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Frequency of copy number abnormalities in common genes associated with B-cell precursor acute lymphoblastic leukemia cytogenetic subtypes in Brazilian children

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Copy number alterations (CNAs) in genes committed to B-cell precursors have been associated with poor survival in subgroups of patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL). We investigated submicroscopic alterations in a series of 274 Brazilian children with BCP-ALL by multiplex ligation-dependent probe amplification and evaluated their correlation with clinical and laboratory features. The relevance of overlapping CNA abnormalities was also explored. Deletions/amplifications in at least one gene were identified in 83% of the total series. In children older than 2 years, there was a predominance of CNAs involving deletions in *IKZF1*, *CDKN2A*, and *CDKN2B*, whereas the pseudoautosomal region 1 (PAR1) had deletions that were found more frequently in infants ($P < 0.05$). Based on the cytogenetic subgroups, favorable cytogenetic subgroups showed more deletions than other subgroups that occurred simultaneously, specifically *ETV6* deletions ($P < 0.05$). *TCF3-PBX1* was frequently deleted in *RB1*, and an absence of deletions was observed in *IKZF1* and genes localized to the PAR1 region. The results corroborate with previous genome-wide studies and aggregate new markers for risk stratification of BCP-ALL in Brazil.

Keywords Acute lymphoblastic leukemia, *IKZF1*, MLPA, multiplex ligation-dependent probe amplification, copy number alterations

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is characterized by recurrent chromosomal abnormalities in approximately 75% of patients. Many of these alterations have a significant impact on prognosis among the risk classifications in several clinical trials. The presence of the *MLL-AFF1* or *BCR-ABL1* fusion gene stratifies patients into poor-outcome risk groups, whereas the presence of a high number of chromosome gains and the *ETV6-RUNX1* fusion implies good prognosis risk groups (1,2). These aberrations are important initiating events in leukemogenesis pathway mechanisms, as well as in making therapeutic decisions. Approximately 20–25% of all BCP-ALL cases are still classified as having an intermediate prognosis requiring further investigation for a clearer outcome association (3,4).

The advent of high resolution genome-wide (GW) technology to measure gene expression, DNA copy number alterations (CNAs), and loss of heterozygosity have led to the detection of many novel genetic abnormalities, which have

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refined the prognostic models for BCP-ALL. GW profiling detected multiple submicroscopic CNAs not visible by cytogenetic analysis, providing new insights into the genetic basis of ALL (5–7). Some studies have identified numerous microdeletions that specifically target genes involved in signaling pathways of B-cell differentiation (e.g., *IKZF1*, *ETV6*, *PAX5*, and *EBF1*), genes involved in cell cycle control and tumoral suppression (e.g., *CDKN2A/2B*, *BTG1*, and *RB1*), and cytokine receptors localized to the pseudoautosomal region 1 (PAR1) (e.g., *CRLF2*, *IL3RA*, *CSF2RA*, and *SHOX*) (5–8). The CNA vary according to the primary genetic abnormalities of the BCP-ALL subtypes (9).

The prognostic relevance of these abnormalities in BCP-ALL has been the subject of previous reports on cohorts from Europe, the United States, and Japan (10–13). *IKZF1* deletions, among other gene mutations, have been associated with poor prognosis in BCP-ALL, though this association is dependent on other clinical features. *IKZF1* deletions have been cited as the strongest predictor of relapse at the time of diagnosis and confers a threefold increased risk of relapse in *BCR-ABL1* ALL patients (12–18). As mentioned previously, the relevance of these gene aberrations may vary according to other features, such as demography, molecular-cytogenetic subgroups, and concomitant occurrence of other genetic deletions. Therefore, the aim of this study was to determine the frequency of gene deletions and the significance of copy number alterations in *IKZF1*, *CDKN2A*, *CDKN2B*, *PAX5*, *EBF1*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region (*SHOX*, *CSF2RA*, *IL3RA,* and *CRLF2* genes) within a cohort of Brazilian pediatric patients.

Material and methods

Participants

A series of 274 bone marrow (BM) aspirates or peripheral blood (PB) samples from patients with BCP-ALL were analyzed prior to any oncological treatment (period 2004–2011). The selection criteria included the quality of the frozen diagnostic material, having at least 30% blast cells, and that patients were 18 years old or younger at the time of BCP-ALL diagnosis. The exclusion criteria were acute leukemia in children with Down syndrome, samples collected in heparin, and DNA of insufficient quality. The diagnosis and characterization of leukemia were established by morphology, immunophenotyping, and molecular-cytogenetic analysis as recommended by the World Health Organization classification (19). DNA content in a propidium iodide–stained index was performed by flow cytometry (DNA index, DI). A DI ratio greater than 1.16 was used as a proxy for hyperploid cases (20). *ETV6-RUNX1*, *TCF3-PBX1*, and *BCR-ABL1* fusion genes were analyzed by reverse transcription PCR as part of the diagnostic procedures. Detection of an *MLL*-rearrangement (*MLL*-r) was performed by reverse transcription PCR and by fluorescence in situ hybridization (FISH) (Vysis LSI MLL dual-color break apart rearrangement probe, Abbott Molecular, Abbott Park, IL) as previously described (21).

Clinical and demographic data are summarized (Supplementary Table S1) according to the BCP-ALL subtypes pro-B ALL, c-ALL, and pre-B ALL. The frequency of CNA-tested patients in each subgroup of BCP-ALL reflects the profile of the leftover biological materials.

The age at diagnosis and white blood cell (WBC) count were the criteria for assigning prognostic risk of ALL, according to the National Cancer Institute (NCI): (1) high risk (NCI-HR), WBC count greater than 50×10^9 cells/μL, age 1 year or younger, or age 10 years or older, and (2) standard risk (NCI-SR), WBC count 50×10^9 cells/μL or less, or between 1 and 10 years of age. Additionally, any patients with the *BCR-ABL1* or *MLL-AFF1* gene fusions were assigned to the NCI-HR group.

Patients were classified into six subgroups according to the presence of fusion genes and/or chromosomal abnormalities that define cytogenetic risk groups—hyperdiploidy, *ETV6-RUNX1*, *TCF3-PBX1*, *MLL*-r, and *BCR-ABL1*—and one additional subgroup entitled *Other* that comprised cases that were absent of any of the abnormalities listed.

Informed consent was obtained in accordance with the Declaration of Helsinki, and both data collection and laboratory procedures were evaluated and approved by the research ethics committee of Instituto Nacional de Câncer–INCA (#005/06 and 116/12).

Copy number alteration analysis

DNA obtained from BM or PB samples was isolated using the DNA MiniBlood kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. DNA was analyzed by the SALSA multiplex ligation-dependent probe amplification (MLPA) kit (P335-A4) (MRC-Holland, Amsterdam, the Netherlands) as previously described (22). This kit includes probes for *IKZF1* (7p12.2, exons 1 to 8), *CDKN2A* (9p21.3, exons 2 and 4), *CDKN2B* (9p21.3, exon 2), *PAX5* (9p13.2, exons 1, 2, 5, 6, 8, and 10), *EBF1* (5q33.3, exons 1, 10, 14, and 16), *ETV6* (12p13.2, exons 1, 2, 3, 5, and 8), *BTG1* (12.q22, exon 1 and 2), *RB1* (13q14.2*,* exons 6, 14, 19, 24, and 29), *SHOX* (Xp22.33)*, CSF2RA* (Xp22.33, exon 16), *IL3RA* (Xp22.33, exon 1), and *CRLF2* (Xp22.33, exon 4). The MLPA probes sizes, chromosomal position and sequences are provided in Supplementary Table S2. The sensitivity of MLPA kit P335-A4 was tested by Schwab et al. using serial dilutions, and the results showed that the MLPA assay detects deletions in samples in which the proportion of cell line DNA was greater than 30%. Furthermore, the MLPA kit is able to detect both monoallelic and biallelic in those samples in which the proportion of cell line DNA was greater than 50% and 75%, respectively (22). The PCR fragments were separated by capillary electrophoresis on a Life Technologies 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). MLPA data were analyzed using GeneMarker version 2.2.0 (SoftGenetics, State College, PA). The relative copy number was obtained after the normalization of peaks against controls. Values between 0.75 and 1.3 were considered to be within the normal range. Values below 0.75 or above 1.3 indicated deletion or gain, respectively. Values below 0.25 indicated biallelic deletion. *IKZF1* deletion results were confirmed using a P202 *IKZF1* SALSA MLPA kit. Status of *CDKN2A* and *CDKN2B* were combined to *CDKN2A/2B*, and we considered at least one probe gene deleted as *CDKN2A/2B* deleted. *CRLF2*, *CSF2RA*, *IL3RA,* and *SHOX* were combined to PAR1 region deletion.

Statistical analysis

To compare the distribution of alterations between cytogenetic subgroups and clinical variables, we used the χ^2 test (two-sided) or the Fisher exact test when the expected values were less than 5. *P* values less than 0.05 were considered statistically significant. The disease risk associated with CNA occurrence across overall and subgroups of patients was determined by calculating odds ratios (ORs) with a 95% confidence interval (CI). All statistical analyses were performed using the Statistical Product and Services Solutions (SPSS) statistical package, version 18.0 (IBM, Armonk, NY).

Results

Frequency of copy number abnormalities in children with BCP-ALL

The demographic, clinical, and laboratory characteristics of 274 children with BCP-ALL are shown in Supplementary Table S1. Participants were distributed equally with respect to gender, predominance of CD10+ settings, low WBC count ($P < 0.01$), and NCI-SR group ($P < 0.01$). The median age of the cohort was 4.7 years (range < 0.1–17.6 y); 89.4% of the patients were over 1 year of age. The molecular tests performed were those standardized for BCP-ALL diagnosis according to, as follows: *ETV6-RUNX1* (n = 226), *TCF3-PBX1* (n = 215), *BCR-ABL1* (n = 229), DI/hyperdiploidy (n = 155), and *MLL*-r (n = 144). The number of cases in which each abnormality was tested differed because of the mutual exclusiveness of abnormalities (for instance, *ETV6-RUNX1*–positive cases are unlikely to harbor *BCR-ABL1*), and in a few cases there was only limited material available for all testing. DI analyses and *MLL*-r detection were performed in fewer cases because of the dependence of viable cells for cytometry or FISH, respectively.

Overall, 83% of the patients tested (228/274) harbored at least one abnormality (either deletion or amplification) in the following genes—*IKZF1*, *CDKN2A*/*2B*, *PAX5*, *EBF1*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region—whereas the remaining 17% (46/274) of the patients had none of these abnormalities. Deletions were more frequent than amplifications (39.0% vs. 25.4%, respectively). Simultaneous aberrations in different genes were observed. Both gene amplifications and deletions were found in 15% of the cases (two abnormalities), and 51.5% had alterations in three or more of the genes listed (Supplementary Figure S1).

The occurrence of gene deletions according to age, WBC count and NCI risk group are shown in Figures 1A–1C. More than one gene deletion occurred simultaneously in the same case. Three or more deletions were significantly more frequent in children older than 10 years ($P = 0.03$) with a high WBC count ($P = 0.02$).

The frequency of deletions in *IKZF1*, *CDKN2A*/*2B*, *PAX5*, *EBF1*, *ETV6*, *BTG1*, *RB1*, and the PAR1 region according to demography, percentage of blast cells, NCI risk group, and known molecular-cytogenetic abnormalities are shown in Tables 1 and 2. Overall, deletions of *CDKN2A/2B* (31.8%), *PAX5* (25.2%), *ETV6* (22.6%), and *IKZF1* (19.3%) were the most frequent, whereas deletions of the PAR1 region (2.9%) were the least frequent. The *IKZF1* and *CDKN2A/2B* frequencies were higher in older children (34.1%, $P = 0.022$; 47.7%, $P = 0.003$, respectively), whereas PAR1 deletions were absent in children older than 10 years ($P = 0.029$). There were no differences in the frequency of gene deletions according to the percentage of blast cells. Comparing the distribution of the deletions between NCI-SR and NCI-HR frequencies of the specific genes, *IKZF1* and *EBF1* deletions were more frequent in the NCI-HR group (26.5% and 7.6%, respectively, $P < 0.05$), whereas *ETV6* deletions were mainly found in patients classified as NCI-SR (28.9%) ($P = 0.01$). Both *IKZF1* and *EBF1* deletions were associated with high WBC count ($P < 0.02$). The frequency of specific gene deletions according to demographic features is represented by Circos plots (23) (Figures 1D–1F).

Deletions in key functional pathways in relation to demography, clinical and cytogenetic features

The demographic and clinical characteristics of BCP-ALL cases and their association risks according to the presence or absence of gene deletions are shown in Table 3. The risk of harboring deletions increases with age (OR 4.36; 95% CI 1.60–11.88) and the presence of CD10 positivity (OR 2.73; 95% CI 1.37–5.46). The magnitude of risk association varies according to deletions in lymphoid differentiation genes (*IKZF1*, *PAX5*, *ETV6*, *EBF1*), cell cycle regulation genes (*CDKN2A/2B*, *BTG1*, *RB1)*, cytokine receptor genes (*CRLF2*, *IL3RA*, *CSF2RA*, *SHOX*), and BCP-ALL with chromosomal abnormalities (*ETV6-RUNX1* and DI/hyperdiploidy). *ETV6-RUNX1* and DI/hyperdiploidy subgroups displayed an increased risk of harboring deletions in lymphoid differentiation genes, whereas patients classified into the *Other* subgroup were negatively associated with deletions in the cytokine receptor genes (OR 0.41; 95% CI 0.20–0.83). BCP-ALL cases with *MLL*-r were significantly less prone to harbor deletions (OR 0.29; 95% CI 0.13–0.68).

Concomitance of gene aberrations in BCP-ALL

Multiple gene deletions occurred in the same patients (shown in Supplementary Figure S1), and the profiles of simultaneous deletions are shown in Figure 2. Deletions of *CDKN2A/2B* coincided significantly with deletions in *PAX5* ($P < 0.01$) and *IKZF1* ($P < 0.05$). *IKZF1* deletions were also found concomitantly with *BTG1* and *EBF1* deletions ($P < 0.05$ and $P < 0.01$, respectively). *ETV6* deletions overlapped with *PAX5*, *BTG1*, and *EBF1* deletions ($P < 0.01$). The overlap between the presence of *RB1* deletions and other abnormalities was significantly correlated only in patients with *EBF1* and PAR1 region deletions ($P < 0.01$).

In Figure 2 and Table 2, the frequencies of gene deletions according to molecular-cytogenetic subgroups are shown. Patients with *ETV6-RUNX1* or DI/hyperdiploidy and those classified as *Other* present a similar frequency profile of gene deletions. Patients with *TCF3-PBX1* present with deletions mainly in *CDKN2A/2B* (30.8%), *PAX5* (30.8%), and *RB1* (15.4%), whereas BCP-ALL with *BCR-ABL1* have a predominance of deletions in *IKZF1, PAX5,* and *CDKN2A/2B* (33.3%). *ETV6* deletions were more frequent in patients with *ETV6-RUNX1* ($P < 0.01$). *MLL*-r was mutually exclusive with *EBF1* deletions ($P < 0.01$). *MLL*-r patients had a lower frequency of

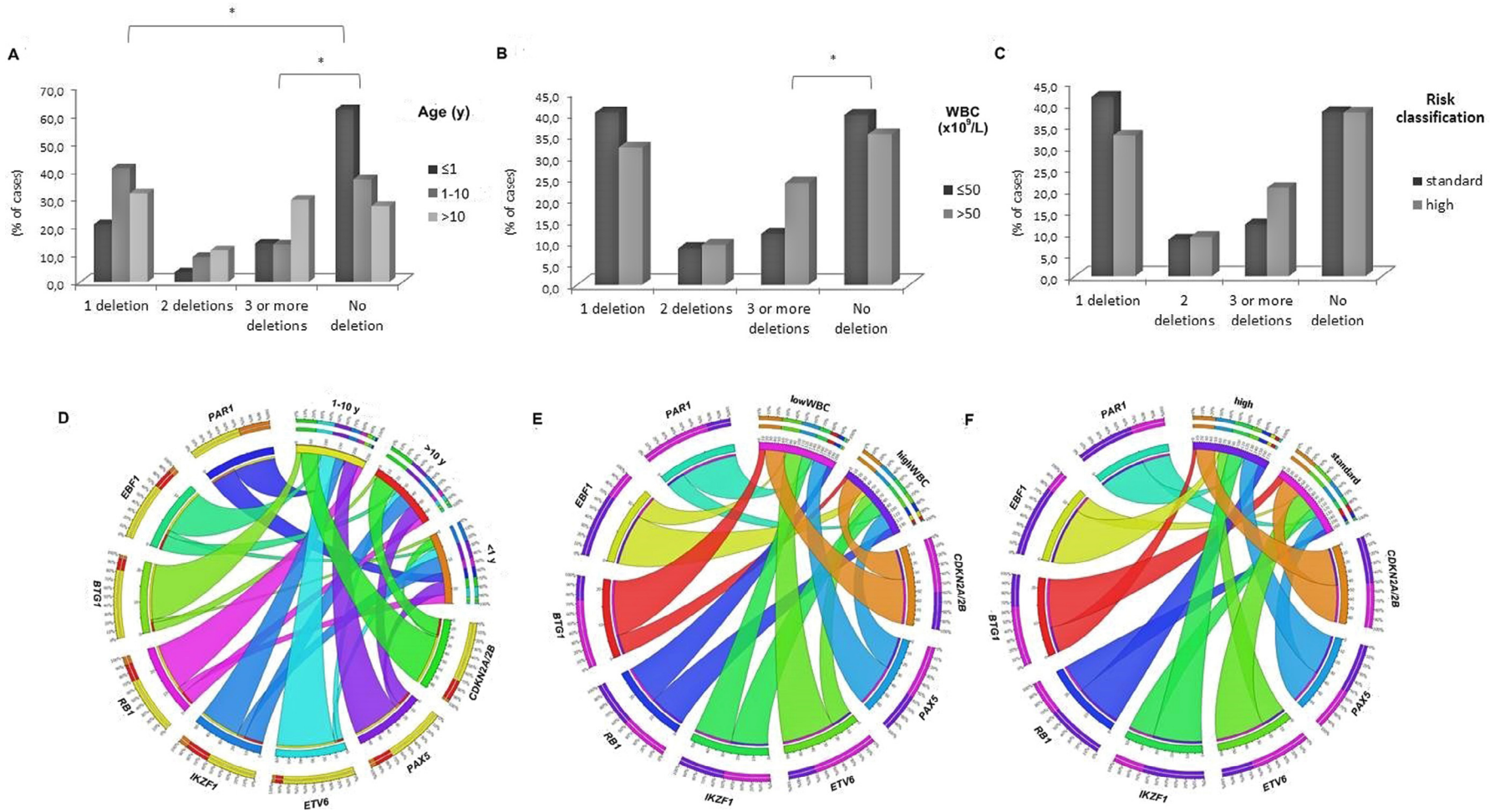

Figure 1 Frequency of patients (in percentage) with gene deletions according to age, WBC count, and the NCI risk group. The occurrence of specific gene deletions according to (A and D) age at diagnosis, (B and E) WBC count, and (C and F) risk classification is visualized using Circos software (23). *Variables significantly correlated.

Table 1 Frequency of specific gene deletions according to demographic characteristics of patients, Brazil, 2004–2011

		Age, y				WBC count			Blast cells count			NCI risk group		
	Total n (%)	≤1 n (%)	1–10 n (%)	≥10 n (%)	χ^2	Low n (%)	High n (%)	χ^2	≤50% n (%)	>50% n (%)	χ^2	SR n (%)	HR n (%)	χ^2
IKZF1					0.022			0.021			0.561	18 (12.7)	35 (26.5)	0.004
deleted	53 (19.3)	6 (20.7)	32 (15.9)	15 (34.1)		27 (15.3)	26 (26.8)		16 (21.6)	37 (18.5)		124 (87.3)	97 (73.5)	
not deleted	221 (80.7)	23 (79.3)	169 (84.1)	29 (65.9)		150 (84.7)	71 (73.2)		58 (78.4)	163 (81.5)				
CDKN2A/2B					0.003			0.254			0.307			0.588
deleted	87 (31.8)	3 (10.3)	63 (31.3)	21 (47.7)		52 (29.4)	35 (36.1)		20 (27.0)	67 (33.5)		43 (30.3)	44 (33.3)	
not deleted	187 (68.2)	26 (89.7)	138 (68.7)	23 (52.3)		125 (70.6)	62 (63.9)		54 (73.0)	133 (66.5)		99 (69.7)	88 (66.7)	
Type of deletion					<0.001			0.341			0.568			0.110
monoallelic	80 (92.0)	3 (100.0)	62 (98.4)	15 (71.4)		49 (94.2)	31 (88.6)		19 (95.0)	61 (91.0)		42 (86.4)	38 (86.4)	
biallelic	7 (8.0)	0 (0.0)	1 (1.6)	6 (28.6)		3 (5.8)	4 (11.4)		1 (5.0)	6 (9.0)		1 (2.3)	6 (13.6)	
PAX5					0.235			0.183			0.608			0.295
deleted	69 (25.2)	5 (17.2)	49 (24.4)	15 (34.1)		40 (22.6)	29 (29.9)		17 (23.0)	52 (26.0)		32 (22.5)	37 (28.0)	
not deleted	205 (74.8)	24 (82.8)	152 (75.6)	29 (65.9)		137 (77.4)	68 (70.1)		57 (77.0)	148 (74.0)		110 (77.5)	95 (72.0)	
PAR1 region					0.029			0.532			0.897			0.540
deleted[a]	8 (2.9)	3 (10.3)	5 (2.5)	0 (0.0)		6 (3.4)	2 (2.1)		2 (2.7)	6 (3.0)		3 (2.3)	5 (3.5)	
not deleted	266 (97.1)	26 (89.7)	196 (97.5)	44 (100.0)		171 (96.6)	95 (97.9)		72 (97.3)	194 (97.0)		137 (97.7)	137 (96.5)	
EBF1					0.756			0.009			0.744			0.034
deleted	13 (4.7)	1 (3.4)	9 (4.5)	3 (6.8)		4(2.3)	9 (9.3)		3 (4.1)	10 (5.0)		3 (2.1)	10 (7.6)	
not deleted	261 (95.3)	28 (96.6)	192 (95.5)	41 (93.2)		173 (97.7)	88 (90.7)		71 (95.9)	190 (95.0)		139 (97.9)	122 (92.4)	
ETV6					0.021			0.233			0.934			0.010
deleted	62 (22.6)	3 (10.3)	54 (26.9)	5 (11.4)		44 (24.9)	18 (18.6)		17 (23.0)	45 (22.5)		41 (28.9)	21 (15.9)	
not deleted	212 (77.4)	26 (89.7)	147 (73.1)	39 (88.6)		133 (75.1)	79 (81.4)		57 (77.0)	155 (77.5)		101 (71.1)	111 (84.1)	
BTG1					0.507			0.329			0.917			0.179
deleted	23 (8.4)	1 (3.4)	19 (9.5)	3 (6.8)		17 (9.6)	6 (6.2)		6 (8.1)	17 (8.5)		15 (10.6)	8 (6.1)	
not deleted	251 (91.6)	28 (96.6)	182 (90.5)	41 (93.2)		160 (90.4)	91 (93.8)		68 (91.9)	183 (91.5)		127 (89.4)	124 (93.9)	
RB1					0.771			0.125			0.941			0.113
deleted	29 (10.6)	3 (10.3)	20 (10.0)	6 (13.6)		15 (8.5)	14 (14.4)		8 (10.8)	21 (10.5)		11 (7.7)	18 (13.6)	
not deleted	245 (89.4)	26 (89.7)	181 (90.0)	38 (86.4)		162 (91.5)	83 (85.6)		66 (89.2)	179 (89.5)		131 (92.3)	114 (86.4)	
Total	274	29	201	44		177	97		74	200		142	132	

[a] These results are suggestive of the presence of *P2RY8-CRLF2* fusion. Abbreviations: n, number; HR, high risk; SR, standard risk. Percentages were calculated with the values of the column. WBC count was stratified as low ($\leq 50 \times 10^9$ cells/L) and high ($>50 \times 10^9$ cells/L).

Table 2 Frequency of specific gene deletions according to molecular-cytogenetic subgroups of patients, Brazil, 2004–2011

	Cytogenetic subgroups											
	ETV6-RUNX1		DI/hyperdiploidy		*TCF3-PBX1*		*Other*		*BCR-ABL1*		*MLL*-r	
	Presence	Absence	Presence	Absence	Presence	Absence	Yes	No	Presence	Absence	Presence	Absence
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
IKZF1												
deleted	6 (17.6)	37 (19.3)	7 (25.0)	23 (18.1)	0 (0.0)	42 (20.5)	22 (23.2)	19 (16.5)	3 (33.3)	41 (18.6)	3 (9.7)	21 (18.6)
not deleted	28 (82.4)	155 (80.7)	21 (75.0)	104 (81.9)	13 (100.0)	163 (79.5)	73 (76.8)	96 (83.5)	6 (66.7)	179 (81.4)	28 (90.3)	92 (81.4)
CDKN2A/2B												
deleted	13 (38.2)	58 (30.2)	8 (28.6)	37 (29.1)	4 (30.8)	65 (31.7)	36 (37.9)	31 (27.0)	3 (33.3)	71 (32.3)	3 (9.7)	37 (32.7)
not deleted	21 (61.8)	134 (69.8)	20 (71.4)	90 (70.9)	9 (69.2)	140 (68.3)	59 (62.1)	84 (73.0)	6 (66.7)	149 (67.7)	28 (90.3)	76 (67.3)
Type of deletion												
Monoallelic	13 (100.0)	52 (89.7)	6 (75.0)	37 (100.0)	4 (100.0)	59 (90.8)	37 (100.0)	6 (75.0)	3 (100.0)	64 (90.1)	3 (100.0)	35 (94.6)
Biallelic	0 (0.0)	6 (10.3)	2 (25.0)	0 (0.0)	0 (0.0)	6 (9.2)	0 (0.0)	2 (25.0)	0 (0.0)	7 (9.9)	0 (0.0)	2 (5.4)
PAX5												
deleted	8 (23.5)	49 (25.5)	10 (35.7)	33 (25.0)	4 (30.8)	52 (25.4)	31 (32.6)	26 (22.6)	3 (33.3)	56 (25.5)	1 (3.2)	31 (27.4)
not deleted	26 (76.5)	143 (74.5)	18 (64.3)	94 (74.0)	9 (69.2)	153 (74.6)	64 (67.4)	89 (77.4)	6 (66.7)	164 (74.5)	30 (96.8)	82 (72.6)
PAR1 region												
deleted[a]	1 (2.9)	6 (3.1)	0 (0.0)	3 (2.4)	0 (0.0)	6 (2.9)	2 (3.2)	3 (2.6)	0 (0.0)	8 (3.6)	2 (6.5)	4 (3.5)
not deleted	33 (97.1)	186 (96.9)	28 (100.0)	124 (97.6)	13 (100.0)	199 (97.1)	92 (96.8)	112 (97.4)	9 (100.0)	212 (96.4)	29 (93.5)	109 (96.5)
EBF1												
deleted	2 (5.9)	10 (5.2)	0 (0.0)	5 (3.9)	0 (0.0)	11 (5.4)	9 (9.5)	2 (1.7)	0 (0.0)	12 (5.5)	0 (0.0)	3 (2.7)
not deleted	32 (94.1)	182 (94.8)	28 (100.0)	122 (96.1)	13 (100.0)	194 (94.6)	86 (90.5)	113 (98.3)	9 (100.0)	208 (94.5)	31 (100.0)	110 (97.3)
ETV6												
deleted	18 (52.9)	31 (16.1)	7 (25.0)	34 (26.8)	0 (0.0)	49 (23.9)	21 (22.1)	28 (24.3)	0 (0.0)	52 (23.6)	3 (9.7)	28 (24.8)
not deleted	16 (47.1)	161 (83.9)	21 (75.0)	93 (73.2)	13 (100.0)	156 (76.1)	74 (77.9)	87 (75.7)	9 (100.0)	168 (76.4)	28 (90.3)	85 (75.2)
BTG1												
deleted	4 (11.8)	14 (7.3)	1 (3.6)	21 (16.5)	1 (7.7)	17 (8.3)	10 (10.5)	7 (6.1)	0 (0.0)	18 (8.2)	1 (3.2)	7 (6.2)
not deleted	30 (88.2)	178 (92.7)	27 (96.4)	106 (83.5)	12 (92.3)	188 (91.7)	85 (89.5)	108 (93.9)	9 (100.0)	202 (91.8)	30 (96.8)	106 (93.8)
RB1												
deleted	2 (5.9)	22 (11.5)	4 (14.3)	11 (8.7)	2 (15.4)	23 (11.2)	13 (13.7)	10 (8.7)	0 (0.0)	25 (11.4)	2 (6.5)	8 (7.1)
not deleted	32 (94.1)	170 (88.5)	24 (85.7)	116 (91.3)	11 (84.6)	182 (88.8)	82 (86.3)	105 (91.3)	9 (100.0)	195 (88.6)	29 (93.5)	105 (92.9)
Total	34	192	28	127	13	205	95	115	9	220	31	113

[a] These results are suggestive of the presence of the *P2RY8-CRLF2* fusion.

Percentages were calculated with the values of the column.

Table 3 Comparisons between the presence of deletions in key functional pathways and demographic and laboratory characteristics of BCP-ALL cases, Brazil, 2004–2011

	No deletions	Deletions		Lymphoid differentiation gene deletions[a]		Cell cycle regulation/tumor gene deletions suppression[b]		Cytokine receptor gene deletions[c]	
	n	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)
Age, y									
≤1	18	11	1[d]	10	1[d]	6	1[d]	5	1[d]
1–10	74	127	2.81 (1.26–6.27)	101	2.46 (1.07–5.63)	91	3.69 (1.39–9.77)	46	2.24 (0.78–6.44)
>10	12	32	4.36 (1.60–11.88)	28	4.20 (1.50–11.73)	28	7.00 (2.23–22.00)	4	1.20 (0.27–5.40)
WBC ($\times 10^9$ cell/L)									
≤10	41	37	1[d]	48	1[d]	35	1[d]	24	1[d]
10–50	28	70	2.77 (1.48–5.17)	61	1.86 (1.01–3.43)	53	2.22 (1.17–4.22)	32	1.95 (0.96–3.99)
≥50	35	63	1.99 (1.09–3.66)	65	1.59 (0.88–2.85)	54	1.81 (0.97–3.36)	28	1.37 (0.67–2.77)
ALL subtype									
CD10-	23	16	1[d]	15	1[d]	9	1[d]	5	1[d]
CD10+	81	154	2.73 (1.37–5.46)	124	2.35 (1.16–4.77)	116	3.66 (1.61–8.31)	50	2.84 (1.01–7.95)
ETV6-RUNX1									
absence	80	112	1[d]	88	1[d]	81	1[d]	38	1[d]
presence	8	26	2.32 (1.00–5.39)	24	2.73 (1.16–6.42)	19	2.35 (0.97–5.67)	7	1.84 (0.62–5.45)
DI/hyperdiploidy									
no	52	75	1[d]	17	1[d]	14	1[d]	9	1[d]
yes	7	21	2.08 (0.82–5.25)	66	28.84 (11.13–74.74)	51	27.06 (10.09–72.55)	23	18.98 (6.30–57.20)
TCF3-PBX1									
absence	75	130	1[d]	106	1[d]	94	1[d]	41	1[d]
presence	8	5	0.36 (0.11–1.14)	4	0.35 (0.10–1.22)	5	0.50 (0.16–1.59)	2	0.46 (0.09–2.25)
Other									
no	48	67	1[d]	53	1[d]	49	1[d]	66	1[d]
yes	30	65	1.55 (0.88–2.74)	57	1.72 (0.95–3.10)	48	1.57 (0.86–2.87)	17	0.41 (0.20–0.83)
MLL-r									
absence	43	70	1[d]	60	1[d]	49	1[d]	21	1[d]
presence	21	10	0.29 (0.13–0.68)	8	0.27 (0.11–0.67)	6	0.25 (0.09–0.68)	6	0.59 (0.21–1.67)
BCR-ABL1									
absence	81	139	1[d]	115	1[d]	102	1[d]	48	1[d]
presence	4	5	0.73 (0.19–2.79)	4	0.70 (0.17–2.90)	4	0.79 (0.19–3.27)	2	0.84 (0.15–4.78)
NCI risk group									
standard	53	89	1[d]	69	1[d]	64	1[d]	32	1[d]
high	51	81	0.95 (058–1.54)	70	1.05 (0.63–1.75)	61	0.99 (0.59–1.67)	23	0.74 (0.39–1.44)

[a] Lymphoid differentiation group included the *IKZF1*, *PAX5*, *ETV6* and *EBF1* genes.
[b] Cell cycle regulation and tumoral suppression group included the *CDKN2A*, *CDKN2B*, *BTG1,* and *RB1* genes.
[c] Cytokine receptors group included the *CRLF2*, *IL3RA*, *CSF2RA* and *SHOX* genes.
[d] Used as reference.

deletions compared with those of the other groups. Among the 10 *MLL*-r patients with deletions, different *MLL* translocation partner genes (TPGs) were found: *MLL-AFF1* (n = 6), *MLL-MLLT1* (n = 1), *MLL-MLLT3* (n = 1), and *MLL-MLLT10* (n = 2). Interestingly, three *MLL*-r patients harbored *IKZF1* deletions, and all three deletions compromised exon 1 of *IKZF1* (Supplementary Table S3); furthermore, the TPG was *MLLT10* in two of the three cases. *EBF1* deletion was significantly associated with the *Other* subgroup ($P < 0.05$).

Discussion

This study presents the result of an analysis on Brazilian BCP-ALL patients in which CNAs in selected genes involved in ALL pathogenesis were investigated using MLPA. The differences in CNAs vary according to each primary genetic abnormality as noted by Schwab et al. (9). The frequency distributions of gene deletions found in our study are rather similar to those of previous reports regarding the associations with cytogenetic subgroups and clinical features but the frequencies vary in some settings (5,9,10,18,24). The most frequent genes with CNAs were *CDKN2A/2B*, *PAX5*, *ETV6*, and *IKZF1*, whereas deletions in the PAR1, *BTG1*, and *EBF1* regions were less frequent. The number of gene deletions was associated with age strata and WBC counts and was also correlated with NCI risk group. *IKZF1* and *EBF1* were frequently found in the NCI-HR group and *ETV6* in the NCI-SR group. The increased risk of harboring deletions for older patients was observed for genes involved in lymphoid differentiation, cell cycle regulation, and tumor gene suppression, but not for the genes grouped as cytokine receptors. The frequency of gene

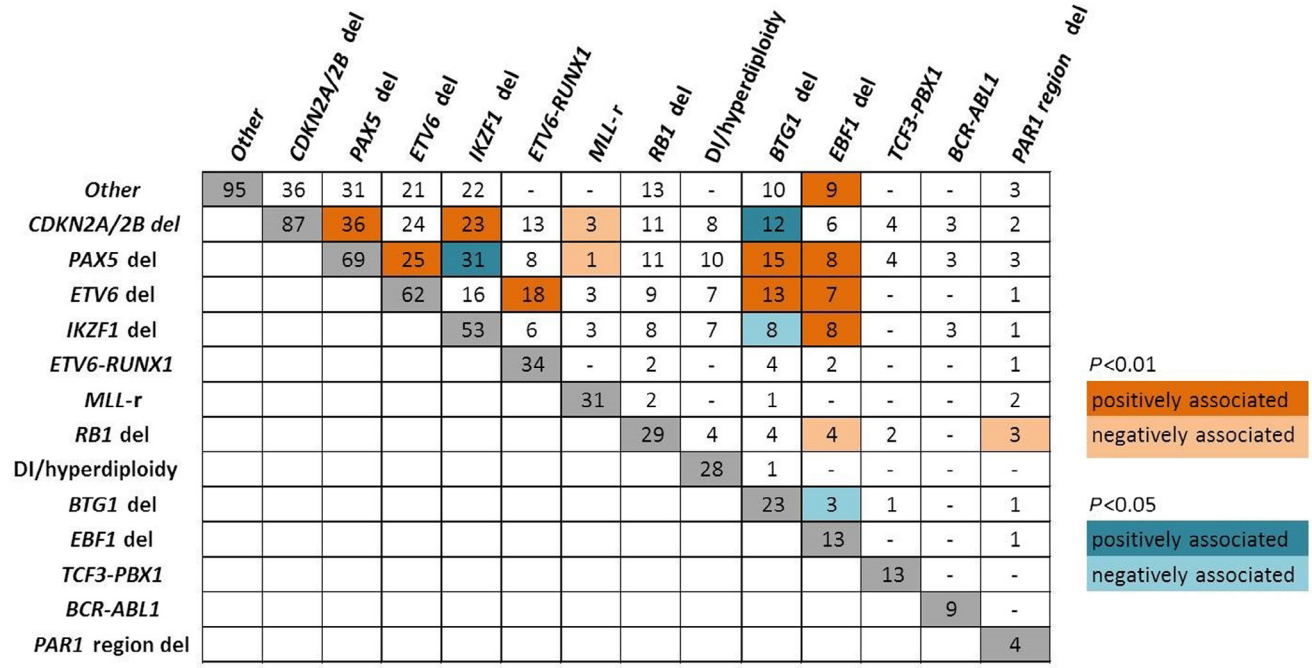

Figure 2 Concomitance of gene deletions in 274 children with BCP-ALL. Grey boxes indicate the total number of patients with a respective genetic alteration. Other boxes indicate the number of patients with two specific abnormalities. Orange or blue boxes indicate a significant overlap association ($P < 0.01$ or $P < 0.05$), respectively.

deletions also varied according to the cytogenetic subgroup. First, we found a high frequency of deletions in patients with favorable cytogenetic subgroups (*ETV6-RUNX1* and DI/hyperdiploidy) often affecting the nonrearranged allele of *ETV6*. The overlap observed between the presence of *ETV6* deletions and the gene fusion *ETV6-RUNX1* confirms that MLPA can be a helpful technique for tracking this alteration at the genomic level in cases of failed detection by FISH or reverse transcription PCR. *CDKN2A/2B*, *PAX5*, and *BTG1* were also found in smaller proportions. These results corroborate previous reports suggesting that this type of alteration, rather than point mutations, collaborate in the development of ALL in these subgroups (5,25–27).

The subgroup defined as *Other* displayed a high frequency of gene deletions, which highlights the importance of screening not only for the common chromosomal abnormalities in BCP-ALL. The identification of *CDKN2A/2B*, *PAX5*, *IKZF1,* or *CRLF2* deletions in these patients may contribute to the characterization of BCP-ALL subgroups that have additional submicroscopic alterations and are associated with relapse and poor outcome (1,12,28). *TCF3-PBX1* patients had a low frequency of CNAs, which were restricted to the *CDKN2A/2B*, *PAX5*, *RB1,* and *BTG1* genes. Compared with the other cytogenetic subgroups, *TCF3-PBX1* patients displayed a high frequency of deletions in cell cycle regulators (*RB1* and *BTG1* genes). These findings align with reports of deletions at 13q and 9p in this cytogenetic subgroup and show that MLPA is also useful to validate data obtained by FISH, comparative genomic hybridization arrays or single nucleotide polymorphism arrays that cannot identify submicroscopic deletions (5,8,29).

Compared with other BCP-ALL genetic subgroups, patients with *MLL*-r are known to harbor a low frequency of DNA copy number aberrations (5,30). This same low frequency of deletions has been observed in our study. However, despite this low frequency, *IKZF1* deletions have been reported in *MLL*-r cases. Mullighan et al. and Schwab et al. reported 9.1% and 8% of *IKZF1* deletions in *MLL*-r cases, respectively, which is similar to that of our study (9.7%). Interestingly, all three deletions found in our study comprised exon 1 of *IKZF1,* and the TPG was *MLLT10* in two of the three cases. Although *IKZF1* deletions affecting only exon 1 are not likely to result in any functional consequence because this is a noncoding exon, it is possible that the normal function of Ikaros as a tumor suppressor is deregulated by the presence of *MLL-MLLT10* in these cases. As suggested by Greif et al, the interaction of Ikaros with *MLLT10* is a pathway that is possibly disturbed in *MLL-MLLT10* leukemias (31).

Overlapping CNA abnormalities that occur simultaneously in the same patients was also explored, and 73.9% of the patients had concomitant gene deletions. *PAX5* deletion was frequently accompanied by deletion in *CDKN2A/2B* on 9p21.3 adjacent to *PAX5* (9p13.2), *IKZF1* (7p12.2), and *ETV6* (12p13.2). Concurrent deletion of *PAX5* and *CDKN2A/2B* suggests that they are common targets in the pathogenesis of BCP-ALL (9,32). The concurrent presence of *IKZF1* deletions and at least one additional deletion in the *PAX5*, *CDKN2A/2B,* or PAR1 region in the absence of *ERG* deletions has been reported. These patients were grouped as IKZF1plus by Dagdan et al. (33). In this study, we confirm the magnitude of the association between *PAX5* or *CDKN2A/2B* and *IKZF1*. In contrast, we found a lower frequency of double-positive for PAR1 region and *IKZF1* deletions; nevertheless, *ERG* deletions have not been analyzed. This study is the first that verifies the significance of submicroscopic alterations on the prognosis of BCP-ALL in Brazil. The impact of *IKZF1*

deletions has been extensively studied, and it is increasingly evident that *IKZF1* deletions are predictive of prognosis in BCP-ALL dependent on their association with specific subgroups, such as *BCR-ABL1,* being NCI-HR (13,17,18), or even dependent on the presence of other concomitant deletions (33).

There are limitations in the present analysis that should be mentioned. MLPA assay has a limited sensitivity: the probes are specific for some regions, and some abnormalities such as point mutations cannot be detected by this technique. In this study, cases were selected according to the viability of the samples and a minimum percentage of blast cell count of 30% because of the MLPA detection limit. On the other hand, MLPA has some advantages over other methodologies. It is a multiplex amplification of different regions, which enables the detection of small focal deletions at the exon level through a single reaction. Also, the use of multiple target probes that are located in the same chromosomal region or gene increases the accuracy and reliability of calls and increases the sensitivity for the detection of a CNA in each specific region.

In addition to the limitations cited, after stratifying the BCP-ALL subsets, the representativeness of the subsets affecting the statistical power could be questioned. However, the potential of the results should be considered taking into account that the associations observed are concordant with previously published studies. The data thus indicate good validity and sensitivity of the MLPA tests. As previously noted, the MLPA results do not rule out the value of somatic alterations already validated through cytogenetic methods (karyotype, FISH, and array comparative genomic hybridization) as prediction markers but rather add more information to the clinical relevance of CNAs in BCP-ALL subtypes.

Summarily, our results corroborate previous GW studies and emphasize the need for including screening of submicroscopic alterations as additional markers for risk stratification, especially in standard risk patients.

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Supplementary data

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