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Research paper

High frequency of intermediate and poor risk copy number abnormalities in pediatric cohort of B-ALL correlate with high MRD post induction

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ABSTRACT

Copy number abnormalities (CNAs) and recurrent fusion transcripts are important genetic events which define and prognosticate B-Cell Acute Lymphoblastic Leukemia (B-ALL). We evaluated CNAs and fusion transcripts in 67 pediatric B-ALL cases and correlated the data with standard risk factors and early treatment outcome parameters. Common fusion transcripts *ETV6-RUNX1, E2A-PBX, BCR-ABL1* and *MLL-AF4* were examined by RT-PCR and noted in 15%, 15%, 13% and 1.4% of all cases respectively. CNAs in *IKZF1, PAX5, EBF1, BTG1, RB1, CDKN2A/B* and genes from *PAR1* region *viz., CSF2RA, IL3RA,P2RY8, SHOX* region and *CRLF2* were analyzed by multiplex ligation dependent probe amplification assay and were detected in 70% (47/67) of cases, with predominantly deletions in *CDKN2A/B* (36%), *PAX5* (18%) and *IKZF1* (16%). A statistically significant association of intermediate/poor risk CNAs was noted with high WBC count ($p = 0.001$), NCI group ($p = 0.02$) and minimal residual disease at Day35 ($p < 0.0001$). *IKZF1* and *CDKN2A/B* deletion revealed poor EFS of 56% at 24 months as compared to EFS of 80% in rest of the cases ($p = 0.05$) suggesting their potential role in early risk stratification.

1. Introduction

B-cell precursor acute lymphoblastic leukemia (B-ALL) comprises 80–85% of all acute lymphoblastic leukemia and is the most common type of childhood leukemia. Age, white blood cell (WBC) count at presentation, minimal residual disease (MRD) post induction and chromosomal translocations are well known prognostic and risk stratification markers in B-ALL. Most developed countries show event free survival (EFS) from 74% to 87% and overall survival (OS) from 85% to 90% [1], while a recently published study from our country shows EFS and OS to be only 66% and 74%, respectively [2].

B-ALL development is proposed to be a two hit genetic event in which primarily recurrent translocations (12;21, 1;19, 4;11, 9;22 *etc.*) and high hyperdiploidy constitute the initiating event. The primary event is usually followed by mutation/copy number abnormalities in genes related to cell cycle, B-cell development, tumor suppressor genes and certain signalling molecules that lead to secondary genetic event, finally resulting in B-ALL. The frequency of recurrent translocations *ETV6-RUNX1, BCR-ABL1* and *MLL* rearrangements and high hyperdiploidy are seen in approximately 25%, 4%, 9% and 30% of in pediatric B-ALL cases, respectively [3]. Recently, copy number abnormalities (CNAs) are gaining importance and studies have provided evidence in supporting their role in better risk stratification along with other genetic alterations [4–7]. These multiple sub-microscopic abnormalities in genes related to B-cell development and differentiation such as *IKZF1, PAX5, EBF1, RAG1&2, FYN, PBEF1, PAG/CBP* and *BTG1* are noted in up to 40% of cases [8–10]. In addition a sub-set of B-ALL cases also harbor deletions in *PAR1* region of Xp22.3/Yp11.3 including genes *CSF2RA, IL3RA, CRLF2* and *P2RY8,* that are reported to be associated with high risk Ph like ALL [11–15].

Prospective studies regarding frequency and outcome analysis of CNAs in B-ALL are very few from developing countries despite a high burden of pediatric B-ALL cases. There is a pressing need to carry out such studies so as to better guide the personalized treatment and prognosis or risk stratification of B-ALL to improve overall survival which is still less than 75% in most of the developing countries. In the current study we analyzed common fusion transcripts and CNAs in prospectively enrolled B-ALL cases (age ≤ 12y) and correlated genetic findings with clinical parameters including MRD status and event free survival (EFS) along with other risk factors important for disease stratification.

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2. Material and methods

2.1. Patients

Newly diagnosed acute leukemia patients were enrolled prospectively for the study from August 2015 to September 2017. B-ALL and blast cell population was confirmed by standard flow-cytometry (immunophenotype of bright CD19, CD10 dual expression, and variable surface CD34 expression along with CD19 + CD10 + CD34 +/−). Patients were categorized into standard and high risk as per National Cancer Institute (NCI) risk criteria depending on age and WBC count. Additional stratification was based on the presence of translocation t (1;19), t(9;22), t(12;21), t(4;11); central nervous system (CNS) status and day-8 prednisolone response. All standard risk patients underwent three drug induction comprising vincristine, L-asparginase and prednisolone and the high risk patients were given daunorubicin in addition, as per the treatment protocol followed in our institute (adapted from UKALL 2003, version 7). An event was defined as a death during treatment, a relapse after remission, or patient leaving treatment against medical advice. Informed consent of the inpatient/guardian was taken in accordance with the Declaration of Helsinki and the study was approved by Institutional Ethics Committee. In addition, approval from departmental board review committee was taken for publication purpose.

2.2. Detection of minimal residual disease

At the end of induction (day 35) bone marrow minimal residual disease (MRD) was assessed by flow cytometry using antibody combination of CD34PE, CD20PerCP, CD19PECy7, CD10APC and CD45APCH7 along with Syto13. All antibodies used were procured from BD biosciences. A minimum of one million events were acquired in BD FACS Canto II flow cytometer and analyzed with BD FACS Diva software. MRD of ≥0.01% was considered positive further categorizing the patients as high risk (HR) while MRD (< 0.01%) were considered negative and low risk (LR).

2.3. RT-PCR

2–3 ml of peripheral blood was taken in EDTA at the time of initial diagnostic work-up and total cellular RNA was extracted using QIAmp RNA Blood Mini Kit (Qiagen, Germany). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's instructions. Common fusion transcripts of *ETV6-RUNX1, E2A-PBX, BCR-ABL1* and *MLL-AF4* were detected by multiplex RT-PCR as described previously [16].

2.4. Detection of CNAs

Genomic DNA was extracted using QIAmp DNA Blood Mini Kit (Qiagen, Germany). CNAs were analyzed using MLPA kit-P335 (MRC-Holland, The Netherlands) according to the manufacturer's instructions. Ten healthy pediatric controls were enrolled in the study, for normalization of peaks and calculation of relative copy number in MLPA run. The FAM-labeled PCR fragments were resolved by capillary electrophoresis on ABI 3130 Genetic Analyzer (Applied Biosystems), and peak intensities were analyzed using Coffalyser.net software (MRC-Holland). CNAs good risk (GR) or intermediate/poor risk (I/PR) were defined according to the report by Moorman et al. [5].

Good risk CNA profile	Intermediate/Poor risk CNA profile
• No deletions in *IKZF1, CDKN2A/B, PAR1, EBF1, BTG1, PAX5, ETV6* or *RB1*. • Isolated deletions of *ETV6, PAX5* or *BTG1*. • *ETV6* deletions with single additional deletion of *BTG1, PAX5* or *CDKN2A/B*.	• Any deletion of *IKZF1, PAR1, EBF1* or *RB1*. • All other copy number profile not mentioned above.

The scoring of copy number abnormalities was done on the basis of dosage quotient as follows:- normal copy number (0.80–1.20); heterozygous deletion (0.40–0.65); homozygous deletion (0.00–0.20); heterozygous duplication (1.3–1.65) and homozygous duplication (1.75–2.15).

2.5. Statistical analysis

Data were presented as median (range) or frequency (%). The association between categorical variables was examined by Chi-Square test. In addition univariate analysis to note association between CNAs and other ALL risk and outcome parameters was done and odds ratio calculated. A p-value ≤ 0.05 was considered as statistically significant. Survival curves were derived using Kaplan-Meier method. Statistical analysis was done using SPSS (2.0) software.

3. Results and discussion

Initially 71 cases were processed for MLPA, but 4 cases did not yield satisfactory result. 67 cases were therefore analyzed for fusion transcripts and copy number abnormalities and correlated with clinical risk factors. Out of 67 patients, 8 left treatment against medical advice (LAMA) during induction phase before the evaluation of MRD and 2 patients died due to treatment related mortality (TRM). Hence, the cohort comprising of 57 cases that included 18 female and 39 male pediatric patients was analyzed for MRD and follow-up data.

3.1. Gene copy number profile

MLPA was performed to assess the copy number abnormalities in *IKZF1, PAX5, EBF1, BTG1, JAK2, RB1, CDKN2A/B, ETV6, ZFY-4* and *PAR1* region genes *viz. SHOX, CRLF2, CSF2RA, IL3RA and P2RY8*. CNAs were detected in 70% (47) of all cases analyzed (Fig. 1) with 34% (23/67) of cases showing variation in 3 or more than 3 genes while only 13% (9/67) and 22% (15/67) of cases showed variation in 2 or 1 gene, respectively. Similar results were reported by Gupta et al. from India where CNAs were detected in 70.4% of cases in a cohort consisting of pediatric and adult B-ALL patients [6]. In our cohort of 67 pediatric cases analyzed, 36% (24/67) showed only deletion while 18% (12/67) and 16% (11/67) cases showed only duplication or both deletion and duplication, in at least one of the aforementioned genes. The most commonly deleted genes were *CDKN2A/B* (36%, n = 24), *PAX5* (18%, n = 12) and *IKZF1* (16%, n = 11). *CDKN2A/B* genes showed only deletion (homozygous in 9 cases and heterozygous in 15 cases) whereas *PAX5* and *IKZF1* also showed duplication in 3 and 2 cases respectively (Fig. 1). The most commonly duplicated genes were *IL3RA* (22%, n = 15), *P2RY8* (21%, n = 14), *SHOX1* (16%, n = 11), *CRLF2* (15%, n = 10) and *CSF2RA* (15%, n = 10). The *RB1* gene which is associated with poor prognosis showed a deletion and duplication in one case each. However the patient showing deletion in *RB1* gene (heterozygous) was also *BCR-ABL1* positive and died before completing the induction phase of treatment.

3.2. Fusion transcript frequency and CNAs

Detection of 4 fusion transcripts *ETV6-RUNX1, E2A-PBX, BCR-ABL1* and *MLL-AF4* was performed by multiplex RT-PCR in 67 pediatric cases.