glycotopes having carbohydrate determinants 9-*O*AcSA α2,6GalNAc. Although both IgG_1 and IgG_2 titers follow the similar trend, distribution of 9-*O*AcSG-specific IgG_2, at clinical remission, showed an interesting profile (Fig. 3A). The clear difference in binding index of IgG_1 and IgG_2 levels at phases D and E (Fig. 3B, 0.93 ± 0.25 vs. 0.54 ± 0.32) indicates that monitoring the antibody titers of 9-*O*AcSG-specific IgG_1 may be the more effective marker for prediction of relapse in ALL patients. This could eventually serve as an alternative approach for universal monitoring of MRD.

ALL patients (n = 17) were longitudinally monitored for antibodies against 9-*O*AcSGs and showed two distinct patterns. Individuals who relapsed during long-term follow-up showed a consistent increase in their antibody titers (Table 1). The other pattern was a decrease in antibody levels following chemotherapy that was maintained while in continual clinical remission (CCR).

On the whole, the data indicate that diverse variations in anti-9-*O*AcSG levels occur, and we propose that sample collection if rigorously undertaken could be an effective tool for prediction of relapse. However, we are limited by the fact that for socioeconomic reasons, most parents are reluctant to regularly attend long-term follow-up clinics.

Although childhood ALL is highly responsive to chemotherapy, patients in remission may harbor residual leukemic blasts, the cause of disease persistence, and resurgence called minimal residual disease (MRD) [30,31]. In an earlier study, we had developed a noninvasive, blood-based lymphoproliferation assay for evaluating the clinical status of ALL patients [9,32,33]. Due to the high expression of cell surface 9-*O*AcSGs at presentation of the disease, a smaller amount of Achatinin-H (0.15–0.25 μg), a 9-*O*AcSA binding lectin, can induce maximal proliferation of lymphoblasts of ALL as compared to normal individuals who require 8.0 μg [9,32,33]. With chemotherapeutic response, the maximal lymphoproliferation dose rose to 2.1 ± 0.6 μg. This increased further to 4.5 ± 1.6 μg during maintenance therapy and reached 5.5 ± 0.8 μg during follow-up. Interestingly, the maximal proliferative dose dropped sharply to 0.25 ± 0.01 μg with relapse of disease corroborating that Achatinin-H-induced lymphoproliferation is indeed a measure of the cell surface 9-*O*AcSA expression. Since the presence of circulating blasts are responsible for antibody

titers, the BSM-ELISA, using Protein A as detecting agent shows great promise in both evaluation of disease progression and assessment of therapeutic effectiveness in ALL especially in the monitoring of minimal residual disease.

Ideally, detection of MRD would be better achieved by molecular techniques that can monitor the presence of lymphoblast-specific antigens that require accessibility to sophisticated equipment and expertise, often not achievable in the developing world. In such a situation, it is relevant that methods be developed where the scales tip in favor of the technology user rather than the technology developer [32,33]. In this regard, this antibody-based assay satisfies these criteria. The cost of the coating antigen is practically negligible, as BSM is abundantly available. To screen approximately 100 samples in duplicate, 200 μg of BSM is required. Generally, 10 g of crude mucin yields approximately 23 mg of semi-purified BSM, having 22% 9-*O*AcSA sufficient for screening 12 000 samples. An additional advantage is the long shelf life of BSM, as it remains stable at −20°C for over 5 years, thereby minimizing batch-to-batch variability. Ideally, production of a monoclonal antibody against this glycotope (9-*O*AcSA α2,6GalNAc) would be the best approach, and research toward this goal is ongoing. Given the socioeconomic scenario in developing countries like India, laboratory tests should be simple yet sensitive and also acceptable to the population tested. We believe that availability of this simple, noninvasive, cost-effective approach satisfies these criteria and would allow for closer monitoring of pediatric ALL patients and may possibly be a step forward in improving long-term follow-up.

Taken together, this study clearly documents the potential of 9-*O*AcSGs as a novel disease marker and hints toward the possibility of BSM-ELISA being an effective alternative tool for monitoring the disease status and possibly aid in detection of MRD. This was indicated by enhanced antibody levels in patients who relapsed and in cases of impending relapse, relative to their low antibody levels following acquisition of remission. Although we have analyzed a large number of samples, long-term monitoring was achievable in 17 patients. However, even in this small patient population, we have successfully detected MRD that hints toward the potentiality of the assay. Therefore, the study needs to be evaluated in a larger sample population and our future endeavor would be to establish whether detection of these antibodies have a better predictive value in predicting/detecting relapse than other current methods of MRD detection; such studies are ongoing.

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