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Research Article

Flowcytometric Analysis of Leukemic Blasts - as Primary Screening Test for *BCR/ABL1* Gene Rearrangement in B- ALL

Raiya Birva,[1] Hemangini Vora,[1] Pina Trivedi,[1,2] Biren Parikh[3]

[1]Division of Immunohistochemistry and Flowcytometry, Gujarat Cancer and Research Institute, Gujarat, India
[2]Division of Cell Biology, Gujarat Cancer and Research Institute, Gujarat, India
[3]Department of Pathology, Gujarat Cancer and Research Institute, Gujarat, India

Abstract

Objectives: Philadelphia chromosome (Ph) is common cytogenetic abnormality in B-ALL. Patients with Ph-chromosome have resistance to chemotherapy treatment with shorter event free survival, however, tyrosine kinase inhibitor (imatinib mesylate) has shown to improve the survival of Ph-positive ALL patients. The aim of the study was to examine immunophenotypic profile associated with *BCR/ABL1* gene rearrangement and to put forward a model for gene rearrangement based on immunophenotypic analysis at diagnosis.

Methods: We had carried out a retrospective analysis of 130 patients with B-ALL diagnosed at The Gujarat Cancer and Research Institute. Immunophenotyping of leukemic blasts was carried out using flowcytometry and *BCR/ABL1* fusion gene was detected by FISH analysis.

Results: *BCR/ABL1* fusion was observed in 24% of B-ALL. All patients with *BCR/ABL1* gene rearrangement were positive for CD10 and Tdt. *BCR/ABL1*-positive cases exhibited a greater MFI value of Tdt, CD10, CD34, CD13, CD33, but a lower MFI value of CD22, CCD79a as compared to *BCR/ABL1* negative cases. Multivariate logistic regression analysis showed that high MFI of Tdt, CD10 and low MFI of CD22 and CCD79a predict the presence of *BCR/ABL1* rearrangement.

Conclusion: Immunophenotypic profile of B-ALL patients can be used as primary screening to predict occurrence of *BCR/ABL1* gene rearrangement.

Keywords: B ALL, *BCR/ABL1* gene rearrangemnt, flowcytometric immunophenotyping

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In B Acute Lymphoblastic Leukemia (B-ALL), maturation arrest of B cells is a usual phenomenon and the immature precursor B cells are accumulated in marrow and/or peripheral blood. These precursor cells are at various stages of maturation which can be studied by flowcytometric analysis. The Immunophenotypic profile of these precursor cells of individual patients may vary and based on their immunophenotypic profile, leukemia can be broadly categorized into Pro-B, Pre-Pre B, Pre-B and Early-B acute lymphocytic leukemia. The primary antigens used in B-ALL Immunophenotyping include cCCD79a, CD19, cCD22 CD34, CD10 and intracellular terminal deoxynucleotidyl transferase (nTdt), these markers are sequentially expressed during B cell maturation process i.e. CD34, CD10, Tdt expressed during initial phase of maturation whereas CD22 expressed in later phase. CCD79a, CD19 are expressed throughout the maturation process.[1]

Address for correspondence: Hemangini Vora, Dr., Associate Professor. Division of Immunohistochemistry and Flowcytometry, Gujarat Cancer and Research Institute, Gujarat, India
Phone: +079-22688377, 079-22688411 **E-mail:** ihcgcri@hotmail.com

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The *BCR-ABL1* gene rearrangement is most common cytogenetic aberrancy found in hematologic malignancies such as chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Approximately 20-30% of B-ALL have translocation t(9;22). This translocation gives rise to fusion gene *BCR/ABL1*. The *ABL1* gene on chromosome 9 is placed in juxtaposition to a lower part of BCR gene on chromosome 22, resulting in a fusion protein that has constitutive tyrosine kinase activity. This fusion protein have different molecular weights, like 190-kDa protein (p190), which is seen solely in Ph-positive ALL, or the 210-kDa protein (p210), which cover 20%-40% of Ph-positive ALL.[2] Clinically Ph-positive B-ALL is an aggressive disease and it is associated with chemotherapy-resistant and poor prognosis.[3–5] Introduction of specific tyrosine kinase inhibitors, such as STI-571 (imatinib mesylate), has significantly improved the survival of Ph-positive ALL patients.[6] Hence, in newly diagnosed ALL patients identification of t(9;22) become a part of routine diagnostic testing. However, FISH analysis is quite time-consuming technique and 5-10% of cases CML and ALL display masked Philadelphia chromosome which can be detected by more sensitive molecular techniques like RT PCR. Earlier studies highlighted an association of *BCR/ABL1* gene rearrangement with CD10, CD34, CD13, CD33, CD66c, CD25 and CD38 expression.[7–10]

At present, in India most immunophenotypic assays detect all the differentiation-associated antigens and still lack data on correlation of *BCR/ABL1* gene rearrangement with immunophenotype of leukemic blasts in large cohort patients. In this hospital based study, we have tried to evaluate immunophenotypic features of leukemic blasts in context with *BCR/ABL1* translocation on larger sample size. Also, we derived a predictive model for *BCR/ABL1* gene rearrangement based on intensity of immunophenotypic markers that have been routinely used in diagnosis of B-ALL.

Methods

In this study, a total 130 B-ALL patients diagnosed at The Gujarat Cancer and Research Institute were retrospectively analyzed for their Immunophenotyping and cytogenetic profile. Detailed Clinical history was collected from medical records of hospital, general inform consent form is taken by Institute and study was approved by Institutional review committee. The diagnosis of Acute Leukemia was carried out on bone marrow aspiration, where more than 20% blasts cell in bone marrow sample were considered as acute Leukemia. Out of 130 patients 52% (68) were in pediatric age group (1-13 years) and 47% (62) were of adult age group (15-65). 66% (87) patients were male and 33% (43) patients were female. Immunophenotyping was performed on initial diagnostic sanples of acute leukemia patients by Flow cytometer BD FACS Canto II, (BD Biosciences (San Jose, USA) using a combination of monoclonal antibodies CD45 V500C (clone 2D1) for gating of blast cells, CD34 PerCP-Cy5.5 (clone 8G12), CD10 APC (clone HI10a), anti terminal deoxynucleotidyl transferase (Tdt) APC (clone E17-1519), HLADR APCH7 (L243)for immature cells, CCD79a PE (clone 2ST8-5H7), CD19APCH7 (clone ST25c1), CD22 FITC (SHCL1) for B cells, anti Myeloperoxidase FITC (clone 5B8), CD13 PE (clone L138), CD33 PECY7 (clone P67.6), CD117(clone 104-D2), for myeloid cells, CD3(clone SK7), CD5(clone L17F12), CD7FITC (clone M-T701) for T cells. All the antibodies and buffers were procured from BD Biosciences (San Jose, USA). For surface markers, (5-10 µl) antibodies were added to the bone marrow or peripheral blood (100µl) sample and incubated for 15 minutes. After incubation, 2 ml of erythrocyte lysing solution (1: 10 dilution with double distilled water) was added and incubated for 10 minutes at room temperature. Then cells were centrifuged at 400g for 5 minutes and supernatant was discarded. Remaining pellet was washed twice with 2 ml PBS and then resuspended in 500 µl of PBS. For intracellular markers, 100 µl sample was lysed using erythrocyte lysing solution (1: 10 dilution). After lysing RBC, sample was incubated with perm wash buffer (1: 10 dilution) for 15 minutes, then cells were centrifuged at 400g for 5 minutes and supernatant was discarded and antibody against intracellular markers (5-10 µl) were added to pellet. After incubation, cells were washed twice with 2 ml PBS and then resuspended in 500µl of PBS. Total 30,000 events were acquired in flowcytometer. Analysis was performed using BD FACS Diva software and CD45 dim population was considered as blasts. Further, in blasts population marker expression was analysed and 20% analyzed events that were brighter than the negative control, was considered to be positive. Marker expression was noted in percent positive cells along with median fluorescence intensity (MFI).

FISH analysis was carried out using Vysis LSI *BCR/ABL1* Dual Color, Dual Fusion Translocation Probe Set [LSI *ABL1* targeting region 9(q34.1) and LSI BCR targeting region 22(q11.2)] (Abbott Molecular Laboratories) at the Cytogenetic Lab of the Institute.

Statistical Analysis

SPSS 19 statistical software was used for analysis. Normality distributions of all variables were tested by Shapiro-Wilk test. Normalized data was used for further analysis. Cut-off point for optimal sensitivity and specificity was determined by receiver operating curves (ROC). A multivariate analysis was performed using a logistic regression model. Strength of association was expressed by Odds Ratio (OR) and 95% confidence interval was reported. P values less than 0.05 were considered statistically significant.

Results

Immunophenotypic Profile of B Acute Lymphoblastic Leukemia

Immunophenotypic expression of leukemic blasts was explored by Acute Leukemia panel which includes B lineage markers, maturation markers, myeloid lineage markers and T lineage markers. In relation to B lineage markers, all the patients were positive for CD19, cCCD79a, while 40% (53) patients were positive for cCD22. In case of maturation markers, all patients were positive for HLADR while 95% (125) patients showed CD10, 90% (117) patients showed Tdt 70% (91) patients exhibited CD34 expression. Regarding myeloid markers, 15% (19) patients showed aberrant expression of CD13 and 11% (14) patients had aberrant CD33 expression. Aberrant marker expression for T cells was not observed in any patients, so B cell marker, maturation markers and myeloid markers were considered for further analysis. Median fluorescent intensity for all markers was calculated, a range of MFI of all markers was noted (Table 1).

BCR/ABL1 Gene Rearrangement Status in B Acute Lymphoblastic Leukemia

BCR/ABL1 gene rearrangement status was analyzed by FISH and fusion gene was observed in 24% (32) patients, whereas normal gene pattern was observed in 75% (98) patients. In relation to Clinical parameters with respect to age, *BCR/ABL1* gene rearrangement was found significantly higher in adult age group 78% (25) as compared to pediatric age group 22% (07, p=0.001) and patients with higher Total WBC count (112093±18365/µl, p=0.0001) as compared to patients with lower WBC count (56730±5730/µl).

Antigen Expression in *BCR/ABL1* Subgroups

Out of 130 total patients, 32 patients were *BCR/ABL1* positive and 98 were *BCR/ABL1* negative. In relation with B cell markers, the entire *BCR/ABL1* positive subgroup showed CD10 and Tdt expression (32/32) while, only 20% (11/32) *BCR/ABL1* positive showed CD22 expression. In case of *BCR/ABL1* negative subgroup, 94% (92/98) patients showed CD10, 87% (85/98) patients showed Tdt, and 80% (42/98) patients showed CD22 expression indicating presence of Tdt, CD10; whereas absence of CD22 associated with *BCR/ABL1* positivity. However, the correlation of CD22 was associated significantly with only with adult patients.

Similarly, 28% BCR/ABL positive patients had CD13 and 20% with BCR/ *ABL1* positive had CD33 expression, whereas only 10% patients with *BCR/ABL1* negativity had CD13 CD33 expression indicating aberrant CD13, CD33 expression is associated with *BCR/ABL1* positivity. CD13 and CD33 known to express aberrantly in pediatric patients. In pediatric age group, only two (2/7) pediatric patients with CD13 have *BCR/ABL1* gene rearrangement and none of the patients with CD33 aberrant expression were positive for *BCR/ABL1* gene rearrangement indicates CD13 and CD33 expression is associated with *BCR/ABL1* gene rearrangement in adult patients (Table 2).

In terms of Median fluorescence Intensity (MFI), *BCR/ABL1* positive patients had significantly high expression of CD10

Table 1. Immunophenotypic profile of B-ALL patients

Marker	Percentage of patients				Median fluorescence intensity (MFI)	
	Positive		Negative		Minimum	Maximum
	n	%	n	%		
B cell lineage						
CD19	130	100	0	0	136	3505
CCD79a	130	100	0	0	120	11490
CD22	77	59	53	41	110	1081
Maturation marker						
CD34	39	30	91	70	24	27791
CD10	124	95	06	04	40	82207
Tdt	117	90	13	10	58	10657
HLADR	130	100	0	0	107	14969
Myeloid lineage						
CD13	19	15	111	85	23	4751
CD33	14	11	116	89	11	5088
T cell lineage						
CD5	0	0	130	100	15	50
CD7	0	0	130	100	10	35
CD3	0	0	130	100	12	60

Table 2. Antigen expression in *BCR/ABL1* subgroups

Markers	*BCR/ABL1* positive patients (n=32)	*BCR/ABL1* negative cases (n=98)	p
	Frequency of positivity	Frequency of positivity	
CD22	20% (11/32)	80% (42/98)	0.39
CD34	81% (26/32)	66% (65/98)	0.10
CD10	100% (32/32)	94% (92/98)	0.09
Tdt	100% (32/32)	87% (85/98)	0.03
CD13	28% (09/32)	10% (10/98)	0.01
CD33	20% (06/32)	08% (08/98)	0.09

(p=0.0001) and Tdt (p=0.0001) and low expression of cCD79a (p=0.01) and CD22 (p=0.004) as compared to *BCR/ABL1* negative patients. Further, a significantly high MFI value of CD13 (p=0.03) while a trend of increased MFI of CD33 was observed in *BCR/ABL1* positive patients as compared to *BCR/ABL1* negative patients (Fig. 1a–c).

ROC Curve Analysis for Diagnostic Accuracy for Significant Markers

In ordered to establish the cut-off points for optimal sensitivity and specificity of all significant markers, receiver operating curves (ROC) were analyzed. Cut off value with maximum sensitivity and specificity and area under curved more than 0.50 was used. Cut off point for B cells markers were >3505 MFI for CD19 (AUC=0.57), <126 MFI for CD22 (AUC=0.66), <508 MFI for cCD79a (AUC=0.61), for maturation markers >27791 for CD34 (AUC=0.58), >21974 MFI for CD10 (AUC=0.73), >4992 MFI for Tdt, for myeloid markers >3110 MFI for CD13 (AUC=0.55) and >5088 MFI for CD33 (AUC=0.60).

In our study, Tdt expression in terms of MFI had maximum sensitivity and specificity to differentiate *BCR/ABL1* positive and negative subgroups. Despite less sensitivity (56%), CD10 positivity in terms of MFI in our study displayed significant high expression in *BCR/ABL1* positive cases, suggesting their possible important role in gene rearrangement prediction (Table 3, Fig., 2–4)

Logistic Regression Model for Prediction of *BCR/ABL1* Gene Rearrangement

Logistic regression analysis of immunophenotypic markers was performed to evaluate predictive values of for *BCR/ABL1* gene rearrangement status. High MFI of Tdt was entered at step 1 (Wald statistic=15.4, df=1, Exp(B)=1.0, p=0.001) followed by high MFI of CD10 (Wald statistic= 6.5, df=1, Exp(B)=1.0, p=0.01) and low MFI of CD22 (Wald statistic= 8.8.4, df=1, Exp(B)=1.0, p=0.003) and cCD79a (Wald statistic=3.8, df=1, Exp(B)=1.0, p=0.05) expression pattern for prediction of BCR/ABL-1 gene rearrangement. The overall rate of correct classification provided by this model was estimated to be 83% (Table 4 a, b).

Discussion

Discovery of TKI inhibitors adds survival benefits of Philadelphia positive Acute Lymphoblastic Leukemia (ALL) patients. The initial risk stratification in ALL patients is an important step for treatment decision since Ph-positive B-ALL have poor clinical outcome its necessary to detect *BCR/ABL1* gene rearrangement on priority basis so that patient receives TKI inhibitors treatment as early as possible. *BCR/ABL1* translocation is carried out using conventional cytogenetic, FISH and RT PCR.

To date, many attempts have been made to correlate immunophenotypic features of Ph-positive ALL with *BCR/ABL1* gene rearrangement status. Tabernero et al.[7] demonstrated expression of CD10, CD13, CD34 and CD38 is associated with Ph-positive patients. They observed high and homogenous expression of CD10 and CD34 but a low and heterogeneous expression of CD38 along with aberrant CD13 expression is a distinctive feature of Ph-positive patients. Some other studies have demonstrated an association of aberrant myeloid marker CD66c expression with *BCR/ABL1* abnormality.[8-10] Schultz et al. and Fuster et al. have analyzed CD25 (interleukin-2 receptor alpha chain) expression in Ph-positive B-ALL and they found high CD25 expression as a surrogate marker in adult acute lymphoblastic leukemia predicts the presence of *BCR/ABL1* fusion transcripts.[11, 12] In contrast to these studies, in the present study, overall immuophenotype of Leukemic blasts including B cell, Myeloid cell, T cell, and non-lineage markers expression has been assessed on larger sample size of B-ALL patients.

In the present study, all the patients expressed CD19, cCCD79a and HLADR, while none of the patients express T cell markers and myeloid marker MPO. 15% elderly patients expressed myeloid marker CD13 and 11% patients expressed CD33. In the case of maturation markers, 95% patients expressed CD10, 90% patients expressed Tdt and 70% patients expressed CD34. Among these patients, *BCR/ABL1* fusion gene was observed in 24% patients, which is in accordance with global incidence (15-20%).[13] Further, *BCR/ABL1* fusion gene was found high in adult compared to pediatric age group and in patients with high WBC count which is in accordance with previous reports.[14-17]

Further, majority of *BCR/ABL1* positive patients expressed high CD10, Tdt, CD13 and CD33 and low CCD79a and CD22 suggest B cell differentiation process is arrested at Pre Pre B and Pre B stage of B cell maturation in *BCR/ABL1* positive

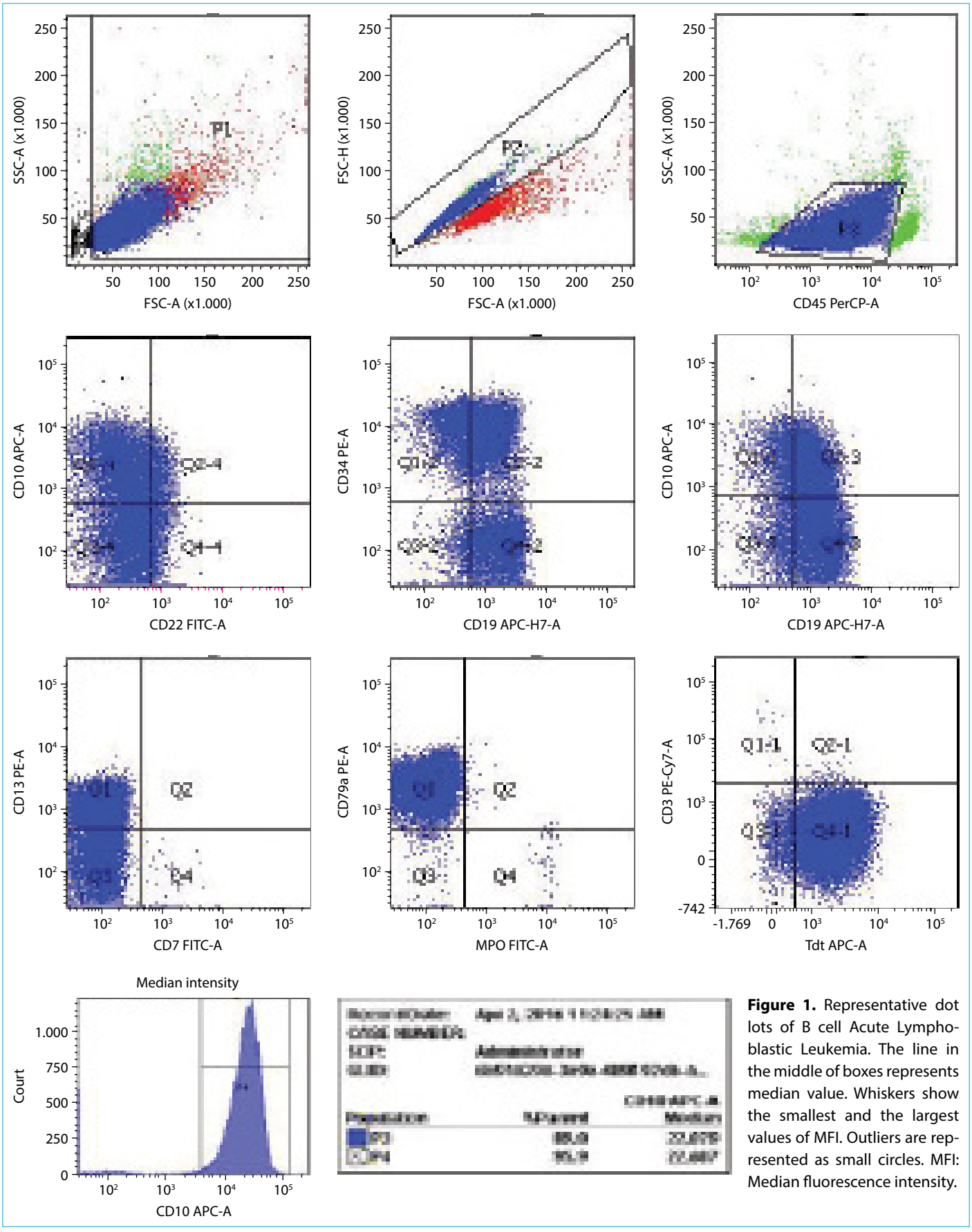

Figure 1. Representative dot lots of B cell Acute Lymphoblastic Leukemia. The line in the middle of boxes represents median value. Whiskers show the smallest and the largest values of MFI. Outliers are represented as small circles. MFI: Median fluorescence intensity.

Table 3. ROC curve analysis for diagnostic accuracy for immunophenotypic markers

Immunophenotypic marker	MFI cutt-off	Sensitivity	Specificity	PPV	NPV
CD22	<126	96.8	29.5	36.2	81.38
CCD79a	<508	37.5	87.76	48.1	80.8
CD10	>21974	56.25	82.65	58.7	82.2
Tdt	>4992	75.0	73.4	72.7	79.4
CD34	>27791	71.87	55.10	34.3	85.7
CD13	>3110	65.6	48.98	29.5	81.3
CD33	>5088	62.5	63.27	35.6	83.7

ROC: Receiver operating characteristic; MFI: Median fluorescence intensity; PPV: Positive predictive value; NPV: Negative predictive value.

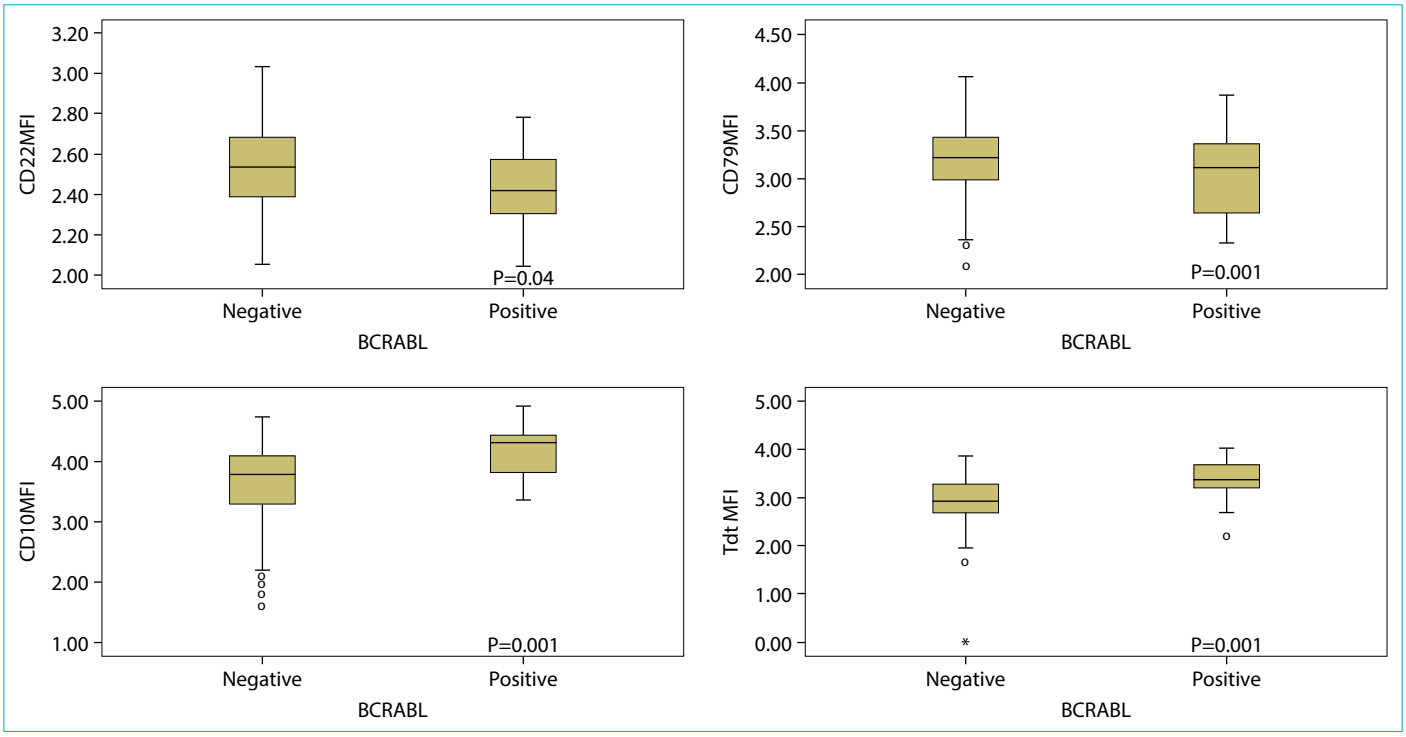

Figure 2. Box plots of B cell marker expression in terms of Median fluorescence intensity.

Table 4a. Logistic regression analysis for prediction of *BCR/ABL1* gene rearrangement

Immunophenotypic marker	B	SE	Wald	df	P	EXP
Step 1 Tdt MFI	0.0001	0.0001	15.446	1	0.0001	1.0
Step 2 CD10 MFI	0.0001	0.0001	6.56	1	0.01	1.0
Step 3 CD22 MFI	-0.006	0.002	8.83	1	0.03	0.99
Step 4 CCD79a MFI	0.0001	0.0001	3.8	1	0.05	1.0

B: Coefficient for the constant; SE: Standard error for the constant; df: degree of foredoom; EXP: odds ratio.

B-ALL which obstruct the down regulation of CD10 and Tdt and up regulation of CD22 which is seen in later part of B cell maturation process. CD10 act as peptidase enzyme could be involved in hindering normal B cell differentiation through the degradation of a protein that involved in B cell differentiation.[7] It has been observed that expression of myeloid markers CD13 and CD33 have been tightly regulated during the normal B cell maturation process, hematopoietic stem cells those are committed into the B-lymphoid lineage have low expression of CD13 and CD33.[18, 19] In this context, aberrantly high expression of myeloid markers CD13, CD33 in Ph-positive B-ALL patients appears to be abnormally regulated among Ph patients.

Along with marker positivity, analysis of Median Fluorescence Intensity (MFI) of each Immunophenotypic markers helped to differentiate in *BCR/ABL1* positive patients. In the present study high MFI value of CD10, Tdt, CD13 and CD33 was associated with *BCR/ABL1* gene rearrangement. Predictive value for each marker was validated using ROC

Table 4b. Classification percentage provided by predictive model

Observed	Predicted		
	***BCR/ABL1* Negative**	***BCR/ABL1* Positive**	**Percentage correct**
Step 1			
Tdt MFI			
BCR/ABL1 negative	91	5	94.8
BCR/ABL1 positive	23	8	25.8
Step 2			
CD10 MFI			
BCR/ABL1 negative	92	4	95.8
BCR/ABL1 positive	22	9	29.0
Step 3			
CD22 MFI			
BCR/ABL1 negative	90	6	93.8
BCR/ABL1 positive	17	14	45.2
Step 4			
CCD79a MFI			
BCR/ABL1 negative	90	6	93.8
BCR/ABL1 positive	16	15	48.4
Overall percentage			82.7

MFI: Median fluorescence intensity.

curve analysis; Tdt expression in terms of MFI had maximum sensitivity and specificity to differentiate *BCR/ABL1* positive and negative groups. Despite the less sensitivity, CD10 positivity, in our study displayed significantly high expression, in *BCR/ABL1*- positive cases, suggesting their possible important role in gene rearrangement prediction. Our predictive values were based on MFI and therefore, precaution must be taken in the selection of flour chrome and instrument settings must be validated in case of proposing a predictive value, so every center should establish their own MFI cutoff value. Our findings are consistent with previous reports demonstrating high expression of CD10, CD13 and CD33in terms of MFI can be used for predicting *BCR/ABL1* gene rearrangement status.[7, 18]

According to logistic regression analysis, high MFI of Tdt, CD10 and low MFI of CD22 and CCD79a had the greatest predictive value for *BCR/ABL1* gene rearrangement. Overall rate of correct classification provided by this model was estimated to be 83%. Quite a few B-ALL patients have masked *BCR/ABL1* fusion gene, which is not detected using FISH method, in such patients we emphasize that patients with the higher predicted probabilities for *BCR/ABL1* gene rearrangement i.e. high MFI of Tdt and CD10 with absence of CD22 should be checked gene rearrangement status using RT-PCR for rapid TKI inhibitor therapy.

In summary, we evaluated association of immunopheno-

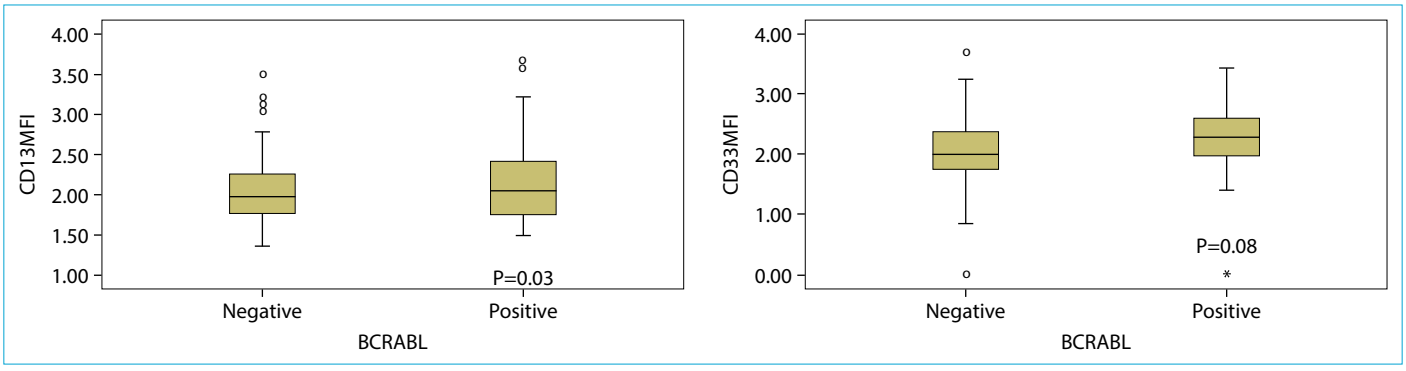

Figure 3. Box plots of myeloid expression in terms of Median fluorescence intensity. The line in the middle of boxes represents median value. Whiskers show the smallest and the largest values of MFI. Outliers are represented as small circles. MFI: median fluorescence intensity.

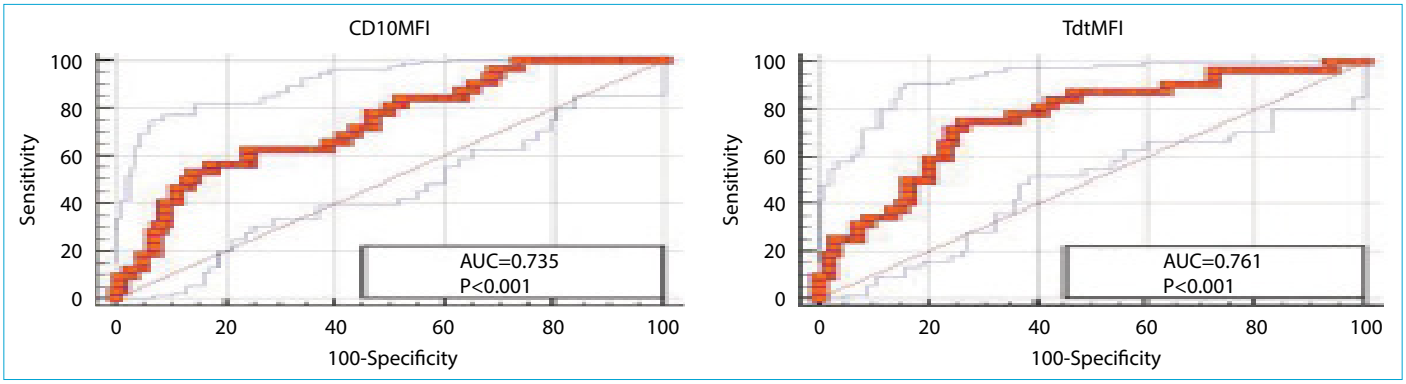

Figure 4. ROC curve analysis of significant B cell markers that discriminate *BCR/ABL1* positive patients from *BCR/ABL1* negative patients.

typic markers in B cell ALL with the presence of *BCR/ABL1* gene rearrangement. Immunophenotypic profile of Ph-positive patients suggests that *BCR/ABL1* gene rearrangement occurs at Pre Pre B and Pre B stage of B cell maturation process. Logistic regression model reveals that high expression of Tdt and CD10 in terms of MFI along with low expression of cCD79a and CD22 are most informative markers for the presence or absence of gene rearrangement at the time of diagnosis. We consider the importance of FISH technique in detection of Ph chromosome which cannot be replaced by flowcytometric immunophenotypic, but it will definitely provide an early clue of *BCR/ABL1* gene rearrangement which can support in initial risk stratification prior to frontline TKI therapy.

Disclosures

Ethics Committee Approval: The study was approved by the Local Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – B.R.; Design – B.R.; Materials – B.R.; Data collection &/or processing – B.R.; Analysis and/or interpretation – B.R., B.P., P.T.; Writing – B.R.; Critical review – H.V.

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