Characterization of Immunophenotypic Aberrancies with Respect to Common Fusion Transcripts in B-Cell Precursor Acute Lymphoblastic Leukemia: A Report of 986 Indian Patients

Objective: Based on the immunophenotype, acute lymphoblastic leukemia (ALL) can be categorized into B-cell or T-cell lineages. B-cell precursor ALL (BCP-ALL) cases show various genetic/molecular abnormalities, and varying frequencies of chimeric fusion transcripts in BCP-ALL cases are reported from different parts of the world. We studied the immunophenotypic aberrancy profiles of a large number of BCP-ALL cases with respect to various common chimeric fusion transcripts.

Results: Among 986 BCP-ALL cases, the incidence of various fusion transcripts was 38.36% in adult cases and 20.68% in pediatric cases. Adult BCP-ALL patients with t(9;22)(BCR-ABL1) fusion transcripts and expression of aberrant myeloid markers were significantly older at presentation (p=0.0218) with male preponderance (p=0.0246) compared to those without aberrant myeloid expression. In pediatric patients with the t(12;21)(ETV6-RUNX1) chimeric fusion transcript, aberrant expression of CD13 was observed in 39.13%, CD33 in 36.95%, and CD117 in 8.69% of patients, respectively. Pediatric BCP-ALL patients with the ETV6-RUNX1 fusion transcript and expression of aberrant myeloid markers were not significantly different compared to those with respect to demographic and clinical/hematological characteristics (p=0.5955). Aberrant myeloid markers were rarely or never expressed in pediatric and adult BCP-ALL patients with the t(4;11)(KMT2A-AF4) and t(1;19)(TCF3-PBX1) fusion transcripts.

Conclusion: Aberrant myeloid markers were frequently expressed among BCP-ALL patients with the t(9;22)(BCR-ABL1) and t(12;21)(ETV6-RUNX1) fusion transcripts. However, BCP-ALL patients with t(9;22)(BCR-ABL1) fusion transcript and expression of aberrant myeloid markers were significantly older at presentation (p=0.0218) with male preponderance (p=0.0246) compared to those without aberrant myeloid expression. In pediatric patients with the t(12;21)(ETV6-RUNX1) chimeric fusion transcript, aberrant expression of CD13 was observed in 39.13%, CD33 in 36.95%, and CD117 in 8.69% of patients, respectively. Pediatric BCP-ALL patients with the ETV6-RUNX1 fusion transcript and expression of aberrant myeloid markers were not significantly different compared to those with respect to demographic and clinical/hematological characteristics (p=0.5955). Aberrant myeloid markers were rarely or never expressed in pediatric and adult BCP-ALL patients with the t(4;11)(KMT2A-AF4) and t(1;19)(TCF3-PBX1) fusion transcripts. However, BCP-ALL patients with t(9;22)(BCR-ABL1) fusion transcript and expression of aberrant myeloid markers were significantly older at presentation (p=0.0218) with male preponderance (p=0.0246) compared to those without aberrant myeloid expression. In pediatric patients with the t(12;21)(ETV6-RUNX1) chimeric fusion transcript, aberrant expression of CD13 was observed in 39.13%, CD33 in 36.95%, and CD117 in 8.69% of patients, respectively. Pediatric BCP-ALL patients with the ETV6-RUNX1 fusion transcript and expression of aberrant myeloid markers were not significantly different compared to those with respect to demographic and clinical/hematological characteristics (p=0.5955). Aberrant myeloid markers were rarely or never expressed in pediatric and adult BCP-ALL patients with the t(4;11)(KMT2A-AF4) and t(1;19)(TCF3-PBX1) fusion transcripts. However, BCP-ALL patients with t(9;22)(BCR-ABL1) fusion transcript and expression of aberrant myeloid markers were significantly older at presentation (p=0.0218) with male preponderance (p=0.0246) compared to those without aberrant myeloid expression. In pediatric patients with the t(12;21)(ETV6-RUNX1) chimeric fusion transcript, aberrant expression of CD13 was observed in 39.13%, CD33 in 36.95%, and CD117 in 8.69% of patients, respectively. Pediatric BCP-ALL patients with the ETV6-RUNX1 fusion transcript and expression of aberrant myeloid markers were not significantly different compared to those with respect to demographic and clinical/hematological characteristics (p=0.5955). Aberrant myeloid markers were rarely or never expressed in pediatric and adult BCP-ALL patients with the t(4;11)(KMT2A-AF4) and t(1;19)(TCF3-PBX1) fusion transcripts.

Amaç: Akut lenfoblastik lösemi (ALL), immünofenotipe dayalı olarak, B-hücre veya T-hücre kökenli olarak kategorize edilebilir. B-hücre prekürsör ALL (BCP-ALL) olguları çeşitli genetik/moleküler anormallikler gösterir ve BCP-ALL olgularındaki kimerik füzyon transkriptlerinin değişik frekansları dünyanın farklı yerlerinden rapor edilmiştir. Çok sayıda BCP-ALL olgusunun ortaktakı kimerik füzyon transkriptlerine göre immünofenotipik anormallik profillerini inceledik.

Gereç ve Yöntemler: 986 BCP-ALL olgusu için akım sitometri immünofenotipelme ve multiples ters-transkripsiyon polimeraz zincir reaksiyonu çalışmaları yapıldı.

Bulgular: 986 BCP-ALL olgusu arasında çeşitli füzyon transkriptlerinin insidansı yetişkin olgulara %38,36 ve pediatrisk olgularda %20,68 idi. t(9;22)(BCR-ABL1) fusion transkripti ve anormal myeloid belirteç ifadesi olan yetişkin BCP-ALL hastaların, anormal myeloid ifadesi olmayan hastalarla karşılaştırıldığında anlamlı olarak daha yaşlıydı (p=0,0218) ve erkek çoğunluğu vardı (p=0,0246). t(12;21)(ETV6-RUNX1) kimerik füzyon transkripti olan pediyatrik hastalarda, sırasıyla anormallik CD13 ifadesi %39,13, CD33 %36,95 ve CD117 %8,69 hasta dağıldı. ETV6-RUNX1 fusion transkripti olan anormal myeloid belirteç ifadesi olan pediyatrik BCP-ALL hastaları, olmayanlara kıyasla demografik ve klinik/hematolojik özellikler açısından farklıydı (p=0,5955). t(4;11)(KMT2A-AF4) ve t(1;19)(TCF3-PBX1) fusion transkriptlerine sahip pediyatrik ve yetişkin BCP-ALL hastalarında anormallik myeloid belirteçler radenin veya hiç ifade edilmedi.

Sonuç: Anormal myeloid belirteçler, t(9;22)(BCR-ABL1) ve t(12;21)(ETV6-RUNX1) fusion transkriptleri olan BCP-ALL hastalarında siklikta ifade edilirdi. Bununla birlikte, t(4;11)(KMT2A-AF4) ve t(1;19)(TCF3-PBX1) fusion transkriptlerine sahip BCP-ALL hastalarında anormallik myeloid belirteçler radenin veya hiç ifade edilmedi.
Abstract

the t(4;11)(KMT2A-AF4) and t(1;19)(TCF3-PBX1) fusion transcripts rarely or never expressed aberrant myeloid markers. Aberrant myeloid CD markers can be used in predicting chimeric fusion transcripts at baseline so as to plan appropriate tyrosine kinase inhibitor therapy in cases of B-ALL with specific chimeric fusion transcripts. This study has delineated the relationship of chimeric fusion transcripts with the aberrant expression of myeloid markers in a large cohort of B-ALL cases.

Keywords: Acute leukemia, Acute lymphoblastic leukemias, Molecular biology, Molecular hematology, Neoplasia

Introduction

Acute lymphoblastic leukemia (ALL) is a blood disorder with uncontrolled clonal proliferation of lymphoblasts in the bone marrow, blood, and/or tissues. Based on flow cytometric immunophenotyping (FCM-IP), ALL can be classified as B-cell or T-cell lineage [1]. ALL is a genetically heterogeneous disease characterized by chromosomal rearrangements, structural variations, copy number variations, and sequence mutations [2,3,4,5]. Pediatric B−cell precursor ALL (BCP-ALL) cases are often associated with a number of cytogenetic/molecular rearrangements, including hyperdiploidy, hypodiploidy, t(9;22) (BCR-ABL1), t(12;21)(ETV6-RUNX1), t(4;11)(KMT2A-AF4), t(1;19)(TCF3-PBX1), and rearrangement of the CRLF2 and KMT2A genes. Different age groups have varying frequencies of these genetic subtypes. Hyperdiploidy (>50 chromosomes) and t(12;21)(ETV6-RUNX1) are observed less commonly in adults compared to pediatric cases and the incidence of Ph positivity increases with advancing age. Treatment outcomes of adult ALL patients are far inferior to those of pediatric ALL cases, possible reasons being the reduced incidence of cytogenetic/molecular abnormalities associated with favorable outcome (e.g., more frequent hyperdiploidy and ETV6-RUNX1 translocation in pediatric ALL) and the rising incidence of BCR-ABL1 positivity [3,6,7]. Authors around the globe have reported high incidences (7%-34.4%) of t(12;21)(ETV6-RUNX1) and low incidences (2%-11.9%) of t(9;22)(BCR-ABL1) in pediatric B-ALL and high incidences (3.2%-37%) of t(9;22)(BCR-ABL1) in adult B-ALL [8,9,10,11,12,13,14,15,16,17,18,19,20,21]. Few authors have studied B-ALL for immunophenotypic abnormalities, with reported expression of CD13 in 5.88%-56.1%, CD33 in 2.47%-49%, and CD117 in 0.68%-22% of cases [18,22,23,24,25,26].

Earlier we reported our experience of detection of chimeric fusion transcripts in B-cell ALL [9] and immunophenotypic aberrancies in ALL [18] in two different sets of patients at different time points. The presence of aberrant myeloid-associated markers in B-ALLs might necessitate the search for poor prognostic parameters, e.g., t(9;22)(BCR-ABL1), which are likely to impact case management [27]. In the present study, we have tried to delineate the relationship between chimeric fusion transcripts and expression of aberrant myeloid-associated markers among 986 BCP-ALL cases. To date, few studies have addressed this issue, with much smaller numbers of patients harboring various fusion transcripts.

Materials and Methods

The present study was conducted in the Department of Hematology of the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. This study was approved by the Institutional Ethics Committee (No. INT/IEC/2017/191 dated August 23, 2017). In our institute, patients up to 12 years of age are managed by the pediatric hematology oncology unit of the department of pediatrics and the rest are managed by the adult hematology unit of the department of internal medicine. We examined 986 BCP-ALL cases, including 522 pediatric and 464 adult cases. Complete blood count (CBC), bone marrow (BM) examination, FCM-IP, and multiplex reverse-transcription polymerase chain reaction (RT-PCR) assay were performed.

Sample Collection

Samples of 2-3 mL of aspirated BM and 3-5 mL of peripheral blood (PB) were collected in EDTA-coated vials after obtaining the signature of the patient or the patient’s guardian on an informed consent form as approved by the Human Institutional Ethics Committee of PGIMER. PB samples were processed for various hematological tests, including CBC and PB film examination. Furthermore, May–Grunwald–Giems and cytochemical staining including myeloperoxidase and periodic acid–Schiff were performed on PB/BM smears. Finally, PB/BM samples were processed for FCM-IP and molecular diagnostic testing.

Flow Cytometric Immunophenotyping (FCM-IP)

After morphological assessment, PB/BM samples were processed for FCM-IP to diagnose BCP-ALL cases according to the protocol
standardized and practiced in the department [18], presently consisting of a panel of 42 monoclonal antibodies (Table 1). At least 1x10^6 events were acquired on a ten-color/twelve-parameter flow cytometer (Navios EX, Beckman Coulter, Chino, CA, USA) and analyzed using Kaluza 2.1 software.

Briefly, the blasts were gated on a plot of CD45 versus side scatter (SS), with blasts mostly representing CD45-dim and SS-low events. Expression of B-lineage-associated markers (CD19, CD10, cytoplasmic CD79a, cytoplasmic CD22) and immaturity-associated markers (CD34, CD38, TdT) confirmed the presence of B-lineage blasts. Expression of myeloid lineage-associated markers including CD13, CD33, and CD117 was also noted. B-lineage blasts were considered to express aberrant myeloid markers if >20% blasts showed positivity for the marker (Figure 1). In addition, absence of cytoplasmic anti-myeloperoxidase and cytoplasmic CD3 was noted to exclude mixed phenotypic acute leukemia.

### Multiplex RT-PCR for Chimeric Fusion Transcripts

Total RNA was isolated using the commercially available QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, followed by NanoDrop quantification (Thermo Scientific, Waltham, MA, USA). Thereafter, cDNA was prepared from 1 µg of RNA using a cDNA synthesis kit (Thermo Scientific). The quality of the cDNA was assessed by running 2% agarose gel electrophoresis for the PCR product of β-actin as the housekeeping gene. Finally, 1 µg of cDNA was used for multiplex RT-PCR assay with specific primers for various fusion transcripts according to the protocol followed in our department [28]. Details of primers for major and minor fusion transcripts are shown in Table 2.

### Statistical Analysis

Data are represented as mean, median, and range in this study. The normality of the data was determined by Shapiro-Wilk test. For continuous variables and uniformly distributed data, the independent t-test was applied, and for non-uniformly distributed data, the Mann-Whitney U test was applied to rule out variability in the data. Statistical analysis was performed using GraphPad Prism 7.1 and values of p<0.05 were taken as significant.

### Results

#### Patients’ Characteristics

Among 986 BCP-ALL patients, 610 were male and 376 were female (male-to-female ratio: 1.62:1), with a median age of 11 years (minimum–maximum: 1–85 years). Males predominated significantly in both adult and pediatric groups. CBC results showed median hemoglobin (Hb) of 7.8 (minimum–maximum: 2.4–15.5 g/dL), median total leukocyte count (TLC) of 12.4 (minimum–maximum: 0.3–576x10^9/L), and median platelet count of 27 (minimum–maximum: 1.7–703x10^9/L) in BCP-ALL patients, as shown in Table 3. Hb and platelet counts were significantly lower while TLC was significantly higher in adults compared to the pediatric group (p<0.0001).

#### Multiplex RT-PCR Results

Multiplex RT-PCR assays revealed recurrent genetic abnormalities in 38.36% (178/464) adult and 20.68% (108/522) pediatric BCP-ALL cases. The BCR-ABL1 chimeric fusion transcript was observed in 31.68% (147/464) adult and 7.08% (37/522) pediatric ALL cases, being significantly more frequent in adults compared to children (p<0.0001).

**BCR-ABL1** fusion transcripts result from the following breakpoints: a minor breakpoint (e1a2, p190 kDa), major

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**Table 1. Panel of 42 monoclonal antibodies used for diagnosis of acute leukemias.**

| Tube no. | FITC | PE  | APC  | PC5.5 | PC7  | ECD | APC-AF700 | APC-AF750 | PB | KrO |
|----------|------|-----|------|-------|------|-----|-----------|-----------|----|-----|
| 1        | Neg  | -   | -    | -     | -    | -   | -         | -         | -  | CD45|
| 2        | CD81 | CD58| CD38 | CD86  | CD123| CD10| CD34      | CD20      | CD19| CD45|
| 3        | CD64 | CD117| CD38 | CD33  | CD123| CD14| CD34      | HLA-DR    | CD36| CD45|
| 4        | CD15 | CD13| CD38 | CD117 | CD56 | CD19| CD7       | HLA-DR    | CD34| CD45|
| 5        | CD38 | CD34| cCD3 | CD8   | CD5  | CD16/CD56| CD7       | CD4       | CD3 | CD45|
| 6        | nuTDT | cMPO| cCD22| cCD79a| CD41 | CD38| CD34      | CD25      | cCD3| CD45|
| Additional tubes |      |     |      |       |      |     |           |           |     |      |
| 7        | nuTDT | CD7 | cCD3 | CD1a  | CD13/33| CD16/56| CD34      | CD3       | CD38| CD45|
| 8        | CD235/CD61| CD71| CD41a| CD117 | CD14  | CD38| CD34      | HLA-DR    | CD36| CD45|
breakpoints (b2a2, p210 kDa; b3a2, p210 kDa), and a micro breakpoint (e19a2, p230 kDa). Among patients with BCR-ABL1 fusion transcripts, the minor (e1a2) transcript was observed in 46.93% (69/147) adult and 59.45% (22/37) pediatric cases, a major (b2a2) transcript in 25.85% (38/147) adult and 29.72% (11/37) pediatric cases, and the other major (b3a2) transcript in 27.21% (40/147) adult and 10.81% (4/37) pediatric cases; no micro (e19a2) transcript was observed in this study. The e1a2 and b2a2 fusion transcripts were significantly more frequent in pediatric BCP-ALL cases, whereas the b3a2 fusion transcripts were significantly more frequent in adult BCP-ALL cases (p<0.0001). The t(4;11)(KMT2A-AF4) fusion transcript

Figure 1. Flow cytometric immunophenotyping of newly diagnosed adult B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients harboring the BCR-ABL1 fusion transcript with expression of aberrant myeloid markers. The blasts were gated on plots of CD45 versus side scatter (SS) (red populations) and were confirmed to be B-lineage blasts based on expression of B-lineage-associated markers (CD19, CD10, cytoplasmic CD79a). The gated blasts showed expression of myeloid-lineage associated markers (CD13 and CD33), which was considered aberrant expression of myeloid markers in cases of B-ALL.
was observed in 1.93% (9/464) adult and 0.57% (3/522) pediatric cases, being significantly more frequent in adult cases (p=0.0027). The t(1;19)(TCF3-PBX) fusion transcript was observed in 3.66% (17/464) adult and 4.21% (22/522) pediatric cases, being significantly more frequent in pediatric cases (p<0.0001). The t(12;21)(ETV6-RUNX1) fusion transcript was observed in 1.07% (5/464) adult and 8.81% (46/522) pediatric cases, being significantly more frequent in pediatric cases (p<0.0001). Representative chimeric transcripts in BCP-ALL are shown in Figures 2 and 3. The frequency of chimeric transcripts in adult and pediatric BCP-ALL cases is shown in Table 4.

**Flow Cytometric Immunophenotyping Results**

Aberrant expression of myeloid markers CD13, CD33, and CD117 was significantly more frequent in adults compared to the pediatric group (p<0.0001) (Table 3). Immunophenotypic aberrancies of myeloid markers including CD13, CD33, and CD117 in BCP-ALL cases with various genetic subtypes are shown in Table 4. Aberrant expression of CD13, CD33, and CD117 in adults with the BCR-ABL1 minor (e1a2) transcript was observed in 31.88%, 28.98%, and 1.44% of cases, respectively. In pediatric cases with the BCR-ABL1 minor (e1a2) transcript, aberrant expression of CD13, CD33, and CD117 was observed in 22.72%, 18.18%, and 4.54% of cases, respectively. Aberrant expression of CD13, CD33, and CD117 in adults with the BCR-ABL1 major (b2a2) transcript was observed in 28.94%, 7.7%, and 2.6% of cases. On the other hand, aberrant expression of CD13, CD33, and CD117 was observed in 18.18%, 18.18%, and 9.09% of pediatric cases with the BCR-ABL1 major (b2a2) transcript. Aberrant expression of CD13 and CD33 with the BCR-ABL1 major (b3a2) transcript was observed in 35%, 36.84%, and 5% of cases. Aberrant expression of CD13 and CD33 was observed in 25% and 25% of pediatric cases with the e1a2 and b2a2 transcripts (but not b3a2) compared to pediatric patients with these transcripts.

### Table 2. Primers for major fusion and micro fusion transcripts.

|   | Primers                        |
|---|--------------------------------|
| 1 | t(9;22)(BCR-ABL1) (p210 kDa) b2a2:  |
|   | BCR-5’-ACAGAATCTCGGATCAATGAAG-3’  |
|   | ABL-5’-TGTTGACTGGCGTGATGTAGTTGG-3’ |
| 2 | t(9;22)(BCR-ABL1) (p210 kDa) b3a2:  |
|   | BCR-5’-ACAGAATCTCGGACATCAATAG-3’  |
|   | ABL-5’-TGTTGACTGGCGTGATGTAGTTGG-3’ |
| 3 | (9;22)(BCR-ABL1) (p230 kDa) e19a2:  |
|   | BCR-5’-GAAGAGATGTTTCAAGAGCTCCTCC-3’  |
|   | ABL-5’-TGTTGATTATAGCCTAAGACCCGAG-3’ |

### Table 3. Biological and hematological features of 986 BCP-ALL cases.

| Parameter          | All cases (n=986) | Pediatric BCP-ALL cases, ≤12 years (n=522) | Adult BCP-ALL cases, >12 years (n=464) | p  |
|--------------------|------------------|-------------------------------------------|--------------------------------------|----|
| Age (years)        |                  |                                           |                                      |    |
| Median             | 11 (1-85)        | 4 (1-12)                                  | 26 (13-85)                           | ***|
| Age groups         |                  |                                           |                                      |    |
| ≤12 years          | 52.94% (522/986) | 52.94% (522/986)                          | 47.05% (464/986)                     |    |
| >12 years          | 47.05% (464/986) | 47.05% (464/986)                          | 52.94% (522/986)                     |    |
| Males, n (%)       | 61.86% (610/986) | 63.79% (333/522)                          | 59.69% (277/464)                     | ***|
| Females, n (%)     | 38.13% (376/986) | 36.20% (189/522)                          | 40.30% (187/464)                     | ***|
| Hb, g/L            |                  |                                           |                                      |    |
| Median             | 7.8 (2.4-15.5)   | 8.87 (3-15.2)                             | 7.50 (2.4-15.5)                      | ***|
| TLC, x10⁹/L        |                  |                                           |                                      |    |
| Median             | 12.4 (0.3-576)   | 19.4 (0.4-521)                            | 19.4 (0.3-576)                       | ***|
| Platelets, x10⁹/L  |                  |                                           |                                      |    |
| Median             | 27 (1.3-703)     | 29 (1.9-597)                              | 24 (1.7-703)                         | 0.0046 **|
| CD13               | 20.58% (203/986) | 18.96% (99/522)                           | 22.41% (104/464)                     | ***|
| CD33               | 18.66% (184/986) | 13.02% (68/522)                           | 25% (116/464)                        | ***|
| CD117              | 3.34% (33/986)   | 3.44% (18/522)                            | 3.23% (15/464)                       | ***|

BCP-ALL: B-cell precursor acute lymphoblastic leukemia; Hb: hemoglobin; TLC: total leukocyte count; ***: significant (p<0.0001).
In patients with the t(12;21)(ETV6-RUNX1) transcript, aberrant expression of CD13 and CD33 was observed in 40% and 20% of adult cases. Aberrant expression of CD13, CD33, and CD117 was respectively observed in 39.13%, 36.95%, and 8.69% of pediatric cases with the t(12;21)(ETV6-RUNX1) transcript. In patients with the t(1:19)(TCF3-PBX1) transcript, aberrant expression of CD13 and CD33 was observed in 5.88% and 5.88% of adult cases. Aberrant expression of CD13 was observed in 13.63% of pediatric cases with the t(1:19)(TCF3-PBX1) transcript. In patients with the t(4;11)(KMT2A-AF4) transcript, no aberrant myeloid marker expression was observed in adult or pediatric cases. Expression of CD13 and CD33 myeloid markers was not significantly different in adult cases with t(12;21)(ETV6-RUNX1), t(1:19)(TCF3-PBX1), or t(4;11)(KMT2A-AF4) transcripts compared to pediatric BCP-ALL cases with these transcripts. Clinical and biological characteristics of patients harboring chimeric fusion transcripts and aberrant expressions of myeloid markers in adult and pediatric BCP-ALL cases are shown in Tables 5 and 6.

Discussion

Among 986 patients with BCP-ALL, the overall incidence of four fusion transcripts was found to be 38.36% in adults and 20.68% in pediatric cases. Table 7 compares several studies from India as well as research from Western countries, showing a high incidence of t(12;21)(ETV6-RUNX1) (range: 7%-34.4%) and low incidence of the t(9;22)(BCR-ABL1) fusion transcript (range: 2%-7.3%) in pediatric B-ALL cases [9,10,11,12,14,15,16,19,21].

Figure 2. Multiplex assay showing presence of minor transcript (e1a2, p190 kDa) and major transcripts (b2a2, b3a2, p210 kDa) of BCR-ABL1 in adult BCP-ALL cases. Lane 1 shows minor transcript-positive control/internal reference genes (e1a2) (521 bp), lane 7 shows positive minor transcript in a patient, lane 10 shows major transcript-positive control/internal reference genes (b2a2) (310 bp), lanes 12 and 13 show positive major transcripts (b2a2) in two patients, lane 15 shows major transcript-positive control/internal reference genes (b3a2) (385 bp), and lanes 17 and 18 show positive major transcripts (b3a2) in two patients.

Figure 3. Multiplex assay showing presence of t(4:11), t(1:19), and t(12:21) in adult BCP-ALL cases. Lane 1 of panel a shows t(4:11)(KMT2A-AF4)-positive control/internal reference genes (511 bp), lane 6 shows positive t(4:11)(KMT2A-AF4), and lane 3 shows t(1:19)(373 bp) in patients. Lane 1 of panel b shows t(12;21)(ETV6-RUNX1)-positive control/internal reference genes, lane 3 shows t(1:19)(TCF3-PBX1)-positive control/internal reference genes, and lane 4 shows t(12;21)(ETV6-RUNX1) (373 bp) transcripts.
Table 4. Four fusion transcripts in adult and pediatric BCP-ALL cases and expression of aberrant myeloid markers.

| Chimeric transcripts | Incidence in adult cases | Incidence in pediatric cases | Sig. | Aberrant myeloid markers |
|----------------------|--------------------------|-------------------------------|------|--------------------------|
|                      |                          |                               |      | CD13, adult              | CD13, pediatric | CD33, adult | CD33, pediatric | CD117, adult | CD117, pediatric | Sig. |
| (e1/a2, p190 kDa)    | 69/147 (46.93%)          | 22/37 (59.45%)               | ***  | 22/69 (31.88%)           | 5/22 (22.72%)   | 20/69 (28.98%) | 4/22 (18.18%)   | ***          | 1/69 (1.44%) | 1/22 (4.54%) | ns   |
| (b2/a2, p210 kDa)    | 38/147 (25.85%)          | 11/37 (29.72%)               | ***  | 11/38 (28.94%)           | 2/11 (18.18%)   | 14/38 (7.7%)  | 2/11 (18.18%)   | 0.0083       | 1/38 (2.6%)  | 1/11 (9.09%) | ns   |
| (b3/a2, p210 kDa)    | 40/147 (27.21%)          | 4/37 (10.81%)                | 0.001| 14/40 (35%)              | 1/4 (25%)       | 16/40 (36.84%)| 1/4 (25%)       | ns           | 2/40 (5%)    | 0/4 (0%)    | ns   |
| t(9;22)(BCR-ABL1)    | 147/464 (31.68%)         | 37/522 (7.08%)               | ***  | 47/147 (31.97%)          | 8/37 (21.62%)   | 50/147 (34.01%)| 7/37 (18.91%)   | ***          | 4/147 (2.72%)| 2/37 (5.40%)| ns   |
| t(12;21)(ETV6-RUNX1) | 5/464 (1.07%)            | 46/522 (8.81%)               | ***  | 2/5 (40%)                | 18/46 (39.13%)  | ns           | 1/5 (20%)       | ns           | 0/5 (0%)     | 4/46 (8.69%)| ns   |
| t(1;19)(TCF3-PBX1)   | 17/464 (3.66%)           | 22/522 (4.21%)               | ***  | 1/17 (5.88%)             | 3/22 (13.63%)   | ns           | 1/17 (5.88%)    | ns           | 0/17 (0%)    | 0/22 (0%)   | ns   |
| t(4;11)(KMT2A-AF4)   | 9/464 (1.93%)            | 3/522 (0.57%)                | 0.0027| 0/9 (0%)                 | 0/3 (0%)        | ns           | 0/9 (0%)        | ns           | 0/9 (0%)     | 0/3 (0%)    | ns   |
| Total                | 178/464 (38.36%)         | 108/522 (20.68%)             |      | 50/178 (28.08%)          | 29/108 (26.85%) | 52/178 (29.21%)| 24/108 (22.22%) | 4/178 (2.24%)| 6/108 (5.59%)|      |

BCP-ALL: B-cell precursor acute lymphoblastic leukemia. ***: Significant (p<0.0001).
### Table 5. Clinical and biological features of adult BCP-ALL cases with four fusion transcripts and expression of aberrant myeloid markers.

| Parameter          | t(9;22)\((BCR-ABL1)\) n=147 | t(1:19)\((TCF3-PBX1)\) n=17 | t(4;11)\((KTM2A-AF4)\) n=9 | t(12;21)\((ETV6-RUNX1)\) n=5 |
|--------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|
| Status             | My+ (72)                   | My- (15)                   | My+ (0)                    | My+ (2)                     |
| My-                | My+ (2)                    | My- (15)                   | My- (9)                    | My- (3)                     |
| Age, years Median (minimum–maximum) | 39.5 (13–68) | 35 (13–66) | 0.0218* | 40 (35–45) | 19.13 (13–27) | ns | NA | 26.77 (14–44) | ns |
| Male               | 33/72                      | 44/75                      | 0.0246*                    | 1/17                        |
| Female             | 39/72                      | 31/75                      | ns                         | 1/17                        |
| Hb, g/dL Median (minimum–maximum) | 7.59 (2.7–11.7) | 7.75 (3.9–14.1) | ns | 7.6 (6.7–8.5) | 6.73 (3.7–10.8) | ns | NA | 6.35 (4.3–9) | ns |
| TLC, x10⁹/L Median (minimum–maximum) | 23.9 (0.9–359.9) | 59.8 (0.8–576) | ns | 53.05 (11.4–94.7) | 43 (1.7–301.6) | ns | NA | 150.3 (2.2–450.2) | ns |
| Platelets, x10⁹/L Median (minimum–maximum) | 31 (3–310) | 22 (2–217) | ns | 10 (8–12) | 33.51 (1.7–112) | ns | NA | 34.88 (10–113) | ns |

Non-normally distributed data: median (range); normally distributed data: mean.

BCP-ALL: B-cell precursor acute lymphoblastic leukemia; TLC: total leukocyte count; Hb: hemoglobin; My+: expression of myeloid markers; My-: no expression of myeloid markers; NA: not available; ns: nonsignificant.

### Table 6. Clinical and biological features of pediatric BCP-ALL cases harboring four fusion transcripts with expression of aberrant myeloid markers.

| Parameter          | t(9;22)\((BCR-ABL1)\) n=37 | t(1:19)\((TCF3-PBX1)\) n=22 | t(4;11)\((KTM2A-AF4)\) n=3 | t(12;21)\((ETV6-RUNX1)\) n=46 |
|--------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|
| Status             | My+ (13)                    | My- (24)                   | My+ (0)                    | My+ (23)                    |
| My-                | My+ (3)                    | My- (19)                   | My- (3)                    | My- (23)                    |
| Age, years Median (minimum–maximum) | 8.23 (1–11) | 5.8 (1–12) | ns | 5 (3–7) | 6.23 (1–12) | ns | 5.3 (2–9) | 5 (1–11) |
| Male               | 5/13                       | 18/24                      | ns                         | 3/3                        |
| Female             | 8/13                       | 6/24                       | ns                         | 0/3                        |
| Hb, g/dL Median (minimum–maximum) | 9.63 (7.4–13.8) | 8.08 (3.9–11.6) | 0.0218* | 9.6 (8.3–11) | 8.1 (4.9–12.4) | ns | NA | 9.2 (6.1–13.3) | ns |
| TLC, x10⁹/L Median (minimum–maximum) | 9.4 (1.5–370) | 15.6 (0.7–521) | ns | 18.16 (5–42.8) | 12.7 (2.5–200) | ns | NA | 142 (28.2–323) | ns |
| Platelets, x10⁹/L Median (minimum–maximum) | 34 (19–182) | 43.5 (5–467) | ns | 31.33 (15–51) | 40.12 (3.4–208) | ns | NA | 27.66 (15–35) | ns |

Non-normally distributed data: median (range); normally distributed data: mean.

BCP-ALL: B-cell precursor acute lymphoblastic leukemia; TLC: total leukocyte count; Hb: hemoglobin; My+: expression of myeloid markers; My-: no expression of myeloid markers; NA: not available; ns: nonsignificant.
A comparison of several such studies of adult B-ALLs from India and Western countries is shown in Table 8. The reported range of incidence of t(9;22)(BCR-ABL1) was 3.2%-37%, that of t(1:19) (E2A-PBX1) was 2.5%-6.25%, that of t(4:11)(KMT2A-AF4) was 0%-4.2%, and that of t(12;21)(ETV6-RUNX1) was 0%-14.9% [8,9,11,13,16,19,20]. Our RT-PCR findings in pediatric and adult BCP-ALL cases align well with these earlier studies. However, the e1a2 and b2a2 fusion transcripts were significantly more frequent in pediatric BCP-ALL cases whereas b3a2 fusion transcripts were significantly more frequent in adult BCP-ALL cases (p<0.0001), a finding that has not been described in detail in earlier studies.

Myeloid-associated markers such as CD13, CD33, and CD117 were the most frequent immunophenotypic aberrancies among BCP-ALL patients with chimeric fusion transcripts (Figure 3). Several studies have shown the presence of myeloid-lineage specific markers in B-lineage ALL cases from India and from Western nations, without or with information regarding genetic subtypes. Gupta et al. [24], from Rohtak, India, reported CD13 expression in 50%, CD33 in 2.6%, and CD117 in 5.3% of 38 B-ALL cases. Sharma et al. [18] from our institute reviewed 303 ALL cases and reported CD33 expression in 26.2% of pediatric and 32.9% of adult B-ALL cases, CD13 expression in 32.9% of pediatric and 34.5% of adult B-ALL cases, and CD117 expression in 16.3% of pediatric and 22% of adult B-ALL cases. Den Boer et al. [22] from the Netherlands reported the expression of myeloid markers in 37.06% of 143 pediatric B-ALL cases. Seegmiller et al. from the United States examined 200 B-ALL cases and reported CD33 expression in 40.2% of pediatric and 49% of adult, CD13 expression in 56.1% of pediatric and 51% of adult, and CD117 expression in 0% of pediatric and 1% of adult cases [25]. Supriyadi et al. [26] from Indonesia reported CD13 expression in 21%, CD33 in 10%, and CD117 in 4% of 239 childhood B-ALL cases. Gujral et al. [23] from Mumbai, India, examined 1120 B-ALL cases and reported CD13 expression in 5.88%, CD33 expression in 2.47%, and CD117 expression in 0.68% of 10 Ph-positive B-ALL cases; CD13 expression in 0% of 10 Ph-positive B-ALL cases; CD13 expression in 50%, CD33

| Authors, year [Ref.] | t(9:22)(BCR-ABL1) | t(12:21)(ETV6-RUNX1) | Country       |
|-----------------------|------------------|---------------------|---------------|
| Borkhardt et al. [10] | -                | 18.9% (63/334)      | Italy         |
| Trka et al. [21]      | -                | 34.4% (15/54)       | Czech Republic|
| Liang et al. [15]     | 6% (9/165)       | 18% (30/165)        | Taiwan        |
| Siraj et al. [19]     | 5% (14/259)      | 7% (18/259)         | India         |
| Chung et al. [12]     | -                | 17.1% (21/123)      | Korea         |
| Bhatia et al. [9]     | 5.35% (3/56)     | 16% (9/56)          | India         |
| Martinez-Mancilla et al. [16] | 7.3% (19/261) | 14.9% (39/261) | Mexico       |
| Kerketta et al. [14]  | 2.2% (2/90)      | 28% (25/90)         | India         |
| Chopra et al. [11]    | 11.9% (26/218)   | 7.3% (16/218)       | India         |
| Present study         | 7.08% (37/522)   | 8.81% (46/522)      | India         |

B-ALL: B-cell acute lymphoblastic leukemia.

| Authors, year [Ref.] | t(9:22)(BCR-ABL1) | t(12:21)(ETV6-RUNX1) | t(1:19)(TCF3-PBX1) | t(4:11)(KMT2A-AF4) | Country       |
|----------------------|------------------|---------------------|-------------------|-------------------|---------------|
| Bao et al. [8]       | 37% (47/127)     | -                   | 3.14% (4/127)     | China             |
| Sudhakar et al. [20] | 3.2% (2/64)      | 6.25% (4/64)        | 1.56% (1/64)      | India             |
| Bhatia et al. [9]    | 25.64% (10/39)   | 5.12% (2/39)        | 2.56% (1/39)      | India             |
| Martinez-Mancilla et al. [16] | 7.3% (19/261) | 14.9% (39/261) | 4.2% (11/261) | Mexico           |
| Chopra et al. [11]   | 28.3% (15/53)    | 0% (0/53)           | 0% (0/53)         | India             |
| Gupta et al. [13]    | 8.8% (22/249; 169 pediatric, 104 adult) | 5.5% (15/270) | Not done | Not done | India |
| Present study        | 31.68% (147/464) | 1.07% (5/464)       | 3.66% (17/464)    | 1.93% (9/464)     | India         |

B-ALL: B-cell acute lymphoblastic leukemia.
expression in 0%, and CD117 expression in 0% of 2 KMT2A-rearranged B-ALL cases; and CD13, CD33, and CD117 expression in none of 4 ETV6-RUNX1-rearranged B-ALL cases. Gupta et al. [13] from Kolkata, India, reported CD13 expression in 40.9%, CD33 expression in 36.9%, and co-expression of CD13/CD33 in 22 Ph-positive patients from among 273 (169 pediatric and 104 adult) B-ALL cases. CD13 was the most frequent aberrantly expressed marker (73.3%, 11/15) among the 5.5% (15/270) of patients with t(12;21) detected by fluorescence in situ hybridization.

In summary, the authors referenced above evaluated from 38 to 1120 B-ALL cases in their respective studies, reporting aberrant expression of CD13 in 5.88%-56.1%, CD33 in 2.47%-49%, and CD117 in 0.68%-22% of those B-ALL cases. As shown in Table 9, aberrant immunophenotypes in the context of multiple genetic abnormalities have been analyzed by only a few authors, including Gupta et al. [13] [22 Ph-positive patients and 15 patients with t(12;21)] and Gujral et al. [23] (10 Ph-positive, 2 KMT2A-rearranged, and 4 ETV6-RUNX1-rearranged B-ALL cases). However, the numbers of their patients with specific genetic abnormalities are very small compared to our study cohort (Table 9).

Among our adult BCP-ALL cases, the incidence of chimeric fusion transcripts was 38.36%, with aberrant myeloid-associated marker CD13 expressed in 28.08%, CD33 in 29.21%, and CD117 in 2.24% of cases (Table 4). Furthermore, we also observed that adult patients harboring BCR-ABL1 fusion transcripts and expressing aberrant myeloid markers were significantly older at presentation (p=0.0218) with male preponderance (p=0.0246). We tested the age factor (continuous variable) for significance in males and females separately, not together. In the case of male patients, we found age to be significant, but not so in female patients.

Our adult patients with t(9;22)(BCR-ABL1) fusion transcripts showed the highest incidence of aberrant expression of myeloid markers with CD13 in 31.97%, CD33 in 34.01%, and CD117 in 2.72% of cases. In pediatric BCP-ALL cases, the incidence of chimeric fusion transcripts was 20.68% with aberrant expression of CD13 observed in 26.85%, CD33 in 22.22%, and CD117 in 5.55% of cases. Pediatric patients with t(12;21)(ETV6-RUNX1) fusion transcripts showed the highest incidence of aberrant myeloid marker expression with CD13 in 39.13%, CD33 in 36.95%, and CD117 in 8.69% of cases. Among adult and pediatric BCP-ALL cases with t(12;21)(ETV6-RUNX1), t(1:19)(TCF3-PBX1), and t(4;11)(KMT2A-AF4) transcripts, the incidence of expression of CD13 and CD33 myeloid markers was not significantly different.

**Study Limitations**

Our observations of a large cohort of BCP-ALL cases have further expanded the knowledge of the complex interrelationships among genetic subtypes and immunophenotypic aberrancies. However, we studied the relationships between only four specific chimeric fusion transcripts and expressions of myeloid markers in this large cohort of BCP-ALL patients. In the future, we plan to study a wider spectrum of genetic/molecular abnormalities including BCR-ABL1-like ALL in relation to immunophenotypic aberrancies in BCP-ALL patients.

**Conclusion**

t(9;22)(BCR-ABL1) and t(12;21)(ETV6-RUNX1) were the most frequent chimeric fusion transcripts seen in our adult and pediatric B-ALL cases, respectively. Aberrant expression of CD13, CD33, and CD117 myeloid markers was significantly more frequent in adults compared to the pediatric group (p<0.0001). The e1a2 and b2a2 fusion transcripts were significantly more frequent in pediatric BCP-ALL cases, whereas b3a2

### Table 9. Comparison of studies of B-ALL cases describing both genetic abnormalities and aberrant immunophenotypes.

| Authors, year [Ref.] | t(9;22)(BCR-ABL1) | t(12;21)(ETV6-RUNX1) | t(1:19)(TCF3-PBX1) | t(4:11)(KMT2A-AF4) | Country |
|----------------------|------------------|----------------------|-------------------|-------------------|---------|
| Borkhardt et al. [10]| Not mentioned    | 18.9% (63/334 pediatric patients) | Not mentioned    | Not mentioned    | Italy   |
| Gupta et al. [13]    | 8.8% (22/249; 169 pediatric, 104 adult) | 5.5% (15/270) | Not done | Not done | India   |
| Gujral et al. [23]   | 10/1120 (717 pediatric, 396 adult) | 4 (ETV6-RUNX1) | Not done | 2 (KMT2A gene rearranged) | India   |
| Raiya et al. [29]    | 24% (32/130; 68 pediatric, 62 adult) | Not done | Not done | Not done | India   |
| Present study, adult patients | 31.68% (147/464) | 1.07% (5/464) | 3.66% (17/464) | 1.93% (9/464) | India   |
| Present study, pediatric patients | 7.08% (37/522) | 8.81% (46/522) | 4.21% (22/522) | 0.57% (3/522) | India   |
fusion transcripts were significantly more frequent in adult BCP-ALL cases, an observation not documented previously in the literature to the best of our knowledge.

Expression of CD13 and CD33 markers was significantly more frequent in adults with e1a2 and b2a2 transcripts (but not b3a2) compared to pediatric patients harboring those transcripts. Aberrant myeloid CD markers can be used to predict chimeric fusion transcripts at baseline, helping to plan appropriate tyrosine kinase inhibitor therapy for BCP-ALL patients with specific chimeric fusion transcripts. The incidence of expression of CD13 and CD33 markers was not significantly different in adult patients with t(12;21)(ETV6-RUNX1), t(1:19)(TCF3-PBX1), and t(4;11)(KMT2A-AF4) transcripts compared to pediatric BCP-ALL patients with those transcripts. Very few studies to date have described such findings in detail.

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Ethics

Ethics Committee Approval: The institutional research committee approved the study (number: INT/IEC/2017/191, dated: 23 August 2017). All experiments or tests performed involving human subjects were conducted according to institutional ethical standards and the Declaration of Helsinki.

Informed Consent: Samples of 2-3 mL of aspirated bone marrow and 3-5 mL of peripheral blood were collected in EDTA-coated vials after obtaining the signature of the patient or the patient’s guardian on an informed consent form as approved by the Human Institutional Ethics Committee of PGIMER.

Authorship Contributions

Concept: D.G.G., N.V.; Design: D.G.G., N.V.; Data Collection or Processing: D.G.G., J.B., P.B., M.G., P.S., P.R.; Supervision: P.M., A.T., A.K., S.V.; Analysis or Interpretation: D.G.G., N.V., A.K.; Literature Search: D.G.G., N.V.; Writing: D.G.G., N.V., S.N., M.U.S.S.

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