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ORIGINAL PAPER

Molecular profiling of gene copy number abnormalities in key regulatory genes in high-risk B-lineage acute lymphoblastic leukemia: frequency and their association with clinicopathological findings in Indian patients

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Abstract Genes related to key cellular pathways are frequently altered in B cell ALL and are associated with poor survival especially in high-risk (HR) subgroups. We examined gene copy number abnormalities (CNA) in 101 Indian HR B cell ALL patients and their correlation with clinicopathological features by multiplex ligation-dependent probe amplification. Overall, CNA were detected in 59 (59%) cases, with 26, 10 and 23% of cases harboring 1, 2 or +3 CNA. CNA were more prevalent in *BCR-ABL1* (60%), pediatric (64%) and high WCC (WBC count) (63%) patients. Frequent genes deletions included *CDNK2A/B* (26%), *IKZF1* (25%), *PAX5* (14%), *JAK2* (7%), *BTG1* (6%), *RB1* (5%), EBF1 (4%), ETV6 (4%), while *PAR1* region genes were predominantly duplicated (20%). *EBF1* deletions selectively associated with adults, *IKZF1* deletions occurred frequently in high WCC and *BCR-ABL1* cases, while *PAR1* region gains significantly associated with *MLL-AF4* cases. *IKZF1* haploinsufficiency group was predominant, especially in adults (65%), high WCC (60%) patients and *BCR-ABL1*-negative (78%) patients. Most cases harbored multiple concurrent CNA, with *IKZF1* concomitantly occurring with *CDNK2A/B*, *PAX5* and *BTG1*, while *JAK2* occurred with *CDNK2A/B* and *PAX5*. Mutually exclusive CNA included *ETV6* and *IKZF1/RB1*, and *EBF1* and *JAK2*. Our results corroborate with global reports, aggregating molecular markers in Indian HR B-ALL cases. Integration of CNA data from rapid methods like MLPA, onto background of existing gold-standard methods detecting significant chromosomal abnormalities, provides a comprehensive genetic profile in B-ALL.

Keywords Copy number abnormalities (CNA) · High-risk B-ALL · MLPA · *IKZF1*

Introduction

B-lineage acute lymphoblastic leukemia (B-ALL) is a genetically heterogeneous disease, characterized by various recurrent (chromosomal and submicroscopic) alterations. Many of these alterations serve as diagnostic and prognostic factors routinely used in risk stratification. Patients with hypodiploidy, *MLL-AF4* and *BCR-ABL1* stratify into poor-outcome high-risk (HR) groups, while presence of hyperdiploidy and *ETV6-RUNX1* implies good prognosis risk groups [1]. Yet, either several ALL patients, including those with HR features (older age, males, high WBC count), and relapse cases remain genetically undefined or disease etiology remains poorly understood [2]. Subsequently, genomic studies in B-ALL identified recurrent copy number abnormalities (CNA) targeting key genes in B-lymphoid development pathway (*IKZF1*, *ETV6*, *PAX5, EBF1*), cell cycle control, tumor suppression (*RB1*, *CDKN2A/2B*, *BTG1*), and cytokine receptors localized to *PAR1* region (*CRLF2*, *IL3RA*, *CSF2RA*, *P2RY8, SHOX*) [3–5].

Recent studies showed diagnostic or prognostic relevance of some of these CNA, suggesting their potential use in risk stratification [6–9]. Notably, deletions of *CDKN2A/B, CRLF2* and *IKZF1* were associated with high-risk disease and poor outcome [10–12]. Moreover, some CNA represented cooperating aberrations that correlate with

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specific cytogenetic subtypes [6, 12, 13]. Like, *IKZF1* deletions are hallmarks of multiple subtypes showing poor prognosis, including *BCR-ABL1* cases [7, 8, 14]. In fact, *IKZF1* deletions serve as prognostic markers in pediatric B-ALL and as strong predictors of relapse at diagnosis, conferring a threefold increased relapse risk in *BCR-ABL1* ALL patients [15].

Compared to Western patients, the overall outcome in Indian B-ALL patients is poor, and one contributing factor includes higher frequency of poor-risk and fewer good-risk genetic subtypes seen in Indian B-ALL patients [16–18]. Testing for multiple mutations including point mutations, insertions/ deletions and translocations has become a standard of care in leukemias and in turn sheds light on the disease pathogenesis [19]. Use of MLPA, as an adjunct to cytogenetic analysis, provides a rapid, accurate, high-throughput and low-cost approach for uncovering molecular profile of multiple clinically relevant CNA in genes recurrently affected in B-lineage ALL. Therefore, the current study evaluated the frequency and type of CNA in commonly affected genes, and their association with clinicopathological features within high-risk B-ALL patients from India.

Table 1 Clinical characteristics of high-risk B-lineage ALL cohort

Demographic parameters	Total HR, *n* (%)
Total	101 (100)
Age (y), median (range)	16 (1–74)
Pediatric (<18y)	55 (54.5)
Adult (≥18 y)	46 (45.5)
Gender	
Male	57 (56)
Female	44 (44)
WCC (x 10^9/L), median (range)	39 (0.2–468)
Low WCC (<50 x 10^9L)	61 (60)
High WCC (≥50 x 10^9/L)	40 (39)
Hb level (gm/dl), median (range)	6.9 (2–14)
PLT level (×10^9/L), median (range)	40 (0.7–887)
Cytogenetic subgroups[a]	
BCR-ABL1	48 (46)
Hypodiploidy	50 (47)
MLL-AF4	7 (7)

WCC WBC count

[a] Four cases show presence of two cytogenetic abnormalities simultaneously

Materials and methods

Study subjects

The present study was performed at the R&D Division, SRL Ltd., India. High-risk (HR) B-lineage ALL cases ($n = 101$) were subjected to copy number analysis which were selected from the same study cohort which was reported earlier from our group [16]. Inclusion criteria involved cytogenetic, FISH or molecular evidence of unfavorable aberrations like hypodiploidy ($n = 50$), *BCR-ABL1* ($n = 48$) and *MLL-AF4* ($n = 7$), similar to previous HR cohorts [1, 20]. Table 1 summarizes the clinical and demographic data of cohort. Median age was 16 years (1–74) with 55 pediatric (<18 years) and 46 adult (≥18 years) patients. Median WCC (WBC count) was 39×10^9/L (0.2–468) with 61 low WCC and 40 high WCC cases. MLPA controls comprised healthy subjects with no previous or concurrent malignancy [21]. Treatment and outcome were not evaluated. Informed consent was obtained according to the Declaration of Helsinki, and study was approved by the institutional ethics committee.

Copy number abnormalities (CNA) analysis

Genomic DNA was isolated from peripheral blood or bone marrow using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and quantified using NanoDrop Spectrophotometer (Thermo Scientific) for the presence of at least 5 ng/ul DNA. DNA integrity was further checked on 0.8% Agarose gel. Gene CNA were analyzed using MLPA kit (P335) (MRC-Holland, Amsterdam, Netherlands) as per protocol. This kit includes probes for *EBF1* (5q33.3, ex 1, 10, 14, 16), *IKZF1* (7p12.2, ex 1-8), *PAX5* (9p13.2, ex 10, 8-5, 2, 1), *CDKN2A/B* (9p21.3, ex 2a, 5, 2), *JAK2* (9p24.1, ex 23), *ETV6* (12p13.2, ex 1-3, 5, 8), *BTG1/BTG1* downstream region (12q21.33, ex 1-2), *RB1* (13q14.2, ex 6, 14, 19, 24, 26) and *PAR1* region genes (*SHOX* area, *CRLF2*, ex 4, *CSF2RA*, ex 10, *IL3RA*, ex 1, *P2RY8*, ex 2) (Xp22.33/Yp11.32). Complete details are available online (www.mrc-holland.com). The FAM-labeled PCR fragments were resolved by capillary electrophoresis on ABI 3500Dx Genetic Analyzer (Applied Biosystems, Foster City, CA), and peak intensities were analyzed using Coffalyser software (MRC-Holland). Relative copy number was obtained after (intra- and inter-sample) normalizations of peaks against internal reference probes and normal control DNA, respectively. As per manufacturer protocol, copy number ratio of each gene was interpreted as follows: <0.3, as homozygous deletion; ≥0.3 to <0.7, as heterozygous deletion; ≥0.7 to ≤1.3, as normal; >1.3 to <1.7, as heterozygous gain and ≥1.7, as homozygous gain. *IKZF1* deletions were classified by their functional effects into 3 groups: "dominant negative group," including exons 4–7 deletion; "haploinsufficiency group," including whole gene deletions and deletions affecting exon 2; and "miscellaneous group," representing all other deletions, as previously reported [14].

Statistical analysis

Routine statistical analysis, z-test of proportions and Fischer's test were done using SPSS v20 (SPSS Inc., Chicago, IL), and two-sided P value <0.05 was considered significant.

Results

Distribution of copy number abnormalities (CNA) in HR cohort

There was considerable heterogeneity seen in CNA distribution and number of CNA per case (Fig. 1). Overall, 59% ($n = 59/101$) cases showed CNA (i.e., deletion/gain in at least one gene). No CNA were observed in 41% ($n = 42/101$) cases, whereas 26% ($n = 26$), 10% ($n = 10$) and 23% ($n = 23$) of cases had 1, 2 or +3 CNA. Notably, CNA occurred at significantly higher frequencies overall (59%), and in *BCR-ABL1* (60%, $n = 29/48$), pediatric (64%, $n = 35/55$) and high WCC (63%, $n = 25/40$) patients compared to those without CNA (all $P < 0.05$) (Fig. 1).

Gene CNA profile and correlation with clinicopathological features

As shown in Table 2, several gene copy number losses were more frequent than gains ($P < 0.05$). Frequent genes deletions included, *CDNK2A/B* (26%), *IKZF1* (25%), *PAX5* (14%), *JAK2* (7%), *BTG1* (6%), *RB1* (5%), *ETV6* (4%) and *EBF1* (4%), while *PAR1* region genes were chiefly duplicated (20 vs. 1%, $P < 0.05$) (Table 2).

Table 3 depicts correlation of gene CNA with clinicopathological features. *EBF1* deletions exclusively associated with adults (9 vs. 0%, $P = 0.02$) while *ETV6* and *PAR1* deletions were absent in adults. Deletions in *BTG1* (4 to 9%), *RB1* (4 to 7%) and *IKZF1* (20% to 30%) showed an increasing trend with age. No gender-related differences were seen. High WCC patients showed more deletions than low WCC cases, notably higher *IKZF1* deletions (38 vs. 16%, $P = 0.01$). *BCR-ABL1* cases showed more deletions than other subgroups, notably *IKZF1* deletions (33 vs. 6%, $P = 0.057$). Interestingly, *PAR1* region gains significantly associated with *MLL-AF4* cases (71 vs. 16%, $P = 0.0003$), despite lower CNA levels seen in this group. Gains in other genes were too few for statistical analysis.

IKZF1 deletion subgroup profile

As seen in Table 4, *IKZF1* deletions ($n = 25$ cases) were classified into three subgroups and correlated with clinical features. Among these, haploinsufficiency was most frequent (60%, $n = 15$, $P < 0.001$), involving deletions like Δ1–8 ($n = 9$) and Δ1–2, Δ2–3, Δ2–7, Δ2–8, Δ2–3, 5–8 and Δ1–3, 5–7 ($n = 1$ each). Dominant negative deletion Δ4–7

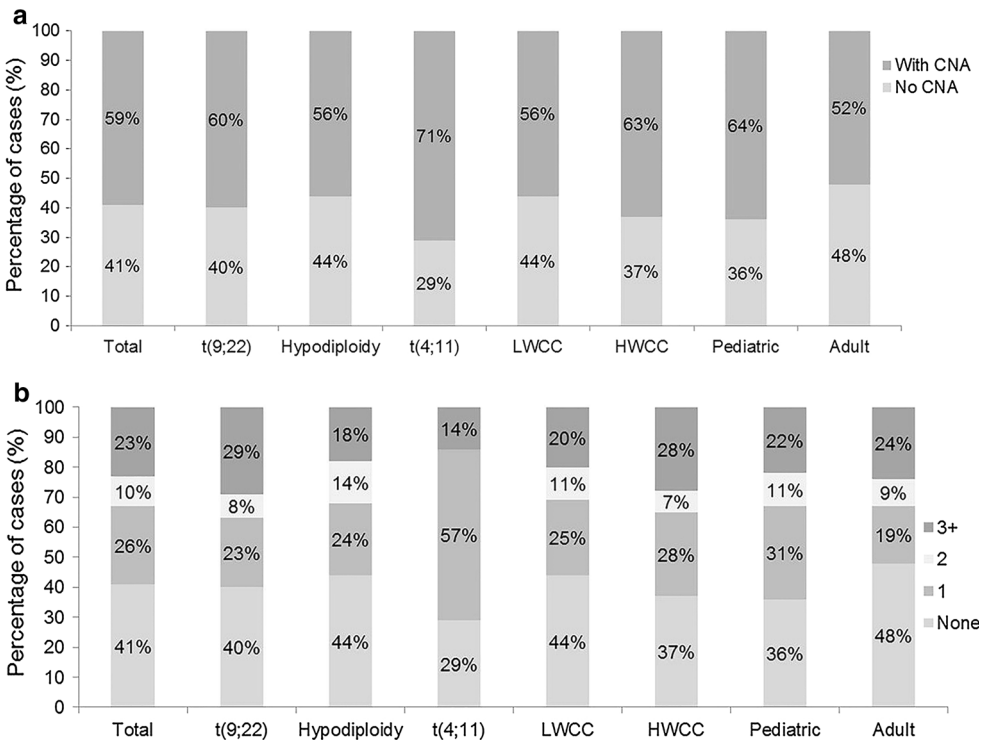

Fig. 1 Distribution of copy number abnormalities (CNA) in HR cohort: **a** percentage of patients with and without CNA; **b** percentage of patients with increasing numbers of CNA

Table 2 Frequency of CNA in HR B-lineage ALL cohort

Genes	Deletion, *n* (%)	Deleted exons (e)/genes, (*n*)	Gain, *n* (%)	Gained exons (e)/genes, (*n*)	Total CNA, *n* (%)
CDKN2A/B[a]	26 (26)*	AB (23); A (3)	2 (2)	AB (1); A (1)	28 (28%)
IKZF1	25 (25)*	e4–7 (7); e1–8 (9); e1–2; e2–3; e2–7; e2–8; e2–3,5–8; e1–3,5–7; e3–7; e4,7; e1,4–7 (1 each)	0 (0)	–	25 (25%)
PAX5	14 (14)	e1–10 (5); e7–10 (2); e1–5,8; e1–6,8; e2–5; e2–7; e5–10; e7–8; e1 (1 each)	7 (7)	e1–10 (1); e1–6,10; e7–10; e6–8; e1–5; e2–5; e1 (1 each)	21 (21%)
JAK2	7 (7)*	e23 (7)	1 (1)	e23 (1)	8 (8%)
BTG1[b]	6 (6)	e2,A2 (3); A1; e1,A1–2; A2 (1 each)	5 (5)	e1; e1–2,A1–2 (2 each); e2,A1–2 (1)	11 (11%)
RB1	5 (5)*	e19–26 (2); e6–14; e6,19–26; e24–26 (1 each)	0 (0)	–	5 (5%)
EBF1	4 (4)	e1–16 (2); e1; e14–16 (1 each)	4 (4)	e1–16 (2); e1 (2)	8 (8%)
ETV6	4 (4)	e1–8 (2); e1–2; e3–8 (1 each)	3 (3)	e1–8 (2); e1–3,8 (1)	7 (7%)
PAR1	1 (1)	*IL3RA*	20 (20)*	All genes (16); *P2RY8* (3); *CRLF2;IL3RA;P2RY8* (1)	21 (21%)

* Deletions versus gains $P < 0.05$, significant difference

[a] A—only *CDKN2A,* AB—both *CDKN2A* and *B*

[b] A1 and A2 refer to the centromeric area probes of *BTG1* (178 and 409 nt, respectively, in kit)

Table 3 Gene CNA Profile in association with clinicopathological features

Category	Total, *N*	*EBF1* deletion		*IKZF1* deletion		*CDKN2A/B* deletion		*PAX5* deletion		*ETV6* deletion		*BTG1* deletion		*RB1* deletion		*JAK2* deletion		*PAR1* gain	
Total *n* (%)	101	4	4%	25	25%	26	26%	14	14%	4	4%	6	6%	5	5%	7	7%	20	20%
Age																			
Pediatric	55	0	–	11	20%	16	29%	8	15%	4	7%	2	4%	2	4%	5	9%	13	24%
Adult	46	4	9%*	14	30%	10	22%	6	13%	0	–	4	9%	3	7%	2	4%	7	15%
Gender																			
M:F	57:44	1:3		13:12		15:11		8:6		2:2		1:5		1:4		4:3		13:7	
(Ratio)	(1.3)	(0.3)		(1.08)		(1.3)		(1.3)		(1)		(0.2)		(0.25)		(1.3)		(1.8)	
WCC																			
Low WCC	61	3	5%	10	16%	13	21%	6	10%	3	5%	3	5%	3	5%	4	7%	11	18%
High WCC	40	1	3%	15	38%*	13	33%	8	20%	1	3%	3	8%	2	5%	3	8%	9	23%
BCR-ABL1																			
Present	48	3	6%	16	33%	14	29%	10	21%	2	4%	5	10%	3	6%	4	8%	9	19%
Absent	53	1	2%	9	6%	12	23%	4	8%	2	4%	1	2%	2	4%	3	6%	11	21%
MLL-AF4																			
Present	7	0	–	1	14%	1	14%	1	14%	0	–	1	14%	0	–	0	–	5	71%*
Absent	94	4	4%	24	26%	25	27%	13	14%	4	4%	5	5%	5	5%	7	7%	15	16%
Hypodiploidy																			
Present	50	2	4%	10	20%	12	24%	4	8%	2	4%	1	2%	2	4%	3	6%	8	16%
Absent	51	2	4%	15	29%	14	27%	10	20%	2	4%	5	10%	3	6%	4	8%	12	24%

* $P < 0.05$, significant difference

(28%, $n = 7$) and other miscellaneous deletions (12%, $n = 3$: Δ3–7, Δ4, 7 and Δ1, 4–7) occurred at lower frequencies.

Further, *IKZF1* haploinsufficiency was significantly higher in *BCR-ABL1*-negative cases (78 vs. 22%), adult cases (65 vs. 35%), high WCC cases (60 vs. 40%), males

Table 4 Correlation of IKZF1 deletion subgroups with clinicopathological features

IKZF1 deletion groups	Age group		WCC		Gender		*BCR-ABL1*		Hypodiploidy		*MLL-AF4*	
	Pediatric $n = 11$	Adult $n = 14$	HWCC $n = 15$	LWCC $n = 10$	Males $n = 13$	Females $n = 12$	Positive $n = 16$	Negative $n = 9$	Positive $n = 11$	Negative $n = 14$	Positive $n = 1$	Negative $n = 24$
Haploinsufficiency	6	9	9	6	8	7	8	7	7	8	0	15
($n = 15$, 60%)*	(55%)	(65%)*	(60%)*	(60%)	(61%)*	(58%)	(50%)	(78%)*	(64%)*	(57%)	–	(63%)
Dominant negative	4	3	4	3	4	3	5	2	3	4	0	7
($n = 7$, 28%)	(36%)	(21%)	(27%)	(30%)	(31%)	(25%)	(31%)	(22%)	(27%)	(29%)	–	(29%)
Miscellaneous	1	2	2	1	1	2	3	0	1	2	1	2
($n = 3$, 12%)	(9%)	(14%)	(13%)	(10%)	(8%)	(17%)	(19%)	–	(9%)	(14%)	(100%)	(8%)

* $P < 0.05$, significant difference

(61 vs. 8%) and hypodiploid patients (64 vs. 9%) (all $P < 0.03$, Table 4).

Gene aberrations types, extent and combinations

As shown in Table 5, ample heterogeneity was seen in the type and extent of genetic change. All losses in *EBF1, PAX5, ETV6* and *JAK2* were heterozygous, while *CDKN2A/B* losses (46%) were mostly homozygous. Among *IKZF1* deletions, 88% were heterozygous, 4% homozygous and rest 8% were mixed ($P < 0.05$). Likewise, *BTG1* (67%) and *RB1* (60%) deletions were chiefly heterozygous. All gains in *JAK2, CDKN2A/B, ETV6* and *BTG1* were heterozygous. Likewise, *PAR1* (85%), *EBF1* (75%) and *PAX5* (71%) gains were chiefly heterozygous (Table 5).

Most cases harbored multiple concurrent CNA, and a total of 59 unique CNA profiles were observed (Table 5). Among these, profiles with single gene aberration (i.e., CNA=1, $n = 26$) involved: *PAR1* ($n = 9$), *IKZF1* ($n = 7$), *CDNK2A/B* ($n = 5$), *RB1* ($n = 2$) and *EBF1, PAX5, ETV6* ($n = 1$ each). Further, simultaneous aberrations in different genes were frequent (i.e., CNA>1, $n = 33$) and involved: *IKZF1, CDKN2A/B, PAX5* ($n = 4$); *IKZF1, CDKN2A/B, PAX5, BTG1* ($n = 2$); *IKZF1, EBF1* ($n = 2$); *IKZF1, PAR1* ($n = 2$); *CDKN2A/B, JAK2, PAX5* ($n = 2$); *CDKN2A/B, ETV6, BTG1* and *PAR1* ($n = 2$), while other rare combinations were seen in single cases ($n = 19$). None of the cases with CNA in *ETV6* showed concomitant CNA in *IKZF1* or *RB1*, while cases with CNA in *EBF1* lacked concomitant CNA in *JAK2*, indicating that these CNA were mutually exclusive.

Discussion

In the present study, several recurrent CNA were identified in genes related to leukemia and key cellular pathways by MLPA-based CNA analysis, consistent with previous studies [6, 12]. Even with a limited panel of tested genes, we detected significantly high levels of CNA (59–64%) in overall, pediatric, *BCR-ABL1* and high WCC patients. These findings suggest aggressive disease biology and corroborate with 57–66% of CNA previously reported in pediatric, *BCR-ABL1* and HR B-ALL patients [6, 11, 12, 14]. Our cohort presented fewer cases without CNA (41 vs. 65%) compared to the UKALLXII/ECOG2993 trial [22] and more cases with ≥3 CNA (23 vs. 9–10%) than the ALL97/99 and UKALL2003 groups [6]. Further, a single gene CNA was noted in 26 (26%) cases, with the other 33 (33%) cases showing CNA in >1 gene, supporting the fact that B-ALL is a multistep process with several cooperating lesions.

Table 5 CNA—types and concomitance observed

CNA types	Total cases, *n*	*EBF1*	*IKZF1*	*CDKN2A/B*	*PAX5*	*ETV6*	*BTG1*	*RB1*	*PAR1*	*JAK2*
Total CNA, *n*	101	8	25	28	21	7	11	5	21	8
Deletion, *n* (%)		4 (4)	25 (25)	26 (26)	14 (14)	4 (4)	6 (6)	5 (5)	1 (1)	7 (7)
Deletion types[a]	HeD	4 (100)	22 (88)	7 (27)	14 (100)	4 (100)	4 (67)	3 (60)	1 (100)	7 (100)
	HoD	–	1 (4)	12 (46)	–	–	2 (33)	2 (40)	–	–
	HeD+ HoD	–	2 (8)	7 (27)	–	–	–	–	–	–
Gain, *n* (%)		4 (4)	0 (0)	2 (2)	7 (7)	3 (3)	5 (5)	0 (0)	20 (20)	1 (1)
Gain types[a]	HeG	3 (75)	–	2 (100)	5 (71)	3 (100)	5 (100)	–	17 (85)	1 (100)
	HoG	1 (25)	–	–	2 (29)	–	–	–	3 (15)	–
CNA combinations observed[b]										
	42	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	1	0
	7	0	1	0	0	0	0	0	0	0
	5	0	0	1	0	0	0	0	0	0
	2	0	0	0	0	0	0	1	0	0
	1	1	0	0	0	0	0	0	0	0
	1	0	0	0	1	0	0	0	0	0
	1	0	0	0	0	1	0	0	0	0
	4	0	1	1	1	0	0	0	0	0
	2	0	1	1	1	0	1	0	0	0
	2	1	1	0	0	0	0	0	0	0
	2	0	1	0	0	0	0	0	1	0
	2	0	0	1	1	0	0	0	0	1
	2	0	0	1	0	1	1	0	1	0
	1	1	0	1	1	0	0	0	0	0
	1	1	0	1	0	0	0	0	1	0
	1	1	0	0	1	0	0	0	1	0
	1	1	0	0	0	1	1	0	1	0
	1	1	0	0	0	0	1	1	0	0
	1	0	1	1	1	0	0	1	1	1
	1	0	1	1	1	0	1	0	0	1
	1	0	1	1	1	0	0	0	0	1
	1	0	1	1	1	0	1	0	1	0
	1	0	1	1	1	0	0	1	0	0
	1	0	1	1	0	0	1	0	0	0
	1	0	1	1	0	0	0	0	0	0
	1	0	1	0	1	0	1	0	0	0
	1	0	0	1	1	1	0	0	0	1
	1	0	0	1	0	1	0	0	0	1
	1	0	0	1	1	0	0	0	1	1
	1	0	0	1	1	0	0	0	1	0
	1	0	0	0	1	0	1	0	0	0
	1	0	0	0	0	1	0	0	1	0

[a] Percentage calculated from total deletion/gain observed in each gene

[b] *0* normal, *1* CNA

Ample heterogeneity was observed in the frequency, extent and type of genetic change. Similar to previous CNA studies, losses were more frequent than gains in lymphoid development (*IKZF1, PAX5, EBF1, ETV6*), cell cycle control and tumor suppression genes (*CDNK2A/B, BTG1, RB1*), but not in cytokine receptor genes (*PAR1*

region) [13, 23]. Strikingly, many genes regulating B-lymphoid development are often affected in HR B-ALL and a greater number of lesions associate to poor outcome, suggesting that degree of "block" in this pathway contributes to both leukaemogenesis and treatment responsiveness [24].

In relation to individual abnormalities, *CDKN2A/B* deletions (26%) were most frequent. *CDKN2A/B* losses, reported earlier in 22–39% B-ALL cases [6, 25], are linked to poor prognosis in adult, *BCR-ABL1*-positive ALL, and rapid relapse in pediatric ALL [5, 10, 26]. *CDKN2A/B* code for tumor suppressors and cell cycle regulator proteins p16, p14 and p15, that inhibit cyclin-dependent kinases [26]. Similar to earlier reports, chiefly both A and B domains were deleted, probably leading to uncontrolled cell cycle progression [27]. Besides, cases with both mono- and biallelic deletions were noted, similar to prior reports [23, 28]. Further as noted earlier, *CDKN2A/B* deletions (9p21.3) co-occurred with abnormalities of 9p (like translocations and deletions) and loss of adjacent *JAK2* (9p24.1) and *PAX5* (9p13.2), suggesting they are common targets in B-ALL pathogenesis [10, 12, 29].

PAX5 showed 14% losses and fewer gains (7%), consistent with 10–25% *PAX5* losses noted earlier in B-ALL [12, 13, 29]. Alternatively, HR-ALL studies using quantitative PCR or DNA chips report more *PAX5* alterations (>30%), probably owing to differences between methods [11, 30–32]. *PAX5* regulates B-lineage commitment by activating B-lineage-specific genes and repressing other-lineage genes; thus, its loss leads to B cell development blockage [30]. In pediatric ALL, CNA in *PAX5* and *IKZF1* alter expression levels and patterns [4, 11]. In adult B-ALL groups (GRAALL and GIMEMA), frequent *PAX5* alterations bared no correlation with outcome [30, 32].

IKZF1 deletions (25%) varied largely in size and were frequent in our high WCC (38%) and *BCR-ABL1* (33%) cases, suggesting a more aggressive disease. Earlier, 14–29% *IKZF1* deletions were reported in pediatric, adult and HR B-ALL patients [6, 11, 12, 22, 33]. Further, prior correlations of *IKZF1* losses with older age, high WCC, *BCR-ABL1* and higher induction failure suggest resistance to standard therapy and need for intensive/alternate therapies [27, 33]. Globally, the independent and adverse impact of *IKZF1* deletions in B-ALL is well documented [7, 9, 11, 14, 34]. They are preserved from diagnosis to relapse and maybe used to identify ALL-relapse and HR cases [31, 35]. *IKZF1* deletions probably block B cell differentiation and activate stem cell gene expression program. Further, biallelic *IKZF1* deletions, reported in 15–20% B-ALL, possibly provide survival advantage to leukemic cells [35, 36].

Classifying *IKZF1* deletions by functional effects is vital in understanding their prognostic relevance. IKZF1 haploinsufficiency (60%) was more predominant than other groups, and significantly higher in our adults (65%), high WCC cases (60%) and *BCR-ABL1*-negative cases (78%). Likewise, *IKZF1* haploinsufficiency was chiefly noted in 57–72% *BCR-ABL1*-negative, HR and adult B-ALL cases [11, 27, 36]. Haploinsufficiency deletions involving all exons or exon 2 result in reduced levels of ikaros protein, as loss of exon 2, harboring the translational start site, prevents *IKZF1* translation, while exon 8 loss affects *IKZF1* dimerization [36]. Dominant negative deletions involving exon 4–7 cause expression of dominant negative isoform Ik6, that lacks the N-terminal DNA binding zinc finger and shows oncogenic activity [35]. Recently, all *IKZF1* deletion groups showed worse or equally poor prognosis in pediatric B-ALL [37].

The anti-proliferative gene, *BTG1*, was deleted in 6% cases and duplicated in 5% cases. Earlier, *BTG1* deletions were noted in 6–10% B-ALL, often involving exon 2 and generating highly instable truncated BTG1 protein [11, 12, 38]. Further, *BTG1* deletions are rare, but commonly occur with *ETV6-RUNX1* and *BCR-ABL1* cases [12, 38, 39], and show no correlation with B-ALL outcome [23].

The cell cycle regulator, *RB1,* only showed deletions (5%). Likewise, *RB1* deletions were earlier reported in 6–11.3% B-ALL [11, 12], with a possible association with poor outcome noted in pediatric B-ALL [40].

EBF1, an essential transcription factor in B-lineage commitment, showed 4% heterozygous deletions only in adults, suggesting its lower incidence in pediatric cases. Likewise, *IKZF1* and *EBF1* deletions occur more frequently in NCI-HR cases that have higher age and WCC [12, 13]. Earlier, *EBF1* deletions were noted in 2–7.7% B-ALL and associated with poor outcome [11, 12, 23]. Also, *EBF1* deletions were more frequent in relapsed B-ALL than at diagnosis, indicating their possible role in disease recurrence [5].

The transcription factor *ETV6*, commonly involved in translocations of B-ALL and other leukemias, showed fewer gains (3%) and 4% heterozygous losses restricted to pediatric cases, suggesting their lower frequency in HR B-ALL cases. In agreement, *ETV6* deletions, seen earlier in 8.2–22% of B-ALL, are frequent in good prognostic *ETV6-RUNX1* and NCI-SR cases and show no impact on B-ALL outcome [6, 11–13].

Notably, gains in *PAR1* region (at Xp22.33/Yp11.32) were significantly higher (20% overall and 71% in *MLL-AF4* cases) than 7.4% *PAR1* gains reported previously [27]. In contrast, prior HR-ALL studies mostly report recurrent *PAR1* deletions or poor prognostic *CRLF2* rearrangements, particularly with concomitant *IKZF1* and *JAK2* alterations [8, 23, 41]. *PAR1* deletions (resulting in *P2RY8-CRLF2* fusion) possibly cause CRLF2 over-expression, which

activates JAK-STAT pathway and unchecked B cell proliferation [8, 23].

Most cases harbored multiple concurrent CNA, in addition to gross chromosomal alterations, as reported earlier [4, 27]. *IKZF1* deletions frequently harbored CNA in *CDNK2A/B, PAX5, BTG1*, while *JAK2* alterations co-occurred with CNA in *CDNK2A/B* and *PAX5*, suggesting these recurrent combinations as partially overlapping CNA in HR B-ALL. In contrast, CNA in *ETV6* and *IKZF1/RB1*, and *EBF1* and *JAK2* were mutually exclusive.

As previously noted, the frequency and type of CNA strongly varied with B-ALL cytogenetic subgroups [12, 13]. Notably, *BCR-ABL1* group harbored more distinct CNA, especially *IKZF1*, *PAX5* and *CDKN2A/B* deletions, consistent with prior studies [4, 12, 31]. In contrast, *MLL-AF4* cases had fewer CNA, corroborating with earlier reports [4, 22, 42]. This finding may be expected due to potency of this chromosomal abnormality to induce leukemia, requiring few cooperating genetic alterations [23, 24]. Among our hypodiploid cases with CNA (56%), 4% were near-haploid (NH, 24–29 chromosomes), 20% were low-hypodiploid (LH, 30–39 chromosomes) and 32% were medium-hypodiploid (MH, 40–45 chromosomes) cases. Agreeing with previous studies, reduced CNA found in hypodiploid group may likely be an artifact of analyzing CNA in context of ploidy changes, probably as neither the genes nor probes tested were located on the affected chromosomes [25]. Besides, prior reports suggest this group is genetically distinct, selectively harboring more *RAS*-activating and IKZF2- and *IKZF3*-inactivating mutations [43].

Summarily, HR B-ALL cases harbor multiple distinct CNA. Our results corroborate previous reports and emphasize screening of submicroscopic alterations as additional markers for risk stratification, especially in HR patients. Nonetheless, differences in frequency of abnormalities may be attributed to smaller size and shorter duration of our study compared to larger studies evaluating data over greater years.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the SRL-Ethics Committee and was in accordance with the Declaration of Helsinki. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all subjects as well as parents/guardians of minor subjects included in the study.

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