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Clinical and Prognostic Impact of Copy Number Alterations and Associated Risk Profiles in a Cohort of Pediatric B-cell Precursor Acute Lymphoblastic Leukemia Cases Treated Under ICiCLe Protocol

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ABSTRACT

Copy number alteration (CNA) status and CNA risk profiles of *IKZF1*^{plus}, UK-ALL CNA risk groups and MRplus scores, were evaluated for clinical and prognostic impact in a cohort of 493 B-cell acute lymphoblastic leukemia cases diagnosed and treated under the Indian Collaborative Childhood Leukemia group (ICiCLe) protocol trial. Overall CNA frequency was 59% with 60% of cases showing 2-loci deletion. *CDKN2A/B* deletion was most common CNA (36.3%), while *IKZF1* deletion and *IKZF1*^{plus} profile were noted in 19.5% and 13.4% of cases, respectively. *IKZF1* deletions and other CNA risk profiles were significantly associated with poor (PR)/high risk (HR) clinical and genetic profile parameters ($P < 0.001$). In addition, the 3-year OS, event-free survival (EFS) was significantly poor with high relapse rate (RR) of 38.6%, 46.5%, and 35.2% for *IKZF1* deletions, *IKZF1*^{plus} profiles, and UK-ALL CNA-intermediate risk (IR)+PR risk groups, respectively ($P < 0.001$). Integrated evaluation of UK-ALL CNA risk profile with ICiCLe trial risk stratification groups revealed a worse overall survival, EFS, and RR of 63.3%, 43.2%, and 35.2% for CNA-IR+PR profile compared to CNA-good risk profile (81.3%, 65.0%, and 21.0%; $P < 0.001$). Hence, routine CNA testing in our setting is must to identify standard risk and IR cases likely to benefit from HR treatment.

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The current study has been approved by the institute ethics committee vide No. PGI/IE/ 2017/87 dated March 2, 2017, and by Departmental review board vide No. DRB-47-22 dated August 06, 2022.
The sampling and testing for CNA had been performed with proper informed and written consent of patients and or their legal guardians.
Most of the study-related data have been adequately provided in the supplementary data section. Raw data are available with corresponding author/s and available on request provided it is stated clearly that same will be utilized for reproducibility or noncommercial purpose only.
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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. With the present multiagent chemotherapy protocols, a cure rate of 85% to 90% has been achieved in developed countries due to the combination of protocol refinement, better risk stratification, and availability of enhanced supportive care.[1] Though survival rates have improved vastly in lower middle income countries (LMICs) and other developing nations including India, treatment-related mortalities (TRMs; 11%–25%) and relapse rates (RRs; 15%–41%) have remained high.[2–5]

Approximately 60% of pediatric ALL cases have been shown to harbor copy number alterations (CNAs) in at least one of the important loci related with cell differentiation, cell cycle control, and apoptosis-related genes that drive leukemogenesis and contribute to relapse.[6] These genetic abnormalities have an influence on the treatment outcome and have been incorporated into integrated risk scoring systems widely in European trials based upon CNA categorization into good risk (GR) and intermediate/poor risk (IR/PR) groups[7] (Table 1). In a recent study by Stanulla et al,[8] *IKZF1*^{plus} profile has been defined and shown to be associated with worse minimal residual disease (MRD), poor prednisolone response (PPR), and high cumulative incidence of relapse (Table 1). Studies from our group have also shown variable risk outcomes of CNAs including the role of MRplus scoring in Ph-negative pediatric B-cell ALL (B-ALL) to better stratify treatment outcomes.[9–12]

In the ICiCLe multicentric collaborative treatment trial (TRI/2015/12/006,434; 2015-2022) involving major oncology

Table 1

Various Genetic Risk Profile and Proposed Risk Definitions Used in the Study Trial

	IKZF1^{Plus} Profile[a]		
	UK-ALL CNA Risk Profile Definitionb7 MRplus Scoring System1[1]		
Score	UK-ALL CNA Risk Profile (n; %)	IKZF1Plus Profile	Final MRplus Group
0	CNA-GR (Score M0) (259; 52.5%)	IKZF1^{Plus}0 (Absent)	MRplus0
1	CNA-IR+PR (Score M1) (168; 34.1%)	IKZF1^{Plus}0 (Absent)	MRplus1
2	CNA-IR+PR (Score M1) (66; 13.4%)	IKZF1^{Plus}1 (Present)	MRplus2
	Proposed final integrated risk stratification involving ICiCLe risk groups and CNA risk profile		
Group	ICiCLe Risk Group (n; %)	UK-ALLCNA Risk Profile (n; %)	Final Risk Category
Group 1	SR (119; 24.1%)	GR (85; 71.4%)	ICiCLe-SR + CNA-GR
Group 2		PR/IR (34; 28.6%)	ICiCLe-SR + CNA-IR+PR
Group 3	IR (159; 32.3%)	GR (83; 52.2%)	ICiCLe-IR + CNA-GR (83; 16.8%)
Group 4		PR/IR (76; 47.8%)	ICiCLe-IR + CNA-IR+PR (76; 15.4%)
Group 5	HR (215; 43.6%)	GR (91; 42.3%)	ICiCLe-HR + CNA-GR (91; 18.4%)
Group 6		PR/IR (124; 57.7%)	ICiCLe-HR + CNA-IR+PR (124; 25.2%)

[a]*IKZF1*^{Plus}profile defined as presence of *IKZF1* deletion co-existing with at least one additional deletion in *CDKN2A*, *CDKN2B* (only homozygous deletion), *PAX5*, or pseudoautosomal region genes (PAR1), in the absence of *ERG* deletion.

[b]UK-ALL CNA risk profile defined as CNA-GR as the absence of any deletion of *IKZF1/CDKN2A/B/PAX5/ETV6/BTG1/EBF1/RB1/*PAR1 or isolated deletions of *PAX5/ETV6/BTG1* or *ETV6* deletions with single additional deletion of *PAX5/CDKN2A/B/BTG1* and CNA-IR+PR as any deletion of *IKZF1*, PAR1, *RB1,* or *EBF1* or all other CNA combinations not included above.

CNA = copy number alteration; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; PR = poor risk; SR = standard risk.

centers in our country, integrated MRD and primary genetic risk stratification of cases is routinely performed.[13] The focus in future trials is shifting toward the development of better risk stratification and prediction scores. With this as aim, we sought to comprehensively evaluate CNA data in a subset of cases, to study their clinical and prognostic impact so as to incorporate relevant testing and risk stratification strategy in the next phase of treatment trial.

MATERIALS AND METHODS

Cohort enrollment

Patients aged 1 to 18 years from 2 major Indian medical institutes AIIMS, New Delhi (center 1) and PGIMER, Chandigarh (center 2) with B-ALL were treated and followed up as per the ICiCLe treatment protocol (Clinical Trials Registry-India number, CTRI/2015/12/006,434)[14] (Suppl. Figure S1). Since the trial has ended new patient recruitment as of April 2022, a retrospective analysis of CNA data and associated risk profiles was planned in a subset of cases (wherever data available in research settings), to evaluate its clinical and prognostic impact for future incorporation into phase 2 of treatment trial. A total of 493 cases (Figure 1, CONSORT flow chart) from the cohort had complete CNA data available for evaluation. Risk stratification was performed upfront based on NCI criteria, prednisolone response at day 8 (PPR if absolute blast count in peripheral blood >1×10^9/L) and primary genetic event. Patients were evaluated for bone marrow remission and flow-based end induction (day 28/day 35) MRD. The final risk stratification at end of induction was based on bone marrow remission status and MRD as ICiCLe standard risk (SR); ICiCLe-IR; and ICiCLe high –risk (HR) (Figure 1 and Suppl. Figure S1).

Diagnostic genetic testing and retrospective screening for CNAs

Diagnostic (2–3 mL) peripheral blood (>60% blasts) and or bone marrow (0.5 mL) EDTA samples from patients were evaluated for CNA by Multiplex Ligation Dependent Probe Amplification (MLPA) assay using probe-sets of P335-ALL-*IKZF1* and P-202 *IKZF1-ERG*. MLPA was performed as per standard protocol standardized and published earlier.[9–11,13] The deletions in the loci of following 9 genes were scored as deleted or nondeleted: *CDKN2A/B*, *IKZF1*, *RB1*, *EBF1*, *ERG*, *PAX5*, PAR1, *BTG1*, and *ETV6*. In all cases, MLPA data were normalized with control samples to calculate the relative copy number. Dosage quotient (DQ) values between 0.75 and 1.3 were considered normal copy number of 2, while any value above or below this threshold was scored as gain or loss and values below 0.25 were considered as biallelic loss (copy number 0). For *CDKN2A/B,* deletion of either locus was considered as deleted. For *PAX5* deletions, intragenic amplifications were scored along with deletions as both are functionally similar.[15]

The cohort enrollment at center 1 was consecutive but enrollment from center 2 was biased since cases with recurrent cytogenetic abnormalities and aneuploidies were excluded for MLPA analysis in initial 1 year of enrollment. Initially, a total of 535 cases were shortlisted, of which 493 patients, that received treatment were further analyzed (Figure 1, CONSORT flow chart). Cases enrolled at center 1 were analyzed by conventional cytogenetics, multiplex RT-PCR, and/or fluorescent in situ hybridization (FISH) for recurrent genetic translocations and aneuploidies including *BCR::ABL1*, *KMT2A* rearrangements, *TCF3::PBX1* and *ETV6::RUNX1*. At center 2, cases that were enrolled after 2018 had RT-PCR and FISH data with additional centromere probes. The primary genetic data evaluation was done as per following groups: good risk (GR) cytogenetics group (*ETV6::RUNX1* and high hyperdiploidy), IR cytogenetics group (cases with either negative RT-PCR/FISH/Ploidy results or other genetic abnormality like *TCF3::PBX1* and *P2RY8::CRLF2* fusion), and high rish (HR) cytogenetics group (*KMT2A-r*, *BCR::ABL1*, hypodiploidy (<45 chromosomes), t(17;19)(q22;p13) and *iAMP21*).

In addition, center 2 also tested a limited number of B-other samples (n = 32) on targeted RNA-NGS Ion Ampliseq panel on Ion Torrent S5 (110 translocations related with B-ALL; mean coverage 500×) and in 7 of the cases noted HR cytogenetic abnormality (*MEF2D::BCL9*, n = 1; *ABL1* kinase fusions, n = 2; *KMT2A-r*, n = 2; *BCR::ABL1*, n = 2). However, since results were available postinduction, the final treatment-based risk stratification continued as per initial categorization.

CNA risk-score definitions

The various CNA risk scoring systems being evaluated in the study trial have been defined in Table 1 and include the *IKZF1*^{plus} profile, UK-ALL CNA risk groups of CNA-GR and CNA-IR+PR,[7] and the MRplus scoring that integrates CNA risk group scores (M0 and M1) along with *IKZF1*^{plus} (1: present; 0: absent) profile[11] to derive 3 categories of MRplus0, MRplus1, and MRplus2. In addition, we evaluated a combined Final risk stratification system integrating CNA risk group scoring with ICiCLe treatment trial risk stratification groups (Table 1).

OUTCOME ASSESSMENT AND STATISTICAL ANALYSIS

Treatment outcome parameters analyzed included event-free survival (EFS)—defined as time from the start of therapy to an event which included relapse or death or refractory disease with

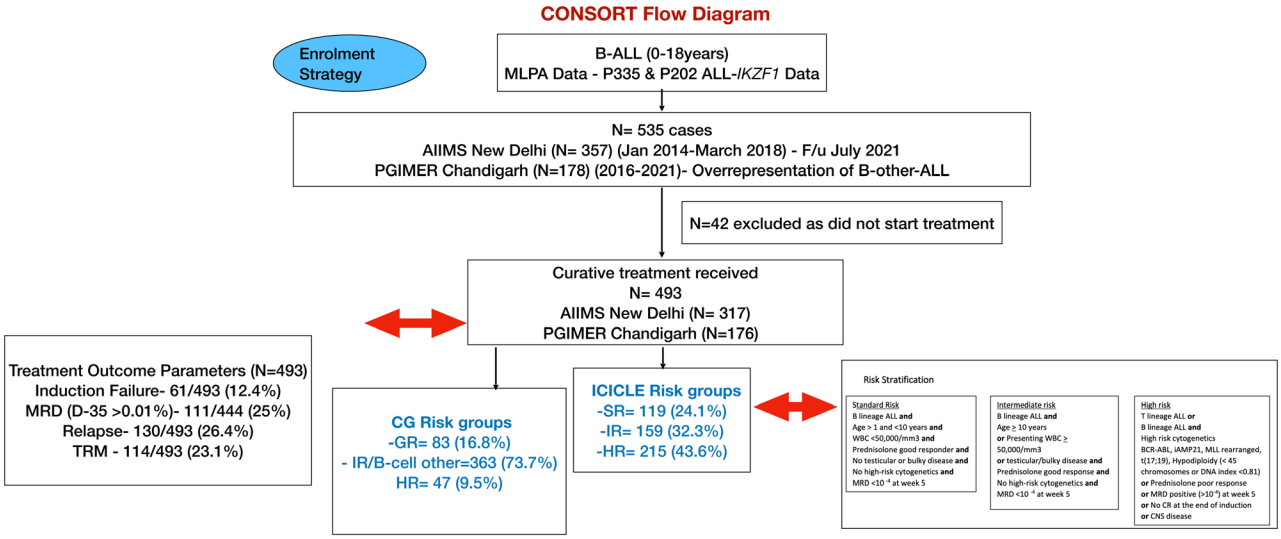

Figure 1. CONSORT flow diagram to highlight case enrollment and treatment trial risk stratification details. B-ALL= B-cell acute lymphoblastic leukemia; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; MRD = minimal residual disease; SR = standard risk; TRM = treatment-related mortality.

censoring at last contact. Relapse-free survival (RFS) or relapse rate (RR) is defined as time period from the onset of therapy to disease relapse for those achieving complete remission (CR) with censoring at death in remission or last contact. Overall survival (OS) is defined as time period from the onset of therapy to death with censoring at the last contact. In addition, TRM was defined as death due to nonrelapse-related causes. Induction failure was defined as per postinduction bone marrow criteria of more than 5% blasts. Cases continuing to be in nonremission status post reinduction therapy were labeled as having refractory disease or treatment failure. A very early relapse was defined as relapse before 18 months post CR, early relapse as between 18 and 36 months and late relapse as relapse occurring after 36 months post CR.

Continuous variables are represented as mean/median (range) and categorical variables as ratio/proportion for whole cohort as well as patient subcategories. The Chi-square test is performed between different clinical, hematological, and treatment outcome parameters and patient subgroups and isolated CNAs as well as CNA risk groups. Survival curves (EFS, RR, OS) and survival rates for overall cohort along with the effect of different ICiCLe risk groups and isolated CNAs, and CNA risk groups and profiles are calculated using Kaplan–Meier methods and log-rank tests. A P value of <0.05 is considered as significant. All statistical analysis have been performed using SPSS v26.0.

RESULTS

Patient demographics and cytogenetic profile

A total of 493 pediatric and adolescent patients (age range 1–18 years) with B-ALL being treated at either center 1 (n = 317) or center 2 (n = 176) under the ICiCLe protocol were included in the study. The median age of the cohort was 5 years with the male-to-female ratio of 1.9:1 (Table 2). The WBC range was between 0.3 and 980×10^9/L. MRD data were available for 444 patients and 111 (25%) had a positive MRD of ≥0.01% postinduction. In addition, 61 of 493 (12.4%) patients had induction failure. According to ICiCLe risk criterion, 119 (24.1%) patients belonged to ICiCLe-SR, 159 (32.3%) ICiCLe-IR, and 215 (43.6%) ICiCLe-HR categories. Eighty-three (16.8%) patients had GR cytogenetics, 363 (73.6%) IR, and 47 (9.5%) HR (details of abnormalities, Figure 2A). The detailed baseline characteristics of the whole cohort (n = 493) as well as center wise breakdown is highlighted in Table 2.

ICICLE risk and primary genetic risk group correlation data

We examined the correlation of ICiCLe risk categories with different clinical and outcome parameters (Suppl. Table S1). The ICiCLe-IR group had 46% (73/159) cases in the poor prognostic age group of 10 to 18 years and 83.6% (133/159) in the NCI-HR group compared to 29% (62/215) and 60.5% (130/215), respectively, in the ICiCLe-HR group. Among the outcome factors, 56% (69/123) of all relapses occurred in the ICiCLe-HR group compared to 22% each in IR and standard ICiCLe risk groups ($P < 0.001$).

In primary genetic subgroups analysis (Suppl. Table S2), HR cytogenetic cases had older age, high white blood cell count (WBC), higher induction failure, MRD positivity, and death rate as compared to those with GR cytogenetics ($P < 0.01$). In addition, nearly 43% (20/47) of all HR cytogenetics cases had a relapse compared to around 23% in each IR and SR cytogenetic groups (P 0.013).

CNA frequency and correlation data with clinical variables and risk stratification groups

Of 493 cases, 291 (59%) harbored a CNA in at least 1 of the 9 loci tested. Of these, 114 (39%) had single loci deletions, followed by 89 (30.5%), 58 (19.9%), and 15 (5.1%) cases with 2, 3, and 4 loci deletion, respectively (Figure 2B). Rare (8/291; 2.7%) cases had 5 or more loci involved.

The most frequent deletion noted in the study group (N = 493) was *CDKN2A/B* (36.3%) followed by *PAX5* (24.7 %) and *IKZF1* (19.5 %). Deletional frequency in *BTG1*, *EBF1*, and *RB1* was 7.7%, 4.1%, and 7.7%, respectively. Data for *ERG* deletions was available for 449 cases and 14 cases (3.1%) were found to harbor *ERG* deletion. The *IKZF1*^{plus} profile was identified in 13.4% (n = 66) of cases. CNAs classified, as per Moorman et al[7] (UK-ALL-CNA), revealed 259 (52.5%) cases in CNA-GR and 234 (47.5%) in CNA-IR+PR groups. As per MRplus scoring system, 259 (52.5%) cases had MRplus0 score, 168 (34.1%) MRplus1, and 66 (13.4%) MRplus 2 score.

To further investigate the correlation of *IKZF1* deletion and other CNA risk profiles with clinical and risk group stratification variables, the chi-square test was performed (Table 3). Significant correlation ($P < 0.05$) of *IKZF1*deletion, *IKZF1*^{plus} profile, CNA-IR+PR, and MRplus2 score with poor prognostic age group 10 to 18 years, high WBC $>50 \times 10^9$/L, high NCI risk, induction failure, and MRD ≥0.01% was observed. On assessment of other CNAs (Suppl. Table S3), *CDKN2A/B*

Table 2

Baseline Clinical, Hematological, and Genetic Characteristics of the Cohort (N = 493)

Variable		Center 1 (N = 317)	Center 2 (N = 176)	Center 1 + 2 (N = 493)
Age (y)		1–18 y (median = 6 y) (64.4% 1–10 y and 35.6% 10–18 y)	1–12 y (median = 5 y) (86.3% 1–10 y and 13.6% 10–18 y)	1–18 y (median = 5) (72.2% 1–10 y and 27.8% 10–18 y)
Gender		201 Males; 116 Females (M:F 1.73:1)	123 Males; 53 Females (M:F 2.3:1)	324 Males; 169 Females (M:F 1.92:1)
WBC (range)		0.3–446 × 10^9/L (mean = 26.3 × 10^9/L)	1.4–980 × 10^9/L	0.3–980 × 10^9/L (mean = 26.3 × 10^9/L)
Day 35 bone marrow not in remission (induction failure)		50 (15.8%)	11 (6.2%)	61 (12.4%)
Minimal residual disease positive (n = 444)		62 (19.6%)	49 (27.8%)	111 (25%)
	Categories	**N (%)**	**N (%)**	**N (%)**
ICiCLe risk category (postinduction)	SR	80 (25.2%)	39	119(24.1%)
	IR	100 (31.5%)	59	159 (32.3%)
	HR	137 (43.2)	78	215 (43.6%)
NCI risk category	SR	154 (47.9%)	76 (42.6%)	230 (46.0%)
	HR	163 (52%)	100 (57.3%)	263 (54.0%)
Cytogenetics	GR	48 (15.14%)	35 (19.9%)	83 (16.8%)
	IR/B-cell-other ALL	235 (74.1%)	128 (72.7%)	363 (73.6%)
	HR	34 (10.7%)	13 (7.4%)	47 (9.5%)
MRplus	GR (MRplus 0)	168 (53%)	91 (51.7%)	259 (52.5%)
	IR (MRplus 1)	110 (34.7%)	58 (33%)	168 (34.1%)
	PR (MRplus 2)	39 (12.3%)	27 (15.3%)	66 (13.4%)
UK-ALL CNA risk criteria	GR	168 (52.5%)	91 (51.7%)	259 (52.5%)
	IR/PR	149 (47.5%)	84 (47.7%)	234 (47.5%)
IKZF1 deletion	Present	56 (17.7%)	40 (22.7%)	96 (19.5%)
	Absent	261 (82.3%)	136 (77.2%)	397 (80.5%)
*IKZF1*plus [2]	Present	39 (12.3%)	27 (15.3%)	66 (13.4%)
	Absent	278 (87.7%)	149 (84.7%)	427 (86.6%)
CDKN2A/B deletion	Present	108 (34%)	71 (40.3%)	179 (36.3%)
	Absent	209 314 (66%)	105 (59.7%)	314 (63.7%)
PAX5 deletion	Present	85 (26.9%)	37 (21%)	122 (24.7%)
	Absent	232 (73.2%)	139 (79%)	371 (75.3%)
BTG1 deletion	Present	23 (7.3%)	15 (8.5%)	38 (7.7%)
	Absent	294(92.7%)	161 (91.5%)	455 (92.3%)
EBF1 deletion	Present	10 (3.2%)	12 (6.8%)	22 (4.5%)
	Absent	307 (96.8%)	164 (93.1%)	471 (95.5%)
RB1 deletion	Present	31 (9.8%)	7 (4%)	38 (7.7%)
	Absent	286 (90.2%)	169 (96%)	455 (92.3%)
ETV6 deletion	Present	38 (12%)	11 (6.2%)	49 (9.9%)
	Absent	279 (988%)	165 (93.8%)	444 (90.1%)
ERG deletion (n=449)	Present	6 (1.9%)	8 (6.1%)	14 (3.1%)
	Absent	311 (98.1%)	124 (93.9%)	435 (96.9%)
PAR deletion	Present	14 (4.4%)	6 (3.4%)	20 (4.1%)
	Absent	303 (95.6%)	170 (96.6%)	473 (95.9%)

CNA = copy number alteration; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; PR = poor risk; SR = standard risk.

deletion was more common in cases with older age group (*P*0.001), high WBC (*P* 0.003), NCI-HR (*P* < 0.001), and ICiCLe-IR/HR (*P* = 0.009) and primary genetic-IR/HR (*P* < 0.001) cases. *RB1* and PAR1 deletions were statistically more common in cases with induction failure (*P* < 0.02 and <0.001). Furthermore, *PAX5* deletions were statistically common in the older age group and NCI-HR (*P* < 0.001). *ERG* deletions were seen primarily in ICiCLe-SR/IR cases and associated with CR status (*P* < 0.01).

Furthermore, we analyzed the proportion of cases harboring a particular CNA in different ICiCLe risk groups (Figure 2C). A statistically significant CNA burden in the ICiCLe-HR group (295 CNA events) compared to SR (84 CNA events) and IR (184 CNA events) groups (*P* < 0.001) was observed. *IKZF1*, *PAX5*, *BTG1*, and *RB1* deletions were seen to increase in proportion from ICiCLe-SR to ICiCLe-HR group while *ERG* deletions were nearly absent from the ICiCLe-HR group. In addition, *IKZF1* deletion, *IKZF1*^{plus}, CNA risk, and MRplus profiles were noted to be significantly more common in the ICiCLe-HR group (Suppl. Table S4; *P* < 0.001). Figure 2D shows correlation matrix highlighting co-occurrence of various CNAs in the whole cohort and reveals that *PAX5* deletions are usually seen along with *CDKN2A/2B* deletions, while deletion of PAR1 region is rarely seen with *IKZF1* or *BTG1* deletions and never with *ETV6* deletion.

The primary genetic risk groups were also analyzed for the presence of CNAs (Figure 2E and Suppl. Table S5). *IKZF1* deletion was noted in 29 of 47 (61.7%) of the HR primary genetic group (*P* < 0.001). Furthermore, 24 of 29 (82.7%) of these *IKZF1*deletion cases had *IKZF1*^{plus} profile (*P* < 0.001). In addition, 76.5% (36/47) of cases with HR cytogenetics had CNA-IR+PR profile and 51.5% (24/47) MRplus2 score. Figure 2E shows that *IKZF1*, *CDKN2A/2B* and *PAX5* deletions increased in proportion from primary genetic GR to HR cytogenetic

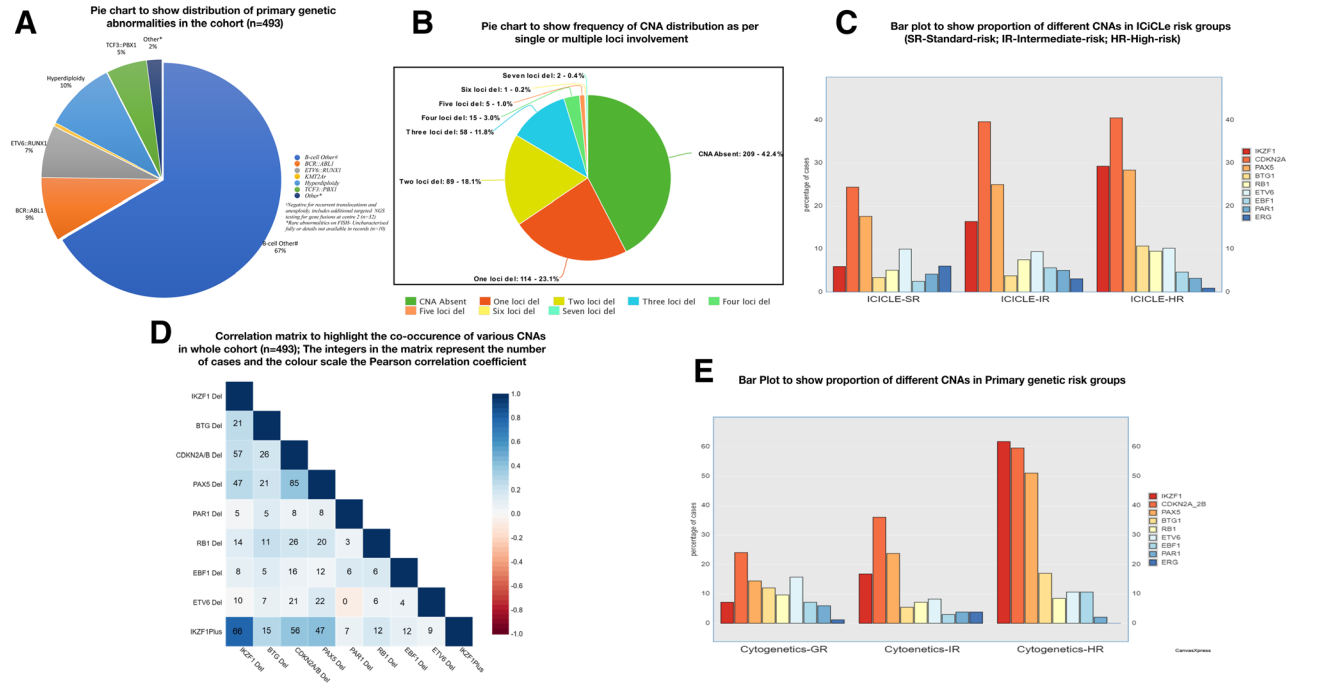

Figure 2. (A) Pie chart to show distribution of primary genetic abnormalities in the cohort (n = 493). (B) Pie chart to show frequency of CNA distribution as per single or multiple loci involvement. (C) Bar plot to show proportion of different CNAs in ICiCLe-SR/IR/HR risk groups. (D) Correlation matrix to highlight the co-occurrence of various CNAs in whole cohort (n = 493). The integers in the matrix represent the number of cases and the color scale the Pearson correlation coefficient. (E) Bar plot to show proportion of different CNAs in primary genetic risk groups. CNA = copy number alteration; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; SR = standard risk; TRM = treatment-related mortality.

groups, while *ERG* deletions were not seen in the HR cytogenetics group.

Outcome analysis of whole cohort, ICiCLe risk stratification groups, CNAs and integrated proposed risk stratification categories

Kaplan–Meyer survival analysis (OS, EFS, RR) and log-rank tests have been performed for the whole cohort, ICiCLe and primary genetic risk groups, *IKZF1* deletion, and *IKZF1*^{plus}, CNA and MRplus profiles. In addition, the survival outcome analysis of the CNAs, that is, *IKZF1* deletion, *IKZF1*^{plus} and CNA risk profiles have been studied with MRD and the three ICiCLe risk groups (SR, IR & HR). The median follow-up for cases in the cohort was 41 months, and hence, 3-year survival analysis data with 95% CI are presented (Tables 4 and 5 and Figure 3A–F) (Suppl. Table S6 and Suppl. Figures S2S8).

The events considered for survival analysis were refractory disease (n = 7), relapse (n = 123), and deaths (n = 131). Among those who relapsed (n = 123), 36.5% (n = 45) cases had very

Table 3

Clinicohematological and Treatment Outcome Parameters in *IKZF1* Deletion and Other CNA Risk Profiles (n = 493)

Category	Total (N)	*IKZF1* del	*P*	*IKZF1*^{Plus}	*P*	CNA-IR+PR	*P*	MRplus0	MRplus1	MRplus2	*P*
1–9 y	358	61	**0.026**	40	**0.023**	153	**<0.0003**	205	113	40	**0.0016**
10–18 y	135	35		26		81		54	55	26	
Male	324	72	**0.03**	52	**0.0162**	160	0.237	164	108	52	**0.054**
Female	169	24		14		74		95	60	14	
<50 × 10^9/L	328	41	**<0.001**	23	**<0.001**	139	**0.0041**	189	116	23	
>50 × 10^9/L	165	55		43		95		70	52	43	**<0.001**
SR	230	19	**<0.001**	8	**<0.001**	80	**<0.001**	150	72	8	**<0.001**
HR	263	77		58		154		109	96	58	
CR	432	74	**<0.001**	52	**0.019**	193	**0.001**	239	141	52	**0.0024**
Not in CR	61	22		14		41		20	27	14	
<0.01%	333	44	**<0.001**	32	**0.0043**	138	**0.001**	215	107	34	**<0.001**
>0.01%	111	36		22		66		44	61	32	
ICiCLe-SR	119	7	**<0.001**	3	**<0.001**	34	**<0.001**	85	31	3	**<0.001**
ICiCLe-IR	159	26		19		76		83	57	19	
ICiCLe-HR	215	63		44		124		91	80	44	
Genetics-GR	83	6	**<0.001**	2	**<0.001**	30	**<0.001**	53	28	2	**<0.001**
Genetics-IR	363	61		40		168		195	128	40	
Genetics-HR	47	29		24		36		11	12	24	

Bolded *P* values <0.05.

CNA = copy number alteration; del = deletion; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; PR = poor risk; SR = standard risk.

Table 4

Highlights 3-year Outcome Analysis of Whole Cohort, Primary Genetic Risk Groups, *IKZF1* Deletion, *IKZF1 plus* Profile, UK-ALL-CNA Profile, MRplus Score, ICiCLe Risk Groups, and Interaction of Combined ICiCLe Groups With CNA-GR and CNA-IR/PR Profile

Category (n)	OS% (Range) (n = Deaths) at 3 y With 95% CI	Log Rank *P*	EFS%, Range (n = Events) at 3 y With 95% CI	Log Rank *P*	RR% (Range) (n = Relapse)[a] at 3 y With 95% CI	Log Rank *P*
Whole cohort	73.1 (70.9–75.3) (116)	–	54.8 (52.4–57.2) (203)	–	26.6 (24.2–29) (92)	–
Genetic GR (83)	82.2 (77.5–86.9) (12)	0.536	68.3 (62.6–74.3) (22)	**<0.001**	16.8 (11.9–21.7) (10)	**<0.001**
Genetic IR (363)	71.9 (69.3–74.5) (88)		54.6 (51.8–57.4) (147)		25.7 (22.8–28.6) (62)	
Genetic HR (47)	63.0 (55.5–70.5) (17)		35.8 (29.3–42.3) (35)		46.9 (39.1–54.7) (20)	
*IKZF1*no del (397)	76.5 (74.2–78.8) (82)	**0.006**	59.0 (56.4–61.6) (149)	**<0.001**	24 (21.4–26.6) (68)	**<0.00**1
*IKZF1*del (96)	57.2 (51.2–63.2) (33)		37.3 (31.9–42.6) (54)		38.6 (32.2–45) (24)	
IKZF1plus absent (427)	75.7 (73.4–78) (92)	**0.003**	58.6 (56.1–61.1) (161)	**<0.001**	23.8 (21.3–26.3) (72)	**<0.001**
IKZF1plus present (66)	53.0 (4505–60.5) (24)		30 (23.9–36.1) (42)		46.5 (38.5–54.5) (20)	
IKZF1plus absent + MRD negative (301)	81.7 (79.2–84.2) (46)	**0.031**	65.2 (62.3–68.1) (93)	**<0.001**	21.7 (19–24.4) (50)	**<0.001**
IKZF1plus absent + MRD positive (89)	75.5 (70.5–80.5) (19)		52.4 (46.8–58) (40)		31.3 (25.4–37.2) (20)	
IKZF1plus present + MRD negative (32)	68.2 (57.8–78.6) (7)		39.8 (30.4–49.2) (17)		42.5 (32–53) (10)	
IKZF1plus present + MRD positive (22)	51 (37.8–64.2) (8)		25.2 (15.1–35.3) (15)		55.6 (41.7–69.5) (9)	
CNA-GR (224)	81.3 (78.7–83.9) (43)	**<0.001**	65 (61.9–68.1) (83)	**<0.001**	20.1 (17.2–23) (39)	**<0.001**
CNA-IR+PR (234)	63.3 (59.7–66.9) (73)		43.2 (39.7–46.7) (120)		35.2 (31.2–39.2) (53)	
MRplus0 (259)	81.3 (78.7–83.9) (43)	**<0.001**	65 (61.9–68.1) (83)	**<0.001**	20.1 (17.2–23) (39)	**<0.001**
MRplus1 (168)	66.7 (62.7–70.7) (49)		48.5 (44.3–52.7) (78)		30.7 (26.2–35.1) (33)	
MRplus2 (66)	53 (45.5–60.5) (24)		30 (23.9–36.1) (42)		46.5 (38.5–54.5) (20)	
ICiCLe-SR (119)	79.3(75.3–83.3) (22)	**0.132**	65.3 (60.6–70) (37)	**0.006**	20.6 (16.2–25) (18)	**0.002**
ICiCLe-IR (159)	69.1 (68.7–73.1) (43)		55.3 (51.1–59.5) (64)		20.9 (16.9–24.9) (22)	
ICiCLe-HR (215)	72.4 (69–75.8) (51)		48.9 (45.3–52.5) (102)		33.9 (30–37.8) (52)	
Combined ICiCLe-SR/IR/HR +CNA-GR Group (Groups 1 + 3+5) (259)	81.3 (78.7–83.9) (43)	**<0.001**	65.0 (61.9–68.1) (76)	**<0.001**	20.1 (17.2–23) (39)	**<0.001**
Combined ICiCLe-SR/IR/HR +CNA-IR+PR Group (Groups 2 + 4+6) (234)	63.3 (59.7–66.9) (73)		43.2 (39.7–46.7) (120)		35.2 (31.2–39.2) (53)	

[a]N = 456 (cases that died during induction were excluded).

Bolded *P* values <0.05.

CI = confidence interval; CNA = copy number alteration; EFS = event-free survival; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; OS = overall survival; PR = poor risk; RR = relapse rate; SR = standard risk.

early relapse, 37.3% (n = 46) early, and 24.2% (n = 30) late relapse. Death due to nonrelapse reasons was 22.9% (113/493), with 38 (33.6) induction deaths and 75 (66.3%) noninduction phase treatment deaths. Eighteen cases (15.9%) had late death posttreatment completion at a median time of 42 months. Induction failure was noted in 61 (12.4%) cases. The majority of these 70.5% (43/61) were NCI-HR as per age and WBC and 18 were females and 43 males. Besides induction failure a subset of patients also showed refractory disease (n = 7) despite reinduction therapy. The TRM data in detail will be reported separately in the report of the entire clinical trial data.

The 3-year OS, EFS, and RR for the cohort is 73.1%, 54.8%, and 26.6%, respectively. Both ICiCLe-HR and primary genetic HR groups had a statistically poor EFS and high RR. Among CNAs, *IKZF1* deletion and *IKZF1*^{plus} profile had significantly poor OS, EFS, and RR at 57.2%, 37.3%, 38.6% and 53.0%, 30.0%, 46.5%, respectively, compared to patients without those deletions/profiles ($P < 0.05$) (Table 5 and Figure 3A and B). In addition, the OS, EFS, and RR of the CNA-IR+PR and MRplus2 score was significantly poor ($P < 0.001$) (Table 5 and Figure 3C and D).

The RR also worsened significantly in cases with MRD positivity (*P* 0.001) when combined with UK-ALL-CNA risk profile, from 29.9% (only MRD) to 41.7% (MRD and CNA-IR+PR). A similar effect on RR was noted when MRD was combined with *IKZF1* deletion 26.1% to 46.7% (*P* 0.002) and *IKZF1*^{plus} profile 31.3% to 55.6% ($P < 0.001$) (Suppl. Table S6).

Survival and outcome analysis for *IKZF1* deletions and the *IKZFI*^{plus} and UK-ALL-CNA risk profiles were also performed in different ICiCLe risk groups as these treatment-based risk stratification groups are already integrated with MRD, NCI, and primary genetic data (Table 5). In ICiCLe-SR, though the number of *IKZF1* deletion and *IKZF1*^{plus} profile cases was low, the OS and EFS were significantly poor in cases with *IKZF1* deletion, CNA-IR+PR, and MRplus2 profiles. In ICiCLe-IR and ICiCLe-HR, *IKZF1*^{plus} profile and UK-ALL-CNA profile (groups 4 and 6) had clear prognostic impact with significantly worse EFS and high RR. *IKZF1* deletions had statistically poor EFS and high RR in the ICiCLe-IR group but was borderline significant in the ICiCLe-HR group. On combining the proposed integrated ICiCLe and UK-ALL-CNA groups with respect to CNA-GR or CNA-IR+PR status (ICiCLe+CNA-GR vs ICiCLe + CNA-IR+PR), significantly improved OS, EFS, and lower RR are noted for CNA-GR compared to CNA-IR+PR profiles ($P < 0.01$) (Table 4 and Figure 3E and F).

DISCUSSION

The present retrospective analysis was initiated to comprehensively investigate and analyze the prognostic role of CNAs in our cohort of pediatric B-ALL cases being treated under the ICiCLe treatment protocol. The objective was to derive clinical and prognostic impact of CNAs and associated risk profiles for future incorporation of routine CNA testing in phase 2 of treatment trial, currently under active consideration. A total of 493 pediatric B-ALL cases were evaluated for nine important loci for CNAs and correlated with clinical and treatment outcome parameters.

The study is limited by restricted primary genetic analysis in B-ALL cases to primarily RT-PCR and or conventional cytogenetics data. Only a limited number of cases, especially from center 2, were evaluated with centromere probes on FISH, flow-based DNA ploidy, and targeted RNA-based NGS panel evaluation. Hence, we focused on the categorization of primary genetic abnormalities as GR, IR, and HR. However, despite this

Table 5

Prognostic Effect of *IKZF1* Deletion and Other CNA Profiles in the ICiCLe-SR/IR/HR Risk Groups

		ICiCLe-SR Group (n = 119)				ICiCLe-IR Group (n = 159)				ICiCLe-HR Group (n = 215)		
Category	**No. Cases (%)**	**RR (95% CI) 3 y (n)**	**EFS (95% CI) 3 y (n)**	**OS (95% CI) 3 y (n)**	**No. Cases (%)**	**RR (95% CI) 3 y (n)**	**EFS (95% CI) 3 y (n)**	**OS (95% CI) 3 y (n)**	**No. Cases (%)**	**RR (95% CI) 3 y (n)**	**EFS (95% CI) 3 y (n)**	**OS (95% CI) 3 y (n)**
IKZF1 NO DEL	112 (94.1%)	20.8 (16.4–25.2) (18)	67.4 (62.7–72.1) (33)	82 (78.21–85.9) (18)	133 (83.6%)	20 (15.9–24.1) (16)	59 (54.4–63.6) (49)	71.7 (67.4–76) (33)	152 (70.7%)	31.4 (26.9–35.9) (34)	52.9 (48.7–57.1) (67)	76.6 (72.9–80.3) (32)
IKZF1 DEL	7 (5.9%)	0 (0)	26.8 (24.7–28.9) (4)	26.8 (24.7–28.9) (4)	26 (16.4%)	37.2 (24.5–49.9) (6)	36.5 (26.2–46.8) (15)	53.4 (41.9–64.9) (10)	63 (29.3%)	39.9 (32.4–47.4) (18)	39.2 (32.7–45.7) (35)	61.6 (54.4–68.8) (19)
P		0.630	**0.001**	**<0.001**		**0.006**	**0.012**	0.176		0.073	0.067	0.285
IKZF1^{Plus} NO DEL	116 (97.5%)	20.6 (16.2–25) (18)	66.3 (61.6–71) (35)	80.6 (76.6–84.6) (20)	140 (88.1%)	17.5 (13.5–21.5) (16)	58.5 (54–63) (52)	70.8 (66.6–75) (36)	171 (79.5%)	30.7 (26.5–34.9) (38)	53.6 (49.6–57.6) (74)	76.2 (72.7–79.7) (36)
IKZF1^{Plus}DEL	3 (2.5%)	0 (0)	33.3 (6–60.4) (2)	33.3 (30.6–36) (2)	19 (11.9%)	45 (30.6–59.4) (6)	33.1 (21.6–44.6) (12)	54.1 (40.5–67.3) (7)	44 (20.5%)	47.4 (37.7–57.1) (14)	29.4 (22–36.8) (28)	54.4 (45.1–63.7) (15)
P		0.841	**0.003**	**0.001**		**0.004**	**0.028**	0.387		**0.006**	**0.001**	**0.033**
CNA-GR	85 (71.4%)	18.8 (14.1–23.5) (13)	72.8 (67.7–77.9) (21)	88 (84.2–91.8) (9)	83 (52.2%)	12.8 (8.2–17) (7)	63.2 (57.5–69) (27)	73.5 (68.2–78.8) (19)	91 (42.3%)	27.3 (21.9–32.7) (19)	59.4 (54.1–64.7) (35)	81.8 (77.5–81.8) (15)
CNA-IR-PR	34 (28.6%)	26.2 (15.7–36.7) (5)	43.6 (33.9–53.3) (16)	53.8 (44–63.6) (13)	76 (47.8%)	30.4 (23.7–37.1) (15)	46.8 (40.7–52.9) (37)	64.9 (58.9–70.9) (24)	124 (57.7%)	39.1 (33.6–44.6) (33)	40.9 (36.2–45.6) (67)	64.9 (60–69.8) (36)
P		0.394	**0.004**	**<0.001**		**0.001**	**0.003**	0.141		**0.020**	**0.004**	0.25
MRPlus0	85 (71.4%)	18.8 (14.1–23.5) (13)	72.8 (67.7–77.9) (21)	88 (84.2–91.8) (8)	83 (52.2%)	12.8 (8.2–17.4) (7)	63.2 (57.5–67.9) (27)	73.5 (68.2–79.3) (19)	91 (42.3%)	27.3 (21.9–32.7) (19)	59.4 (54.1–64.7) (35)	81.8 (77.5–86.1) (15)
MRPlus1	31 (26.1%)	26.2 (15.7–36.7) (5)	46 (36–59) (14)	57 (47–67) (11)	57 (35.8%)	25.9 (18.3–33.5) (9)	51.9 (44.8–59) (25)	67.4 (60.7–74.1) (17)	80 (37.2%)	34.8 (28.3–41.3) (19)	47 (41.1–52.9) (39)	69.5 (63.8–75.2) (21)
MRPlus2	3 (2.5%)	0 (0)	33.3 (6.1–60.5) (2)	33.3 (3.3–60.2) (2)	19 (11.9%)	45 (30.6–59.4) (6)	33.1 (21.6–44.6) (12)	54.1 (40.5–67.7) (7)	44 (20.5%)	47.4 (37.7–57.1) (14)	29.4 (48.4–63.2) (27)	54.4 (45.1–63.7) (15)
P		0.671	**0.001**	**<0.001**		**0.001**	**0.006**	0.322		**0.009**	**0.001**	**0.034**

Bolded *P* values <0.05.

CI = confidence interval; CNA = copy number alteration; EFS = event-free survival; GR = good risk; HR = high risk; ICiCLe = Indian Collaborative Childhood Leukemia group; IR = intermediate risk; OS = overall survival; PR = poor risk; RR = relapse rate; SR = standard risk.

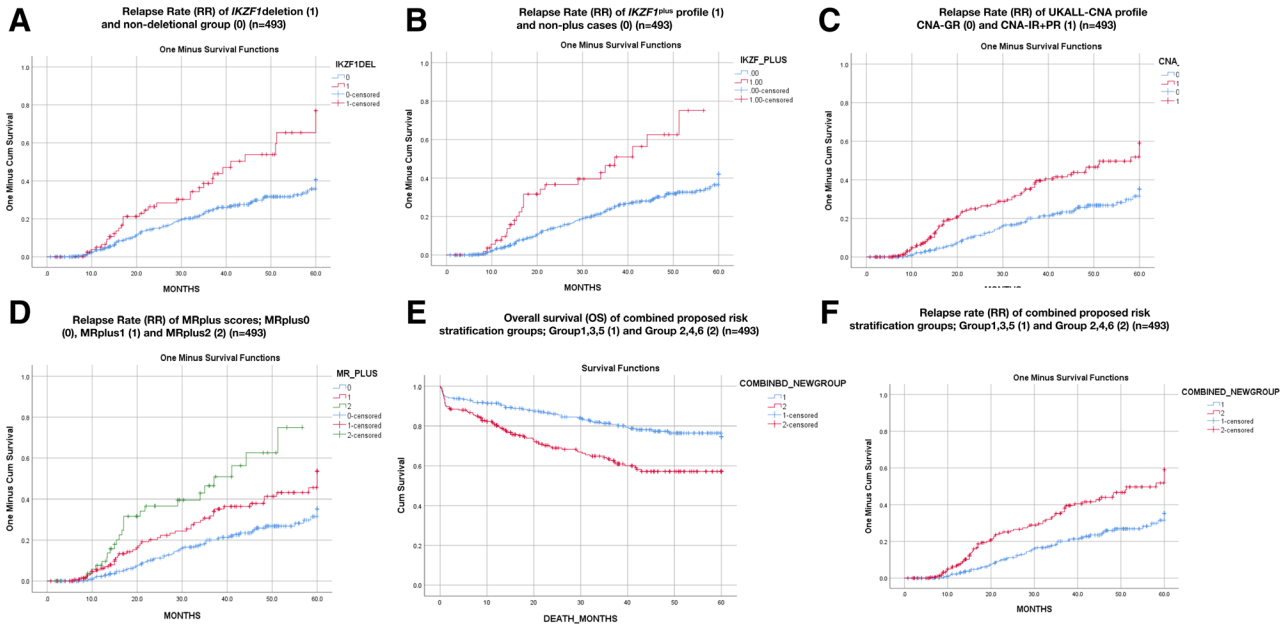

Figure 3. (A) RR of IKZF1deletion (1) and nondeletional group (0) (n = 493). (B) RR of *IKZF1*^{plus} profile (1) and nonplus cases (0) (n = 493). (C) RR of UK-ALL-CNA profile CNA-GR (1) and CNA-IR+PR (0) (n = 493). (D) RR of MRplus scores: MRplus0 (0), MRplus1c (1), and MRplus2 (2) (n = 493). (E) OS of combined proposed risk stratification groups; groups 1, 3, 5 (1) and groups 2, 4, 6 (2) (n = 493). (F) RR of combined proposed risk stratification groups: groups 1, 3, 5 (1) and groups 2, 4, 6 (2) (n = 493). CNA = copy number alteration; GR = group risk; HR = high risk; IR = intermediate risk; OS = overall survival; RR = relapse rate; SR = standard risk.

limitation, the study data on CNA are important to highlight since CNAs have been shown to be independent prognostic factors in many different trials.[14–18]

The 3-year OS, EFS, and RR of the overall cohort was 73.1%, 54.8%, and 26.6%, respectively. Studies from our subcontinent as reviewed by Arora and Arora[19] show improved OS and EFS over the past decade or so to 60% to 80% and >50%, respectively. As highlighted in the result section, the TRM data in detail will not be dealt here and will be a part of the report of the entire clinical trial data.

The majority of the cases (43.6%; 215/493) belonged to the ICiCLe-HR category and only 32.2% and 24.1% to IR and SR. The OS of ICiCLe treatment trial risk stratification groups was not statistically significant, but RR was significantly worse for ICiCLe-HR group compared to ICiCLe-SR & IR groups (33.9% vs. 20.75%; *P* 0.002).

The overall M:F ratio in the cohort was nearly 2:1 and studies previously have shown these to be ranging from 1.8 to even 6.5:1[20–23] in our population suggesting the possibility of geographical male preponderance of disease. However, the existing sociocultural gender bias in India, especially among the lower socioeconomic strata, where boys receive differential treatment, could also be an important factor responsible for this ratio.

The overall frequency of a CNA, in either of the 9 loci tested in our cohort, was 59% with 60% (170/284) showing 2 or more loci deletion. *CDKN2A/B* deletion was the most frequent CNA identified (36.3%), but prognostically, the most significant CNA in our cohort was *IKZF1* deletion (n = 96; 19.5%). The frequency of *IKZF1* deletion in the current study population was consistent with previously published reports (15%–26%).[17,18,24] We noted that *IKZF1* deletion was concentrated in the HR groups (genetic poor risk and ICiCLe-HR) consistent with literature data.[7,25–27]

A highly significant correlation of *IKZF1* deletion (n = 96) was noted with high TLC ($P < 0.001$), NCI-HR ($P < 0.001$), induction failure ($P < 0.001$), and ICiCLe-HR ($P < 0.001$) with primary genetic HR group ($P < 0.001$). Furthermore, when RR was analyzed independently with genetic variables, the RR of *IKZF1* deletion cases was noted to be high (53.8%) compared to wild-type status (27.5%). The association of *IKZF1* deletion with poor overall survival outcome has been reported by many studies from adult and pediatric BCP-ALL cohorts,[7,25,26,28–32] except for the ones with UKALL14 and UKALL60+ adults' cohort.[33–35] A number of studies have also identified the *IKZF1*^{plus} profile to be an independent stronger molecular stratification marker in pediatric population.[6,19] In our study too, when *IKZF1* deletion were further categorized as *IKZF1*^{plus} profile (n = 66), similar associations were observed with different clinical parameters and with relapse (*P* 0.02), events (*P* 0.0001), and death (*P* 0.025). In addition, *IKZF1* deletion and *IKZF1*^{plus} profile were also noted to be independent poor prognostic markers compared to MRD, which is currently the single most important prognostic factor in treatment of ALL.[36–39] A highly significant correlation was observed with *IKZF1* deletion and MRD positivity in our cohort ($P < 0.0001$). Furthermore, the RR was higher in *IKZF1* deletion and plus profile cases with positive MRD and increased dramatically from 26.1% (MRD alone) to 46.7% (*P* 0.002) and 31.3% to 55.6% ($P < 0.0001$), respectively, as noted in other trials.[31,40] We also tried comparing our results with AIEOP-BFM ALL 2000 trial of Stanulla et al and noted that the *IKZF1*^{plus} profile in our cases, even with a MRD <0.01% at day 35, had a very poor EFS of 40% ± 10%, much worse and comparable to the MRD-IR group of their trial rather than the MRD-SR group which had 94% ± 5%.[8] Similarly, RR for *IKZF1*^{plus} profile in our cases, with a MRD <0.01% at day 35, was 40% ± 12%, significantly worse than RR noted in their trial at 6% ± 6%. This suggests underlying biological differences, highlighted in other studies from our subcontinent earlier.

We also classified CNAs as per Moorman et al CNA risk classification (UK-ALL-CNA) into CNA-GR and CNA-IR+PR groups and also scored the CNA risk group with *IKZF1*^{plus} profile as per our published MRplus scoring system. The CNA-GR cases had significantly better OS, EFS, and RFS and this remained unchanged despite MRD positivity suggesting

CNA-GR profile to be an independent good prognostic marker in pediatric B-ALL. MRplus scoring too can be used as a risk stratification strategy as a high score of 2 helped identify a subset of CNA-IR+PR cases that had *IKZF1*^{plus} profile, which clearly showed poor outcome and high RR.

Finally, we also evaluated and proposed an integrated UK-ALL CNA profile and ICiCLe risk group categorization. The outcome analysis, revealed that groups 2 and 4 (ie, ICiCLe-SR and IR with CNA-IR+PR status) behaved similar to group 6 (ICiCLe-HR + CNA-IR+PR) with poor OS, EFS, and RR, and this was statistically different from other groups 1, 3, and 5 with GR CNAs. Overall, in ICiCLe-SR, few cases (7/119; 6%) had *IKZF1* deletion and *IKZF1*^{plus} profile (3/119; 2.5%) and none relapsed; but 28.5% cases (34/119) had CNA-IR+PR profile, of which around 50% (16/34) had an event with 31% relapsed (5/16). In the ICiCLe-IR group, *IKZF1* deletion as well as *IKZF1*^{plus} and UK-ALL-CNA profile were clearly prognostic with twice the number of cases having relapse compared with cases without deletion and plus profile, respectively ($P < 0.001$). The outcome analysis of combined groups 1, 3, 5 with groups 2, 4, 6 also clearly demarcated the prognostic impact of CNAs with CNA-IR+PR profile behaving as an independent poor prognostic marker irrespective of SR, IR, or HR ICiCLe stratification status. This provides strong evidence that CNA testing needs to be incorporated in our prospective enrollment strategy to identify subset of SR/IR cases that would benefit from HR treatment during the consolidation phase.

A recent study had also reported *BTG1* as one of the prognostic markers in acute leukemia.[32] However, in our cohort, no promising correlation was observed for *BTG1* deletion with any of the clinical variables. The most common CNA noted, that is, the deletion of *CDKN2A/B* did show significant correlation with a few variables; however, the significant association with relapse, as reported earlier by our group, could not be substantiated in this cohort.[32] Similar observation had been noted in UKALL14 study except for the trend for biallelic deletions of *CDKN2A/B* in *BCR- ABL1* where they found association with lower EFS and OS.[33]

In conclusion, despite limited primary genetic analysis and slightly biased cohort enrollment with partial exclusion of recurrent GR and HR cytogenetic cases, our study provides strong evidence for CNAs as one of the important independent prognostic factors, in addition to MRD, for better risk stratification of pediatric B-ALL cases. The study data strongly suggest routine screening and prospective testing for CNAs in the next phase of ICiCLe treatment trial. This coupled with extensive primary genetic analysis in our treatment trial can help generate better prospective data on status of *IKZF1* deletion and plus profile in Ph-Like, Ph-positive and Ph-negative groups. The study also proposes integration of CNA risk group status with ICiCLe risk stratification categories in future phase of the trial to define a novel subset of cases in both ICiCLe-SR and IR groups that will behave as HR and would require therapy escalation postinduction.

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AUTHOR CONTRIBUTIONS

SKG and PB designed and conceptualized the study and analyzed the data. MS and PB wrote the manuscript and performed MLPA analysis at center 2; AVM mentored and guided the manuscript writing and data analysis as a subject expert; PHC, AT, DB, RJ, SP, SB, and DP clinicians involved with patient recruitment, enrollment in ICiCLe trial, treatment, and follow-up work at centers 1 and 2. SKG, PS, RT, and MS performed MLPA at centers 1 and 2 and analyzed MLPA data. SS, SG, RG, and MSS primary genetic and MRD analysis at centers 1 and 2, manuscript review and editing.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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