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A Study Evaluating the Ikaros Protein Expression and Ikzf1 mRNA Transcripts in B-Cell Acute Lymphoblastic Leukemia

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Abstract

Background: Ikaros, a zinc finger transcription factor protein encoded by the IKZF1 gene is crucial for hematopoiesis in humans. Loss of function mutations in IKZF1 have been implicated in adult and pediatric B cell acute lymphoblastic leukemia (B-ALL).

Aim: Our aim was to evaluate the incidence of Ikaros protein expression and IKZF1 mRNA transcripts (isoforms) in B-ALL patients in Indian population.

Method: Ikaros protein expression was evaluated in total 67 patients diagnosed as de novo B-ALL using flow cytometry and IKZF1 mRNA transcripts (isoforms) were detected using reverse transcriptase - polymerase chain reaction (RT-PCR) method in 23 patients.

Results: The expression levels of the Ikaros protein and IKZF1 isoforms were analyzed and correlated with clinical and hematological parameters. Most frequently noted isoform was IK6, a dominant negative isoform. B-ALL patients with decreased Ikaros protein expression and presence of dominant negative isoform IK6 were associated with high blast count (>88%, median cutoff), high WBC count, low RBC and low platelet counts as well as low hemoglobin levels along with CD34 progenitor cell marker and leukemia associated phenotype (LAP).

Conclusion: Patients showing association of decreased Ikaros protein and presence of IK6 with abnormal blood count, immature cell and LAP can be considered as high-risk patients. Also, when Ikaros protein was compared with isoforms, decrease in protein expression was noted in patients having dominant negative isoform IK6. However, to confirm clinical relevance of Ikaros more number of patients needs to be enrolled.

Keywords: B-ALL; Ikaros; IKZF1 isoforms

Introduction

Alterations of the transcriptional regulation of lymphoid development is a hallmark of B-cell acute lymphoblastic leukemia (B-ALL), with deletion, sequence mutations, or rearrangements of the transcription factors PAX5, IKZF1, and EBF1 present in more than two-thirds of patients [1,2]. Relatively few of the novel genetic alterations have been found to be reproducibly associated with outcome, with the notable exception of alterations of the lymphoid transcription factor gene IKZF1 (Ikaros) in B-ALL which is associated with a high risk of treatment failure in B-ALL [1].

IKZF1 encodes Ikaros, the founding member of a family of zinc finger transcription factors that is required for the development of all lymphoid lineages [3]. The expression of IKZF1 involves expression of wild type isoforms as well as shorter isoforms lacking DNA binding motifs that leads to loss of function. Loss of function mutations in IKZF1, have been implicated in adult and pediatric B-ALL. These mutations result in haploinsufficiency of the Ikaros gene in approximately half of the cases. The remaining cases contain more severe or compound mutations that lead to the generation of dominant-negative isoforms or complete loss of function [4]. All IKZF1 mutations are associated with a poor prognosis.

At our Institute, according to 2011 data, the incidence rate of lymphoid leukemia in males is 1.9 and in females is 1.3 among all the cancer sites, which is almost similar to the incidence rate of western countries. Further, the Ikaros protein and isoforms have been studied in B-cell ALL in the western population where, it has been suggested that Ikaros variants contributes towards B ALL predisposition. In comparison, only one Indian study has evaluated specific polymorphism of Ikaros along with other genes in B lineage ALL and gave conflicting conclusion that Ikaros variants were associated with reduced disease risk [5]. Therefore, considering the variable clinical significance of Ikaros isoforms in different population and lack of other Indian study on Ikaros, the present study aimed to analyse the protein expression by flow cytometry and IKZF1 isoforms by RT-PCR, correlate them with the clinical and hematological parameters and thus evaluate the clinical significance of Ikaros protein as well as isoforms in B-ALL patients in the Indian population.

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Patients and Methods

In the present study, 67 de novo B-ALL patients diagnosed and treated between 2015 and 2016 at The Gujarat Cancer & Research Institute were enrolled. Ten healthy controls were also included in the study. Bone marrow (BM) or peripheral blood (PB) samples were collected at diagnosis from acute leukemia patients while PB samples of healthy donors were collected as control. The diagnosis and classification of acute leukemia was based on morphology and cytochemistry according to the French-American-British classification and on the presence of maturation and differentiation antigens as determined by diagnostic immunophenotyping criteria of the European Group for the Immunological Characterization of Leukemias [6]. The patients' detailed clinical history (age, gender and hematological findings) were noted from the case files maintained at the Medical Record Department of the Institute. This study was approved by the Institutional Scientific Review Board and Ethics Committee.

Flow cytometry analysis

For flow cytometry study, the number of cells of BM or PB were quantified and adjusted to $1x10^6$ in each tube. The intracellular Ikaros protein expression was analyzed using phycoerythrin (PE) conjugated anti-Ikaros monoclonal antibody (R32-1149). The peridinin-chlorophyll protein (PerCp)-conjugated CD45 monoclonal antibody was added to each tube for gating lymphoblasts. The surface and intracellular antigens related to leukemia associated phenotype (LAP) were analyzed using reagents procured from BD Bioscience (San Jose, CA, USA) and the manufacture's protocol was followed. For lineage assignment, the following panels of monoclonal antibodies were used as the primary panel: CD22/ CD34/ CD45/ CD5/ CD10/ CD19, CD7/ CD13/ CD45/ CD33/ CD117/ HLADR and cMPO/ cCD79a/ CD45/ cCD3/ nTdT. The negative controls for surface and cytoplasmic antigens were simultaneously stained with omission of antibodies except CD45 to gate lymphoblastic cells.

To study the intracellular Ikaros expression, 2 mL Lysing solution (1:10 dilution, BD Biosciences) was added to 1 x 10^6 mononuclear cells (100 µL) and incubated for 15 minutes. Then samples were centrifuged at 400 *g* for 5 minutes. The supernatant was discarded and 1 mL Perm/Wash buffer (1:10 dilution, BD Biosciences) was added followed by 20 minutes incubation. The sample was centrifuged at 400 *g* for 5 minutes and the supernatant was discarded. Antibodies [10 µl (CD45) and 5 µl (Ikaros)] were added to the pellet and incubated for 15 minutes. Two washes of 2 mL phosphate buffered saline (PBS) were given with centrifugation at 400 g for 5 minutes and supernatant was discarded. The pellet was resuspended in 500 µl PBS.

Data acquisition and analysis

The FACSCanto II Flow Cytometer with FACSDiva software (BD Biosciences) was used for the acquisition and analysis of the samples. At least 30,000 events / tube were acquired. For analysis, lymphoblasts were gated on dot plot of side scatter *vs.* CD45 PerCp. If the percentage of positive events were more than 20%, the leukemic samples were considered to be positive for that surface or intracellular marker.

Isoform analysis

IKZF1 isoforms were analyzed by RNA extraction followed by cDNA synthesis and polymerase chain reaction (PCR). RNA was extracted from fresh BM or PB samples using QIAamp RNA mini kit (Qiagen, Germany) according to manufacturer's protocol. cDNA synthesis was done using first strand cDNA synthesis kit (Fermentas, Canada). The procedure of cDNA synthesis was divided into two steps. In step 1, approximately 3 µg of RNA was used with addition of 1 µl random hexamer primers and nuclease free water to make the volume up to 11 µl. The sample was briefly centrifuged, incubated at 65 °C for 5 minutes, spinned down and chilled on ice. In step 2, 4 µl (5X) Reaction buffer, 1 µl Ribo Lock RNase Inhibitor, 2 µl 10 mM dNTP and 2 µl M-MuLV Reverse Transcriptase were mixed and added to the reaction mix from step 1 to make the final volume up to 20 µl. After centrifugation, cDNA synthesis was carried out in Mastercycler gradient (Eppendorf, Germany) with conditions consisting of 25 °C for 5 minutes, 37 °C for 60 minutes followed by 70 °C for 5 minutes. cDNA was amplified by PCR using IKZF1specific primer sets [Exon2 Forward: 5'-CACATAACCTGAGGACCATG-3' and Exon8 Reverse: 5'-AGGGCTTTAGCTCATGTGGA-3'] (Sigma-Aldrich, USA). Approximately 100 ng of cDNA was added to PCR mix containing 5 µl (10X) PCR buffer, 10 µl (5X) Q solution, 2 µl 25 mM MgCl2, 1.5 µl dNTP (10 mM each), 0.5 µlTaq DNA polymerase of PCR core kit (Qiagen, Germany) and 5 µl (0.12 µM final concentration) each of forward as well as reverse primers, to make a final volume of 50 µl per reaction tube. PCR was carried out in Mastercycler gradient (Eppendorf, Germany) with conditions consisting of initial activation step at 95 °C for 2 minutes followed by 40 cycles each of denaturation at 95 °C for 30 seconds, annealing at 56.6 °C for 1 minute and extension at 72 °C for 1 minute followed by final extension step at 72 °C for 10 minutes. Amplified products were resolved on 2% agarose gels stained with ethidium bromide and visualized on Gel Documentation System (Alpha Innotech Corp., USA). The Ikaros isoforms obtained were IK1 at ~1400 bp, IK2/3 at ~1300 / 1200 bp, IK4 at ~1050 bp and IK6 at ~900 bp.

Statistical analysis

Statistical analysis was carried out using SPSS statistical software version 19 (SPSS Inc., USA). Mean, standard error (SE) of mean and median were calculated and Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between the two parameters. In case of patient number less than 5 in the cells of 2 x 2 tables, Yates' continuity correction value along with its significance was taken into consideration. P values ≤0.05 were considered significant.

Results

Ikaros protein was evaluated in 67 de novo B-ALL patients. Of these patients, 23 patients were further evaluated for IKZF1 mRNA transcripts (isoforms). The Ikaros protein and isoforms were correlated with clinical parameters, hematological parameters and LAP.

Ikaros protein in B-ALL

Incidence: Ikaros protein expression was evaluated in 10 healthy controls and 67 de novo B-ALL patients. The Ikaros protein expression ranged from 97 to 100% in normal lymphocytes of healthy controls (Figure 1A) while it ranged from 9 to 100% in blasts of B-ALL patients (Figure 1B). In 67 de novo B-ALL patients, 75% (50/67) patients had Ikaros protein levels lower than 97% while only 4% (03/67) of patients

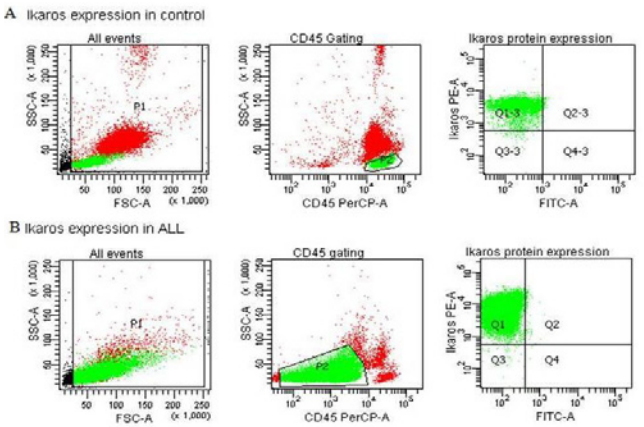

Figure 1: Representative Flow Cytometric Image showing Ikaros Protein over expression in A) Healthy Control and B) B-ALL patient.

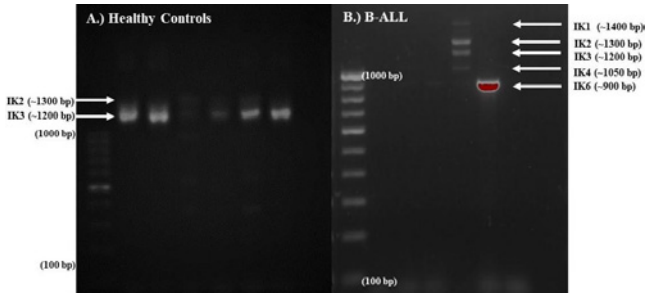

Figure 2: Representative Agarose Gel Image showing IKZF1 Isoforms in A.) Healthy Controls and B) B-ALL patients.
A) IKZF1 isoforms in 6 healthy controls
Lane1: 100 bp ladder, Lane2-7: IK2/3 isoforms (~ 1300 and 1200 bp), Lane8: Negative Control
B) IKZF1 isoforms in 4 B-ALL patients
Lane1: 100 bp ladder, Lane3-4: IK6 (~900 bp), Lane5: IK1 (1400 bp), IK2/3 isoforms (~1300 and 1200 bp), IK4 (1050 bp) and IK6 (900 bp), Lane6: IK6 (900 bp), Lane7: Negative Control.

had protein levels lower than 20%.

Further, mean ± standard error of mean for Ikaros protein expression calculated was 79.85 ± 2.897 and median was 90. For statistical correlation, Ikaros protein expression was sub grouped into low and high groups keeping 50% as arbitrary cutoff (Table 1).

Correlation of Ikaros protein with clinical parameters: With clinical parameters, a trend of high incidence of decreased Ikaros protein was noted in pediatric group (<16 years, 67%, 06/09) as compared to adult group (≥16 years; 33%, 03/09) and in male patients (56%, 05/09) as compared to female patients (44%, 04/09) (Table 1).

Correlation of Ikaros protein with hematological parameters: In case of hematological parameters, data of WBC count, RBC count, platelet count and hemoglobin levels at diagnosis for 5 patients was not available in their respective medical records. Hence, statistical correlation for these parameters was done in 62 patients only. The incidence of decreased Ikaros protein tended to be higher in high blast count (median as cutoff) (≥88%; 67%, 06/09), high WBC count (>$11x10^3$/µl; 50%, 04/08), low RBC count (<$3.8x10^6$/µl; 62%, 05/08), low platelet count (<$1.5x10^5$/µl; 87%, 07/08) and low hemoglobin levels (<9 g/dL; 62%, 05/08) as compared to their respective counterparts (Table 1).

Correlation of Ikaros protein with Philadelphia (Ph) chromosome: Data related to Ph chromosome was available for 60 patients. The incidence of decreased Ikaros protein was higher in Ph- patients (67%, 06/09) as compared to Ph+ (33%, 03/09) (Table 1).

Correlation of Ikaros protein with B-ALL subtypes: The incidence of decreased Ikaros protein was higher in Pre-Pre-B ALL subtype (78%, 07/09) as compared to other subtypes [Pro B (0/09); Pre B (22%, 02/09)]. One patient with Burkitt's ALL showed elevated Ikaros protein (02%, 01/01) (Table 2A).

Correlation of Ikaros protein with LAP: Ikaros protein was correlated with CD34, a progenitor cell marker, where incidence of decreased Ikaros protein was higher in CD34 positive (67%, 06/09) as compared to CD34 negative (33%, 03/09) (Table 2A).

Correlating with B cell Markers: Further, Ikaros protein was correlated with B cell markers (cCD79a, CD19, CD22). All patients with decreased Ikaros protein showed cCD79a positivity (100%, 09/09). A trend of high incidence of decreased Ikaros protein was noted in CD19 positive (89%, 08/09) and CD22 positive (56%, 05/09) as compared to their respective counterparts (Table 2A).

Correlating with Non-Lineage Markers: With non-lineage markers (nTdT, HLA-DR and CD10), all patients with decreased Ikaros protein showed nTdT positivity (100%, 09/09), HLA-DR positivity (100%, 09/09) and CD10 positivity (100%, 09/09) (Table 2B).

Correlating with Aberrant Myeloid and T cell Markers: Ikaros protein was then correlated with the aberrant marker expression of myeloid lineage (CD13, CD33, CD117 and cMPO). CD13 and CD33 were aberrantly expressed in 12 and 10 patients, respectively. However, aberrant CD13 expression showed similar expression of decreased Ikaros (22%, 02/09) while aberrant expression of CD33 showed elevated Ikaros protein (17%, 10/58) (Table 2B).

Similarly, Ikaros protein was then correlated with the aberrant marker expression of T cell lineage (cCD3, CD5 and CD7). CD5 was aberrantly expressed in 01 patient where elevated Ikaros protein was noted (2%, 01/58) (Table 2B).

IKZF1 Isoforms in B-ALL

Incidence: IKZF1 mRNA transcript expression was evaluated in 10 healthy controls and 23 B-ALL patients. The healthy controls showed expression of IK2 and IK3 isoforms (Figure 2A) while the B-ALL patients showed four different isoforms – IK1, IK2/3, IK4 and IK6 (Figure 2B).

IKZF1 Isoforms: The predominantly observed IKZF1 isoform was IK6. All four isoforms (IK1, IK2, IK4 and IK6) were noted in 06 (26%) patients. Single IK6 isoform was noted in 05 (22%) patients. IK6 isoform in combination of IK1, IK2 or IK4 isoforms was noted in 03 (13%) patients while combination of isoforms without IK6 was noted in 06 (26%). Single IK2 isoform was noted in 03 (13%) patients (Table 3).

For statistical analysis, total of 23 patients were divided into two isoform groups: first group of patients had IK6 isoform (alone or in

Table 1: Correlation of Ikaros Protein and Isoforms with Clinical and Hematological Parameters.

PARAMETERS	Total Patients	Ikaros protein N (%)		Total Patients	Ikaros Isoforms N (%)	
	N (%)	Low (<50%)	High (≥50%)	N (%)	IK6	Non IK6
	67 (100)	**09 (13)**	**58 (87)**	**23 (100)**	**14 (61)**	**09 (39)**
Clinical Parameters						
Age						
Pediatric (<16 years)	36 (54)	06 (67)	30 (52)	14 (61)	09 (64)	05 (56)
Adult (≥16 years)	31 (46)	03 (33)	28 (48)	09 (39)	05 (36)	04 (44)
	$X^2 = 0.23$, p = 0.63[a], r = 0.10			$X^2 = 0.00$, p = 1.00[a], r = 0.09		
Gender						
Male	43 (64)	05 (56)	38 (65)	15 (65)	10 (71)	05 (56)
Female	24 (36)	04 (44)	20 (35)	08 (35)	04 (29)	04 (44)
	$X^2 = 0.04$, p = 0.84[a], r = -0.07			$X^2 = 0.11$, p = 0.74[a], r = 0.16		
Hematological Parameters						
Blast (Median = 88%)						
< 88 %	32 (48)	03 (33)	29 (50)	11 (48)	06 (43)	05 (56)
≥ 88%	35 (52)	06 (67)	29 (50)	12 (52)	08 (57)	04 (44)
	$X^2 = 0.33$, p = 0.57[a], r = -0.11			$X^2 = 0.03$, p = 0.87[a], r = -0.12		
	N=62	**08 (13)**	**54 (87)**	**N=20**	**11 (55)**	**09 (45)**
WBC Count						
<3.9 x 10^3/µL	16 (26)	02 (25)	14 (26)	06 (30)	02 (18)	04 (44)
4-11 x 10^3/µL	06 (10)	02 (25)	04 (07)	05 (25)	02 (18)	03 (33)
>11 x 10^3/µL	40 (64)	04 (50)	36 (67)	09 (45)	07 (64)	02 (22)
	$X^2 = 2.53$, p = 0.28, r = 0.06			$X^2 = 3.48$, p = 0.18, r = -0.39		
RBC count						
<3.8 x 10^6/ µL	51 (82)	05 (62)	46 (85)	16 (80)	08 (73)	08 (89)
3.8 – 4.8 x 10^6/ µL	09 (15)	03 (38)	06 (11)	04 (20)	03 (27)	01 (11)
> 4.8 x 10^6/ µL	02 (03)	00 (00)	02 (04)	-	-	-
	$X^2 = 4.07$, p = 0.13, r = -0.13			$X^2 = 0.11$, p = 0.74[a], r = -0.20		
Platelet Count						
<1.5 x 10^5/µL	58 (93)	07 (87)	51 (94)	19 (95)	10 (91)	09 (100)
1.5 - 4.5 x 10^5/µL	03 (05)	01 (13)	02 (04)	01 (05)	01 (09)	00 (00)
> 4.5 x 10^5/ µL	01 (02)	00 (00)	01 (02)	-	-	-
	$X^2 = 1.29$, p = 0.52, r = -0.05			$X^2 = 0.00$, p = 1.00[a], r = -0.21		
Hemoglobin						
< 9 g/dL	48 (77)	05 (62)	43 (80)	16 (80)	08 (73)	08 (89)
≥ 9 g/dL	14 (23)	03 (38)	11 (20)	04 (20)	03 (27)	01 (11)
	$X^2 = 0.39$, p = 0.53[a], r = -0.14			$X^2 = 0.11$, p = 0.74[a], r = -0.20		
	N=60	**09 (15)**	**51 (85)**	**N=20**	**12 (60)**	**08(40)**
Philadelphia chromosome (Ph)						
Negative	48 (80)	06 (67)	42 (82)	16 (80)	09 (75)	07 (88)
Positive	12 (20)	03 (33)	09 (18)	04 (20)	03 (25)	01 (12)
	$X^2 = 0.40$, p = 0.53[a], r = -0.14			$X^2 = 0.01$, p = 0.91[a], r = -0.15		

p value ≤ 0.05 is significant, a= Yates continuity correction for cell volume <5

combination) while second group of patients had combinations of different isoforms without IK6 (Table 3).

Correlation of IKZF1 Isoforms with clinical parameters: In relation to clinical parameters, IKZF1 isoforms were correlated with age and gender. The pediatric patients (<16 years) showed a trend of high incidence of IK6 (64%, 09/14) as compared to adult patients

Table 2A: Correlation of Ikaros protein and isoforms with B-ALL subtypes and LAP.

PARAMETERS	Total Patients	Ikaros protein N (%)		Total Patients	Ikaros Isoforms N (%)	
B-ALL subtypes	N (%)	Low (<50%)	High (≥50%)	N (%)	IK6	Non IK6
	67 (100)	09 (13)	58 (87)	23 (100)	14 (61)	09 (39)
Pro B-ALL	05 (07)	00 (00)	05 (09)	01 (04)	01 (07)	00 (00)
Pre Pre B-ALL	42 (63)	07 (78)	35 (60)	16 (70)	10 (71)	06 (67)
Pre B-ALL	19 (28)	02 (22)	17 (29)	06 (26)	03 (21)	03 (33)
Burkitt's ALL	01 (02)	00 (00)	01 (02)	-	-	-
	X^2 = 1.44, p = 0.69, r = 0.01			X^2 = 0.96, p = 0.62, r = 0.18		
Leukemia Associated Immunophenotype (LAP)						
Progenitor cell marker						
CD34						
Negative	25 (37)	03 (33)	22 (38)	07 (30)	05 (36)	02 (22)
positive	42 (63)	06 (67)	36 (62)	16 (70)	09 (64)	07 (78)
	X^2 = 0.00, p = 1.00[a], r = -0.03			X^2 = 0.05, p = 0.82[a], r = 0.14		
B cell markers						
cCD79a						
Negative	02 (03)	00 (00)	02 (03)	01 (04)	01 (07)	00 (00)
Positive	65 (97)	09 (100)	56 (97)	22 (96)	13 (93)	09 (100)
	X^2 = 0.00, p = 1.00[a], r = -0.07			X^2 = 0.00, p = 1.00[a], r = 0.17		
CD19						
Negative	04 (06)	01 (11)	03 (05)	03 (13)	03 (21)	00 (00)
Positive	63 (94)	08 (89)	55 (95)	20 (87)	11 (79)	09 (100)
	X^2 = 0.00, p = 1.00[a], r = 0.08			X^2 = 0.73, p = 0.39[a], r = 0.31		
CD22						
Negative	36 (54)	04 (44)	32 (55)	14 (61)	09 (64)	05 (56)
Positive	31 (46)	05 (56)	26 (45)	09 (39)	05 (36)	04 (44)
	X^2 = 0.06, p = 0.81[a], r = -0.07			X^2 = 0.00, p = 1.00[a], r = 0.09		

p value ≤ 0.05 is significant, a= Yates continuity correction for cell volume <5

(≥16 years) (36%, 05/14). Similarly, in case of gender, incidence of IK6 was higher in male (71%, 10/14) as compared to female patients (29%, 04/14) (Table 1).

Correlation of IKZF1 Isoforms with hematological parameters: In case of hematological parameters, data of WBC count, hemoglobin levels, platelet counts and RBC counts at diagnosis for 3 patients were not available in the respective medical records. Hence, statistical correlation for these parameters was done in 20 patients. The trend of high incidence of IK6 was noted in high blast count (≥88%; 57%, 08/14), high WBC count (>11 x 10^3/μL; 64%, 07/11), low RBC count (<3.8 x 10^6/ μL; 73%, 08/11), low platelet count (<1.5 x 10^5/μL; 91%, 10/11), low hemoglobin level (<9 g/dL; 73%, 08/11) as compared to their respective counter parts (Table 1).

Correlation of IKZF1 Isoforms with Ph chromosome: Data related to Ph chromosome was available for 20 patients. The Ph- patients showed high incidence of IK6 (75%, 09/12)as compared to Ph+ patients (25%, 03/12) (Table 1).

Correlation of IKZF1Isoforms with B-ALL subtypes: In relation to B-ALL subtypes, Pre-Pre-B ALL showed higher incidence of IK6 (71%, 10/14) than Pro B-ALL (07%, 01/14) and Pre-Pre-B ALL (21%, 03/14) (Table 2A).

Correlation of IKZF1 Isoforms with LAP: IKZF1 isoforms were correlated with CD34, a progenitor cell marker, and CD34 positive patients showed high incidence of IK6 (64%, 09/14)as compared to CD34 negative patients(36%, 05/14) (Table 2A).

Correlating with B cell Markers: Further, IKZF1 isoforms were correlated with B cell markers (CD19, CD22, and cCD79a). Incidence of IK6 was higher in cCD79a positive (93%, 13/14) and CD19 positive (79%, 11/14) as compared to their respective counterparts. However, incidence of IK6 was higher in CD22 negative patients (64%, 09/14) as compared to CD22 positive patients (36%, 05/14) (Table 2A).

Correlating with Non-Lineage Markers: With non-lineage markers (nTdT, HLA-DR and CD10), incidence of IK6 was higher in nTdT positive (93%, 13/14), HLA-DR positive (93%, 13/14) and CD10 positive (93%, 13/14) as compared to their respective counterparts (Table 2B).

Correlating with Aberrant Myeloid and T cell Markers: Ikaros isoforms were then correlated with the aberrant marker expression of myeloid lineage (CD13, CD33, CD117 and cMPO). CD13 and CD33 were aberrantly expressed in 06 and 03 patients, respectively. In

Table 2B: Correlation of Ikaros Protein and Isoforms with LAP.

PARAMETERS	Total Patients	Ikaros protein N (%)		Total Patients	Ikaros Isoforms N (%)	
	N (%)	Low (<50%)	High (≥50%)	N (%)	IK6	Non IK6
	67 (100)	09 (13)	58 (87)	23 (100)	14 (61)	09 (39)
Leukemia Associated Immunophenotype (LAP)						
Non-lineage markers						
nTdT						
Negative	09 (13)	00 (00)	09 (16)	03 (13)	01 (07)	02 (22)
Positive	58 (87)	09 (100)	49 (84)	20 (87)	13 (93)	07 (78)
	$X^2 = 0.55$, p = 0.46[a], r = -0.15			$X^2 = 0.17$, p = 0.68[a], r = -0.22		
HLA-DR						
Negative	03 (05)	00 (00)	03 (05)	01 (04)	01 (07)	00 (00)
Positive	64 (95)	09 (100)	55 (95)	22 (96)	13 (93)	09 (100)
	$X^2 = 0.00$, p = 1.00[a], r = -0.08			$X^2 = 0.00$, p = 1.00[a], r = 0.17		
CD10						
Negative	07 (10)	00 (00)	07 (12)	02 (09)	01 (07)	01 (11)
Positive	60 (90)	09 (100)	51 (88)	21 (91)	13 (93)	08 (89)
	$X^2 = 0.27$, p = 0.61[a], r = -0.13			$X^2 = 0.00$, p = 1.00[a], r = -0.07		
Myeloid markers						
CD13						
Negative	55 (82)	07 (78)	48 (83)	17 (74)	09 (64)	08 (89)
Positive	12 (18)	02 (22)	10 (17)	06 (26)	05 (36)	01 (11)
	$X^2 = 0.00$, p = 1.00[a], r = -0.04			$X^2 = 0.68$, p = 0.41[a], r = -0.27		
CD33						
Negative	57 (85)	09 (100)	48 (83)	20 (87)	12 (86)	08 (89)
Positive	10 (15)	00 (00)	10 (17)	03 (13)	02 (14)	01 (11)
	$X^2 = 0.72$, p = 0.39[a], r = 0.16			$X^2 = 0.00$, p = 1.00[a], r = -0.05		
CD117						
Negative	67 (100)	09 (100)	58 (100)	23 (100)	14 (100)	09 (100)
Positive	00 (00)	00(00)	00(00)	00 (00)	00(00)	00(00)
cMPO						
Negative	67 (100)	09 (100)	58 (100)	23 (100)	14 (100)	09 (100)
Positive	00 (00)	00(00)	00(00)	00 (00)	00(00)	00(00)
T cell markers						
cCD3						
Negative	67 (100)	09 (100)	58 (100)	23 (100)	14 (100)	09 (100)
Positive	00 (00)	00(00)	00(00)	00 (00)	00(00)	00(00)
CD5						
Negative	66 (98)	09 (100)	57 (98)	22 (96)	13 (93)	09 (100)
Positive	01 (02)	00 (00)	01 (02)	01 (04)	01 (07)	00 (00)
	$X^2 = 0.00$, p = 1.00[a], r = 0.05			$X^2 = 0.00$, p = 1.00[a], r = -0.17		
CD7						
Negative	67 (100)	09 (100)	58 (100)	23 (100)	14 (100)	09 (100)
Positive	00 (00)	00(00)	00(00)	00 (00)	00(00)	00(00)

p value ≤ 0.05 is significant, a= Yates continuity correction for cell volume <5

Table 3: Pattern of distribution of IKZF1 Isoforms.

Presence of IKZF1 Isoform	N	Percent
IK1, IK2, IK4, IK6	06	26%
IK6 alone	05	22%
IK6 combined with any isoform	03	13%
Combination of isoforms without IK6	06	26%
IK2 alone	03	13%
Subgroups of IKZF1 Isoforms		
Subgroups	**N**	**Percent**
IK6	14	61%
Non IK6	09	39%

Table 4: Correlation of IKAROS Protein with individual IKZF1 Isoforms and their subgroups.

	Total Patients	IKAROS protein expression (mean)	
	N (%)	Low (<50%) [N(%)]	High (≥50%) [N(%)]
	23 (100)	05 (22)	18 (78)
IK1			
Negative	14 (61)	03 (60)	11 (61)
Positive	09 (39)	02 (40)	07 (39)
		X^2 = 0.00, p = 1.00[a], r = -0.01	
IK2/3			
Negative	07 (30)	03 (60)	04 (22)
Positive	16 (70)	02 (40)	14 (78)
		X^2 = 1.15, p = 0.28[a], r = 0.34	
IK4			
Negative	09 (39)	02 (40)	07 (39)
Positive	14 (61)	03 (60)	11 (61)
		X^2 = 0.00, p = 1.00[a], r = 0.01	
IK6			
Negative	09 (39)	01 (20)	08 (44)
Positive	14 (61)	04 (80)	10 (56)
		X^2 = 0.22, p = 0.64[a], r = -0.21	
IKZF1 Isoform subgroups			
IK6	14 (61)	04 (80)	10 (56)
Non IK6	09 (39)	01 (20)	08 (44)
		X^2 = 0.22, p = 0.64[a], r = 0.21	

p value ≤ 0.05 is significant, a= Yates continuity correction for cell volume <5

aberrantly expressed CD13, incidence of IK6 (36%, 05/14) was higher as compared to non IK6 group (11%, 01/09). However, in aberrantly expressed CD33, incidence of IK6 (14%, 02/14) was similar to the non IK6 group (11%, 01/09) (Table 2B).

Similarly, Ikaros isoforms were then correlated with the aberrant marker expression of T cell lineage (cCD3, CD5 and CD7). CD5 was aberrantly expressed in 01 patient where IK6 was present (07%, 01/14) (Table 2B).

Correlation of Ikaros protein with Individual Isoforms and the Subgroups of IKZF1 Isoforms

When Ikaros protein was correlated with individual isoforms, in relation with wild type isoforms, the incidence of decreased Ikaros protein was higher in patient groups showing absence of IK1 isoform (60%, 03/05) and absence of IK2/3 isoform (60%, 03/05) as compared to their respective counterparts. Further, in relation with mutated/dominant negative isoforms, the incidence of decreased Ikaros protein was higher in patient groups showing presence of IK4 isoform (60%, 03/05) and presence of IK6 isoform (80%, 04/05) as compared to their respective counterparts. Similarly, in relation with Ikaros isoform subgroups, the incidence of decreased Ikaros protein tended to be higher in IK6 group(80%, 04/05) as compared to non IK6 group (20%, 01/05) (Table 4).

Discussion

IKZF1 gene encodes Ikaros protein, the member of a family of zinc finger transcription factors and is required for the development of all lymphoid lineages. The IKZF1 alterations observed in ALL include focal or broad deletions that result in loss of function and/or dominant negative isoforms [1]. The present study was designed to evaluate the incidence of Ikaros protein in 67 B-ALL by flow cytometry method. The Ikaros protein expression ranged from 97 to 100% in normal lymphocytes of healthy controls and from 9 to 100% in blasts of B-ALL patients. In 67 de novo B-ALL patients, 75% (50/67) patients had Ikaros protein levels lower than 97% while only 4% (03/67) of patients had protein levels lower than 20%. In present study, 23 out of 67 patients were further evaluated for IKZF1 isoforms by RT–PCR method. The Ikaros protein and isoforms were further correlated with clinical parameters, hematological parameters and LAP.

Very few studies have evaluated protein and none by flow cytometric method. The methodology used has been western blot and immunofluorescence or immunohistochemistry to quantitate and observe the subcellular localization respectively [7-10]. A previous study found that only wild type IK1 and IK2 isoforms showed normal nuclear protein localization while dominant negative isoforms showed abnormal subcellular compartmentalization patterns [7]. Another study that has evaluated IKZF1 isoform/mutations along with protein expression observed that approximately 55% of B-ALL with IKZF1 mutations showed reduced protein levels [10]. Also, approximately 33% of IKZF1 mutations lead to IK6 isoforms – a dominant negative isoform and the protein is localized in cytoplasm due to loss of DNA binding domain [11-13]. About 12% of B-ALL with IKZF1 mutations have biallelic deletions corresponding to 2 null alleles and that leads to complete absence of Ikaros protein [11,14].

In the present study, the Ikaros protein subgroups were based on 50% arbitrary cutoff and they were correlated with the clinical parameters, age and gender. Ikaros protein was analyzed in 36 pediatric and 31 adults out of 67 B-ALL patients. High incidence of decreased protein was noted in pediatric and male patients as compared to their respective counterparts. Further, when protein expression was correlated with hematological parameters, high incidence of decreased protein was noted in high blast count, high WBC count, low RBC counts, low platelet counts and low hemoglobin levels as compared to their respective counterparts. No study till now has shown correlation of Ikaros protein with clinical and hematological parameters. However, data from other studies have established that Ikaros acts as clinically relevant tumor suppressor in B cell ALL [15].

Hence in the present study, decreased Ikaros protein which is due to the presence of dominant negative isoform IK6 and is associated with abnormal blood count that are conventional prognostic markers for B-ALL.

Further, Ikaros protein was correlated with the LAP, where a trend of high incidence of decreased protein level was noted with immature marker CD34, B- cell markers and non-lineage markers, however no significant correlation was noted with aberrantly expressed myeloid and T cell markers. A trend of high incidence of decreased Ikaros protein was noted in Pre-Pre-B ALL patients through not significant as compared to other subtypes.

Further to evaluate the presence of IKZF1 isoforms and correlate them with the Ikaros protein over expression, IKZF1 mRNA expression was studied in 23 out of 67 patients. Four different IKZF1 isoforms were identified and they were IK1, IK2, IK4 and IK6. The predominantly observed IKZF1 isoform was IK6. All four isoforms (IK1, IK2, IK4, and IK6) were noted in 06 (26%) patients. Single IK6 isoform was noted in 05 (22%) patients while in combination with other IK1, IK2 or IK4 isoforms it was 03 (13%). Combination of isoforms without IK6 was noted in 06 (26%). There are studies that show variable results on the incidence of IKZF1 isoforms in conjunction with Ph chromosome. Some of the initial reports have proposed that genomic IKZF1 deletions are the cause of expression of dominant-negative Ikaros isoforms [14,16]. Mullighan, et al. reported deletions of IKZF1 in 84% of Ph+ B precursor Acute Lymphoblastic Leukemia (BPL), including 76% of pediatric and 91% of adult Ph+ BPL cases [14]. The same authors also reported a >25% frequency of IKZF1 deletions in Ph- high-risk BPL patients [16]. In both studies, IKZF1 deletions included homozygous/biallelic as well as heterozygous/monoallelic deletion of the entire gene locus as well as intragenic deletions [14,16]. Subsequently, Volejnikova, et al. (2012) reported discordant results in 206 children with Ph– ALL. In that study, out of 24 patients with over expression of dominant-negative Ikaros isoforms other than IK6, only one patient had a deletion within the IKZF1 locus and only half of the IK6+ cases were found to have monoallelic IKZF1 deletions. The overall incidence of IKZF1 deletions was only 7% and no patient had homozygous IKZF1 deletions and no patient had evidence of decreased Ikaros protein expression even in the presence of monoallelic IKZF1 deletions [17]. In their most recent paper, Palmi, et al. (2013) documented no homozygous IKZF1 deletions and heterozygous IKZF1 deletions were detected in only ~13% of their Ph- BPL patient population. In approximately half of the cases with deletions (7.1%), the deletion involved the entire IKZF1 locus and in the other half a portion of the IKZF1 gene [18]. IKZF1 deletions (and sequence mutations) are less common, being present in approximately 15% of childhood ALL cases and are hallmark of high-risk ALL [1].

In present study for statistical analysis, the patients were divided into two isoform groups: first group of patients had IK6 isoform (alone or in combination) while second group of patients had combination of isoforms without IK6. When IKZF1 isoforms were correlated with age and gender, the incidence of IK6 presence was higher in pediatric and male patients. Similar to our results, many studies have reported over expression of dominant negative Ikaros isoforms in cases of T and B cell childhood ALL [7,13], while another study found association of these isoforms with adult B cell ALL [19]. Further, when IKZF1 isoforms were correlated with hematological parameters, a trend of high incidence of IK6 was noted in high blast count, high WBC count, low RBC counts, low platelet counts and low hemoglobin levels. Similar to our study, Liu, et al. (2016) showed correlation of IKZF1 deletions with high WBC count [20]. Abnormal WBC count along with other abnormal blood counts are the major clinical feature of prognostic significance in acute leukemias and therefore association of IK6 isoform with the abnormal blood count suggests prognostic significance of the dominant negative isoform.

Further, IKZF1 isoforms were correlated with the LAP, where high incidence of IK6 presence was noted with immature marker CD34, B- cell markers and non-lineage markers positivity with exception of CD22 positivity where incidence of IK6 presence was low. These immature progenitor cell markers are common aberrancies with uniform positive expression in lymphoblasts of B-ALL [21]. Further, the aberrant expression of CD13 showed higher incidence of IK6 presence as compared to non IK6 group while aberrant expression of CD33 showed similar incidence of IK6 and non IK6 combinations.

As discussed earlier, Ikaros gene abnormality is very frequent in Ph chromosome-positive ALL (about 80%) [14] and therefore associated to poor prognosis. However, in the present study, the incidence of both decreased Ikaros protein and presence of IK6 tended to be higher in Ph- patients as compared to Ph+ patients. While Qazi, et al. shows contrary results where they found no evidence for homozygous or heterozygous *IKZF1* deletions in Ph^+ or Ph^- BPL [22]. Also, conventional RT-PCR method has been used in the present study which might not be sufficient to detect the variants and other intragenic mutations that might be present over and above the common deletions associated with isoforms generated by alternative splicing. Ikaros protein was then correlated to the individual isoforms wherein, decreased protein level was tended to be associated with IK4 and IK6 which are isoforms lacking DNA binding motif. In one of the patient we found only 9% of Ikaros protein expression and having both isoforms IK4 and IK6 with absence of normal isoforms. Further, Ikaros protein was correlated with the isoform groups including and excluding IK6 wherein, a trend of high incidence of decreased Ikaros protein was noted in IK6 containing group as compared to the IK6 absent group.

In conclusion, the present study showed that B-ALL patients with decreased Ikaros protein and isoform group with IK6 had abnormal blood count and CD34, B cell markers and non-lineage markers positivity. These being the common aberrancies and conventional prognostic markers, the patients showing decreased Ikaros protein and IK6 isoforms can be considered as high-risk patients. Also, when Ikaros protein was compared with isoforms, decrease in protein expression was noted in patients having dominant negative isoforms. However, to confirm clinical relevance of Ikaros more number of patients needs to be enrolled.

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