Research Article

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IKZF1 Gene in Childhood B-cell Precursor Acute Lymphoblastic Leukemia: Interplay between Genetic Susceptibility and Somatic Abnormalities

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Abstract

SNPs in *IKZF1* are associated with inherited susceptibility to B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Besides, somatic copy number abnormalities (CNA) in genes related to lymphopoiesis (e.g., *IKZF1*, *CDKN2A/B*, *BTG1*) impact patient's outcome. Therefore, this study aimed to investigate an association between germline susceptibility and CNAs in BCP-ALL. The *IKZF1* SNPs (rs11978267 and rs4132601) were genotyped in 276 cases and 467 controls. Bone marrow samples were used to determine the presence of somatic abnormalities. The *IKZF1* transcript levels were quantified and associated with the SNPs and CNAs. Categorical variables were compared by χ^2 test. ORs were estimated with unconditional logistic regression with 95% confidence interval (CI). The variant allele of *IKZF1* rs4132601 conferred increased risk of BCP-ALL (OR, 2.09; 95% CI, 1.16–3.74). Individuals with either rs11978267 or rs4132601 had an increased risk for harboring *IKZF1* deletion (OR, 2.80; 95% CI, 1.25–6.23 and OR, 2.88; 95% CI, 1.24–6.69, respectively). Increased risks were observed for individuals harboring both *IKZF1* and *BTG1* deletions (OR, 4.90; 95% CI, 1.65–14.55, rs11978267 and OR, 5.80; 95% CI, 1.94–17.41, rs4132601). Germline genetic variation increases the risk for childhood ALL in general, but also acts as a susceptibility factor bound for risk of specific somatic alterations. These findings provide new insight into the development of childhood ALL regarding causal variants and the biological basis of the risk association, offering the opportunity for future tailored research. *Cancer Prev Res; 10(12); 738–44. ©2017 AACR.*

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Introduction

Upon the advent of high-resolution genome-wide analysis, focal deletions, amplifications, point mutations, and rearrangements in genes encoding normal B-cell development regulators were identified in approximately 40% of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients (1, 2). Aberrant copy number abnormalities (CNA) located in the *IKZF1* gene have been identified in approximately 15% of BCP-ALL cases; *IKZF1* deletions were consistently associated with two high-risk subtypes, *BCR-ABL1* (3) and *BCR-ABL1*-like cases (4). These aberrations are somatic mutations demonstrated by their absence in remission samples.

On the other hand, common germline allelic variants in *IKZF1* (7p12.2), *ARID5B* (10q21.2), and *CDKN2A* (9p21) have been repeatedly and significantly associated with childhood ALL risk (5–7). Interestingly *IKZF1* rs11978267 and rs4132601 are among the most replicable SNPs to be associated with an increased risk of childhood BCP-ALL (5, 6). Although the *IKZF1* somatic alterations result in dominant negative activity and/or loss-of-function mutations accelerating the onset of BCP-ALL in murine models (8), the functional significance of the *IKZF1* SNP is not fully elucidated. The mRNA expression was evaluated in transformed Epstein–Barr virus lymphocytes, and significantly lower dose-dependent expression was found with each copy of the variant *IKZF1* allele (5).

The *IKZF1* deletions frequently cooccur with other CNAs in BCP-ALL (e.g., *CDKN2A/B*, *PAX5*, pseudoautosomal region 1 (PAR1) region, and *BTG1* deletions; refs. 9, 10), and these frequencies may vary depending on the types of *IKZF1* deletion (e.g., intragenic or complete gene deletion; ref. 11). The latter, *BTG1* (B-cell translocation gene 1), is a transcriptional coregulator that promotes B-cell differentiation and has been shown to contribute to leukemia development (10, 12). In this context, we aimed to investigate a possible association between inherited genetic susceptibility (*IKZF1* rs11978267 and rs4132601), *IKZF1* somatic deletions, and other cooperative somatic deletions in BCP-ALL.

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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Materials and Methods

Subjects

Samples from 743 children (276 BCP-ALL and 467 controls) were ascertained from 2004 to 2011 and included in this study. The eligibility criteria for BCP-ALL diagnosis and case selection have been previously described (10). Patients were 18 years old

or younger at the time of diagnosis. The diagnosis and classification were performed through several methods, including morphologic, multiparametric flow cytometry, and molecular-cytogenetics analyses according to standard criteria recommended by the World Health Organization (13). First, the case–control study included 246 BCP-ALL cases without any further selection. On the basis of the data obtained in the initial analysis regarding *IKZF1* and *BTG1* alterations, we also included an additional series of 31 cases that harbored *BTG1* deletions, aiming to analyze the risk association between genetic variants and acquisition of this specific type of abnormality. These supplementary *BTG1*-deleted cases were ascertained from 2012 to 2014 following the same eligibility criteria of the initial BCP-ALL series of cases.

The controls, which consisted of healthy children enrolled in the Brazilian Collaborative Study Group of Infant Acute Leukemia (BCSGIAL), were genotyped for rs11978267 in a previous study (14). BCSGIAL is a study group that investigates the acute leukemia risk factors linked to maternal environmental exposures, immunologic factors, and genetic susceptibility (15–17). Cases and controls were age-matched and from the same Brazilian regions.

Ethics

Data collection and laboratory procedures were evaluated and approved by the Ethics Committee of all participating hospitals. Data analysis was approved by the Ethics and Scientific Committee of Instituto Nacional de Câncer (Rio de Janeiro, Brazil; #33243214.7.0000.5274; #33709814.7.0000.5274).

Genotyping of *IKZF1* rs11978267 and rs4132601

For cases, DNA isolation was performed either using peripheral blood cells from remission, minimal residual disease, or diagnostic samples, whereas for controls, DNA was isolated from buccal cells. DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen) or with Oragene DNA technology (Genotek), respectively. Genotyping of *IKZF1* rs11978267 and rs4132601 for cases and controls was conducted by TaqMan allelic discrimination assays: C_199413_10 and C_26019772_10, respectively (Applied Biosystems). Genotypes were defined upon allelic discrimination charts in which the germline, heterozygous or mutant patterns were identified by comparison with reference controls for each allele. To ensure quality and reproducibility of the method, 10% of random samples were analyzed in duplicates and concordance was absolute.

The genomic regions of deletion breakpoints within *IKZF1* were described previously (18). On the basis of those breakpoint sequences, we retrieved a list of SNPs surrounding 100 bp of each deletion breakpoint using the UCSC Genome Browser. We analyzed data from LDlink 2.0 to compare the linkage disequilibrium between *IKZF1* rs11978267 or rs4132601 and the remaining SNPs (19).

Detection of gene copy number abnormalities

Genomic DNA was isolated from bone marrow or peripheral blood cells (blast percentage > 30%) of samples at patient diagnosis using the QIAamp DNA Blood Mini Kit (Qiagen). The presence of CNAs in *IKZF1* and other genes, such as *CDKN2A/B*, *PAX5*, *EBF1*, *BTG1*, and pseudoautosomal region 1 (PAR1), was analyzed by multiplex ligation-dependent probe amplification (SALSA MLPA P335, MRC Holland). Data were analyzed using Gene Marker software v2.2.0. The types of *IKZF1* deletions were confirmed using either long distance multiplex PCR, SALSA MLPA P202, or in-house designed MLPA, as described previously (10, 11, 18). *IKZF1* deletions affecting exons 4–7 and exons 4–5 were classified as "dominant-negative", whereas deletions of exons 1 or 2 were classified as "haploinsufficient". The CNA groups were defined as "*IKZF1* plus" (*IKZF1* deletions plus *CDKN2A/B*, *PAX5*, and/or PAR1 deletions) and "9p deletions" (concurrent deletions of *CDKN2A/B* and *PAX5*).

***IKZF1* gene expression analysis**

Total RNA was extracted from bone marrow samples at the time of diagnosis with TRIzol reagent (Life Technologies). After DNase treatment, 2 μg of purified RNA was used to synthesize the cDNA by SuperScript II Reverse Transcriptase (Life Technologies). The oligonucleotides sequences for *IKZF1* are described elsewhere (20). PCR reactions were performed in technical replicates using the BRYT GoTaq qPCR Master Mix (Promega BioSciences, LLC) and the Rotor Gene Q 2plex HRM Platform (QIAGEN). To determine the relative quantification of gene expression, the average quantification cycle (C_q) value of the endogenous control gene (*GAPDH*) was subtracted from the average experimental gene C_q values (ΔC_q). Next, gene expression data were presented as $2^{-\Delta Cq}$, according to previous recommendation (21). Expression level was considered altered when augmented or diminished $\geq$2-fold compared with the reference group (22).

Statistical analysis

The expected SNP frequency was calculated using the Hardy–Weinberg law based on the allele frequency in the control group. To compare the distribution of genotypes between cases and controls, the χ^2 test (two-sided) was used (or Fisher exact test when expected values were less than five). To avoid calculating multiple comparisons, we selected the best genetic model for this case–control study based on a genetic model selection strategy (23). Both *IKZF1* rs11978267 and rs4132601 best fitted the recessive genetic model for analyzing their relevance in BCP-ALL susceptibility. $P < 0.05$ was considered statistically significant. The disease risk associated with SNPs occurrence across overall or subgroups of patients was determined by calculating ORs with 95% confidence interval (CI). All statistical analyses were performed using the Statistical Product and Services Solutions statistical package, version 18.0 (SPSS Inc.).

The gene expression analysis was performed with GraphPad Prism 5 software. First, the Kolmogorov–Smirnov normality test was done for definition of appropriate statistical analyses. Then, expression data were compared according to genotype and demographic variables with the two-tailed Mann–Whitney test.

Results

The distribution of demographic and genetic variant data of controls ($n = 467$) and cases ($n = 246$) is shown in Supplementary Table S1. Control genotypes for both SNP loci were in Hardy–Weinberg equilibrium ($P > 0.05$). Among cases, there was a predominance of boys (54.1%), 1 to 9 years old at diagnosis (73.6%), and the most frequent subtype was CD10$^+$ ALL (86%). The *IKZF1* somatic status was established for cases, being 196 wild-type and 50 with deletions (Supplementary Table S1).

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First, the disease-associated risk has been evaluated (Supplementary Table S2). There was no risk association of developing BCP-ALL in patients with the variant allele of *IKZF1* rs11978267. However, there was a significant increased risk for patients with the homozygous variant allele of *IKZF1* rs4132601 for the codominant and recessive models (OR, 1.98; 95% CI, 1.13–3.48 and OR, 1.94; 95% CI, 1.13–3.34, respectively). As explained in the Materials and Methods section, both *IKZF1* rs11978267 and rs4132601 best fitted the recessive genetic model for analyzing their relevance in BCP-ALL susceptibility and, therefore, all subsequent analyses were performed on the basis of this genetic model.

The risk associations between genetic variants and BCP-ALL were further stratified by age range, as displayed in Table 1. Although the age at diagnosis was not associated with any disease risk for the variant allele of *IKZF1* rs11978267, the variant allele of *IKZF1* rs4132601 conferred a two times risk of developing BCP-ALL between 1 and 9 years old (OR, 2.09; 95% CI, 1.16–3.74).

To evaluate whether such risk associations could be explained by *IKZF1* expression, we quantified *IKZF1* transcripts and compared them with genotypes. Although the relationship between genotype and phenotype indicated that *IKZF1* expression might be influenced by its SNPs in a recessive pattern, our analyses did not show statistical differences between *IKZF1* SNPs and *IKZF1* transcript levels (Supplementary Fig. S1A–S1D).

IKZF1 expression was also tested by age strata and CD10 status. Infants presented 14- and 24-fold higher expression compared with children aged between 1 and 9 years ($P = 0.009$) and those older than 10 years ($P = 0.007$), respectively. Among patients with wild-type *IKZF1*, the same pattern of higher expression in younger children was observed ($P < 0.05$; Supplementary Fig. S1E). The results demonstrated that *IKZF1* transcript level was lower among samples with $CD10^{+}$ in the analysis that included all patients ($P = 0.012$; Supplementary Fig. S1F).

The risk associations between controls and BCP-ALL cases further stratified by *IKZF1* somatic status are shown in Fig. 1. The homozygous variant of *IKZF1* rs11978267 significantly increased the risk for somatic *IKZF1* deletion occurrence (OR, 2.80; 95% CI, 1.25–6.23). We further tested whether this risk allele was associated to a specific phenotypic consequence of *IKZF1* deletion (i.e., expression of dominant-negative isoforms or haploinsufficiency). The results show that individuals with *IKZF1* rs11978267 variant genotype had a higher risk of developing BCP-ALL with *IKZF1* haploinsufficiency in the blast cell (OR, 2.79; 95% CI, 1.14–6.78). A similar result was observed for individuals with *IKZF1* rs4132601 variant allele, who had a significantly increased risk for *IKZF1* deletion occurrence (OR, 2.88; 95% CI, 1.24–6.69). Higher odds of *IKZF1* haploinsufficiency were observed for these individuals with *IKZF1* rs4132601 variant allele (OR, 2.75; 95% CI, 1.06–7.08; Fig. 1). Regardless of *IKZF1* somatic status, the gene expression was similar among all groups (Supplementary Fig. S1G). Then, we tested whether *IKZF1* rs11978267 or rs4132601 were in linkage disequilibrium with SNPs that change recombination signal sequences (RSS) surrounding deletion breakpoints (Supplementary Table S3). Although 27 SNPs were located within breakpoint hotspots of *IKZF1*, 12 occurred at CAC/GTG sites. These SNPs were in linkage equilibrium with rs11978267 and rs4132601, and their minor allele frequency was lower than 0.01%.

We further tested the effect of cumulative variant alleles of both *IKZF1* SNPs in the risk susceptibility to BCP-ALL. Children with the variant genotype for both *IKZF1* rs11978267 and rs4132601 had significant higher odds of harboring an *IKZF1* deletion (OR, 3.73; 95% CI, 1.49–9.33; Supplementary Table S4). Interestingly, when analyzing the cumulative risk associations using a case–case approach (comparing *IKZF1* wild-type vs. deleted cases), individuals with both variant genotypes had increased risk to develop disease with *IKZF1* deletion (OR, 2.71; 95% CI, 1.01–7.33; data not shown).

Because the somatic *IKZF1* deletions frequently occur concomitantly with abnormalities in other genes in BCP-ALL, we also aimed to evaluate whether these risk alleles were associated to a specific combination of genetic alterations (Table 2). The disease risk was increased for individuals categorized as "*IKZF1* plus" with the *IKZF1* rs11978267 homozygous variant genotype (OR, 2.94; 95% CI, 1.13–7.63). The chances of harboring a combination of *IKZF1* and 9p deletions were higher for individuals with the variant allele of *IKZF1* rs11978267 (OR, 3.64; 95% CI, 1.14–11.66) and for those with the variant allele of *IKZF1* rs4132601 (OR, 4.32; 95% CI, 1.34–13.95).

Finally, we observed that patients with the variant allele of *IKZF1* rs4132601 had more than 7 times the risk of developing disease with both *IKZF1* and *BTG1* deletions; however, the number of cases with *BTG1* deletions was very small precluding the opportunity to reach a reliable statistical result (OR, 7.55; 95% CI, 1.33–42.96, recessive model, Table 2). To further explore the magnitude of this risk association, we analyzed an extended cohort consisting of additional 31 cases that harbored *BTG1* deletions (Supplementary Table S1). The results presented in Table 2 show a remarkable risk association for individuals harboring both *IKZF1* and *BTG1* deletions with either the variant allele of *IKZF1* rs11978267 (OR, 4.90; 95% CI, 1.65–14.55) or rs4132601 (OR, 5.80; 95% CI, 1.94–17.41).

Table 1. The risk associations between genetic variants and age at diagnosis, Brazil, 2004–2011

		Age					
	Controls	<1 year old		1–9 years old		>9 years old	
***IKZF1* SNPs**	***n* (%)**	***n* (%)**	**OR (95% CI)**	***n* (%)**	**OR (95% CI)**	***n* (%)**	**OR (95% CI)**
rs11978267							
AA	258 (55.2)	12 (46.2)	1	98 (54.1)	1	23 (59.0)	1
AG	175 (37.5)	11 (42.3)	1.35 (0.58–3.13)	63 (34.8)	0.95 (0.65–1.37)	13 (33.3)	0.83 (0.41–1.69)
GG	34 (7.3)	3 (11.5)	1.90 (0.51–7.06)	20 (11.0)	1.55 (0.85–2.82)	3 (7.7)	0.99 (0.28–3.47)
Recessive model			1.66 (0.47–5.81)		1.58 (0.88–2.83)		1.06 (0.31–3.62)
rs4132601							
TT	246 (52.7)	10 (38.5)	1	90 (49.7)	1	20 (51.3)	1
TG	192 (41.1)	12 (46.2)	1.54 (0.65–3.63)	69 (38.1)	0.98 (0.68–1.42)	17 (43.6)	1.09 (0.55–2.14)
GG	29 (6.2)	4 (15.4)	3.39 (1.00–11.51)	22 (12.2)	2.07 (1.13–3.79)	2 (5.1)	0.85 (0.19–3.82)
Recessive model			2.75 (0.89–8.50)		2.09 (1.16–3.74)		0.82 (0.19–3.56)

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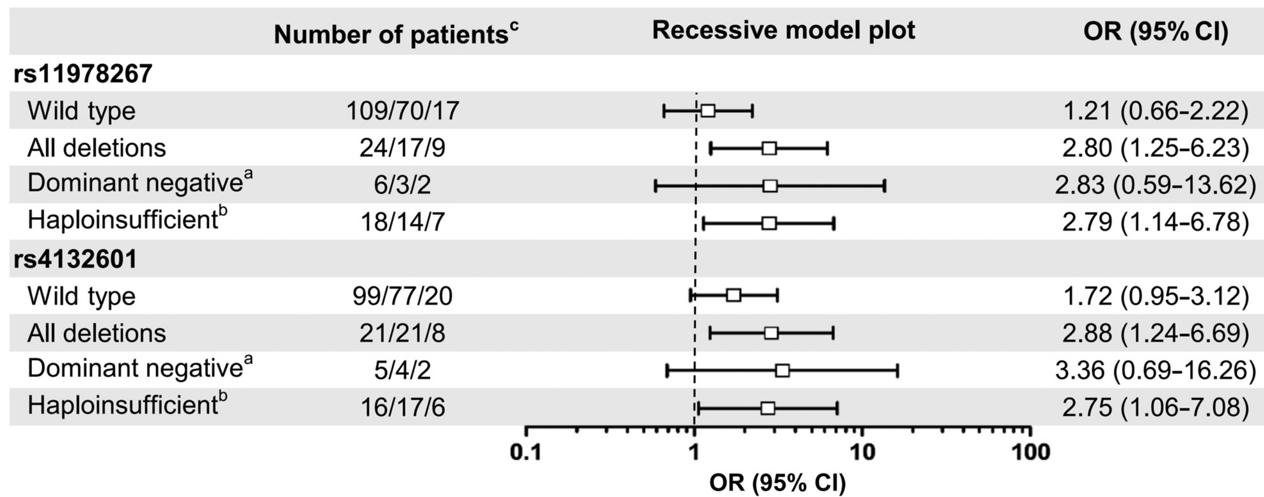

Figure 1.

The risk associations between genetic variants and *IKZF1* somatic status. [a]Patients with *IKZF1* deletions affecting exons 4–7 ($n = 10$) and exons 4–5 ($n = 1$); [b]patients with *IKZF1* deletions affecting exons 1 or 2 ($n = 39$); [c]the number of patients are described for wild-type homozygote/heterozygote/variant homozygote genotypes, respectively.

Discussion

Childhood ALL is a malignancy characterized by the detection of primary genetic abnormalities in more than 75% of cases (24). The understanding of how such changes emerge is still poor. Therefore, despite the fact that ALL cases have long been very well characterized from a genetic point of view, it is still a challenge to associate the risk of childhood ALL with inherited susceptibility. Nevertheless, since the advent of genome-wide technologies, some studies have reported that two independent loci located within *IKZF1* are exclusively associated with the risk of developing ALL (5, 6). After replication of this discovery by other studies, a meta-analysis provided large-scale evidence that both *IKZF1* SNPs

Table 2. The risk associations between genetic variants and concomitant somatic genes abnormalities, Brazil, 2004–2014

IKZF1 genotype	Controls[a]	Cases[a]	OR_{het} (95% CI)	OR_{hom} (95% CI)	OR_{rec} (95% CI)
rs11978267					
Discovery series[b]					
IKZF1 plus	258/175/34	15/11/6	1.08 (0.49–2.41)	3.04 (1.10–8.35)	2.94 (1.13–7.63)
IKZF1 non-plus	258/175/34	9/6/3	0.98 (0.34–2.81)	2.53 (0.65–9.81)	2.55 (0.70–9.23)
$IKZF1_{del}$ + $9p_{del}$	258/175/34	9/5/4	0.82 (0.27–2.49)	3.37 (0.98–11.55)	3.64 (1.14–11.66)
$IKZF1_{wt}$ + $BTG1_{wt}$	258/175/34	101/63/17	0.92 (0.64–1.33)	1.28 (0.68–2.39)	1.32 (0.72–2.43)
$IKZF1_{wt}$ + $BTG1_{del}$	258/175/34	8/7/0	1.29 (0.46–3.62)	—	—
$IKZF1_{del}$ + $BTG1_{wt}$	258/175/34	22/15/7	1.01 (0.51–1.99)	2.41 (0.96–6.08)	2.41 (1.00–5.81)
$IKZF1_{del}$ + $BTG1_{del}$	258/175/34	2/2/2	1.47 (0.21–10.56)	7.59 (1.03–55.64)	6.37 (1.13–36.02)
Additional series[c]					
$IKZF1_{wt}$ + $BTG1_{del}$	258/175/34	17/16/1	1.39 (0.68–2.82)	0.45 (0.06–3.46)	0.39 (0.05–2.91)
$IKZF1_{del}$ + $BTG1_{del}$	258/175/34	9/4/5	0.66 (0.20–2.16)	4.22 (1.34–11.32)	4.90 (1.65–14.55)
rs4132601					
Discovery series[b]					
IKZF1 plus	246/192/29	13/14/5	1.38 (0.63–3.00)	3.26 (1.09–9.81)	2.80 (1.00–7.80)
IKZF1 non-plus	246/192/29	8/7/3	1.12 (0.40–3.15)	3.18 (0.80–12.66)	3.02 (0.83–11.03)
$IKZF1_{del}$ + $9p_{del}$	246/192/29	7/7/4	1.28 (0.44–3.71)	4.85 (1.34–17.56)	4.32 (1.34–13.95)
$IKZF1_{wt}$ + $BTG1_{wt}$	246/192/29	92/69/20	0.96 (0.67–1.38)	1.84 (0.99–3.42)	1.88 (1.03–3.41)
$IKZF1_{wt}$ + $BTG1_{del}$	246/192/29	7/8/0	1.46 (0.52–4.11)	—	—
$IKZF1_{del}$ + $BTG1_{wt}$	246/192/29	19/19/6	1.28 (0.66–2.49)	2.68 (0.99–7.25)	2.39 (0.93–6.10)
$IKZF1_{del}$ + $BTG1_{del}$	246/192/29	2/2/2	1.28 (0.18–9.18)	8.48 (1.15–62.52)	7.55 (1.33–42.96)
Additional series[c]					
$IKZF1_{wt}$ + $BTG1_{del}$	246/192/29	15/17/2	1.45 (0.71–2.98)	1.13 (0.25–5.20)	0.94 (0.22–4.14)
$IKZF1_{del}$ + $BTG1_{del}$	246/192/29	9/4/5	0.57 (0.17–1.88)	4.71 (1.48–15.02)	5.80 (1.94–17.41)

NOTE: The CNA groups were defined as "*IKZF1* plus" [*IKZF1* deletions plus *CDKN2A/B, PAX5* and/or pseudoautosomal region 1 (PAR1) deletions], and "9p deletions" (concurrent deletions of *CDKN2A/2B* and *PAX5*).

Abbreviations: del, deletion; wt, wild-type; OR_{het}, heterozygote versus wild-type homozygote; OR_{hom}, variant homozygote versus wild-type homozygote; OR_{rec}, calculated by recessive model; wt, wild type.

[a]Wild-type homozygote/heterozygote/variant homozygote.

[b]This series includes 246 BCP-ALL cases.

[c]This series includes 31 additional BCP-ALL cases with $BTG1_{del}$.

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contribute to the occurrence of BCP-ALL (25), and our study could clearly confirm the association of *IKZF1* rs4132601 with childhood BCP-ALL.

A striking feature in the risk association involving *IKZF1* is that somatic mutations within the same gene are recurrently detected in cases of BCP-ALL (1, 2), which suggests that germinative variant alleles may favor an intrinsic vulnerability of precursor cells to acquire additional transforming events, such as *IKZF1* somatic deletions. In accordance, the interplay between genetic susceptibility and somatic abnormalities in the etiology of childhood ALL has been previously demonstrated by others. For example, SNPs located at 10q21.2 (*ARID5B*) appear to be highly associated with the risk of developing hyperdiploid ALL (5, 6). Furthermore, an *ARID5B* SNP conferred increased risk to acquire specific *MLL* rearrangements in a cohort of early childhood leukemia (14). In addition, germline variation of *TP63* and *PTPRJ* genes can contribute specifically to the risk of the subset of BCP-ALL with *ETV6-RUNX1* (26). Our findings demonstrate that *IKZF1* SNPs can distinctively contribute to the risk of BCP-ALL with *IKZF1* deletions, especially those leading to haploinsufficiency.

Because breakpoints of intragenic deletions of *IKZF1* are located nearby RSSs (3, 11), which are recognized by the RAG complex for V(D)J recombination, one hypothesis that could explain the relationship between *IKZF1* SNPs and risk of acquiring *IKZF1* deletions is that SNPs could modify RSSs nearby deletion breakpoints, thus influencing RAG complex recognition. To evaluate this hypothesis, we mapped recurrent SNPs located proximal to *IKZF1* breakpoint hotspots and checked whether they altered the minimal recombination motif of RAG. Although our findings showed that some SNPs could change RSSs nearby recurrent deletion breakpoints, the allele frequency was very low to explain this risk association.

The relationship between *IKZF1* SNP and disease risk may be explained by the genotype influence on gene expression. Papaemmanuil and colleagues investigated the relationship between rs4132601 and expression of *IKZF1* transcript in Epstein–Barr virus–transformed lymphocytes. They found an association between mRNA expression and genotype in a dose-dependent manner; the homozygous variant genotype had lower expression levels and therefore might disrupt B-cell differentiation (5). As rs4132601 is not located on the promoter or enhancer region of *IKZF1*, the process linking its SNP and disease risk in not fully understood. One hypothesis is that it could indirectly alter gene expression when in linkage disequilibrium with another SNP. Notably, rs4132601 is in linkage disequilibrium with rs11978267 and rs1110701. The latter lies on enhancer regions of *IKZF1* annotated in GM12878 cell line (19, 27, 28). Thus, our data suggest that haplotypes carrying variant genotypes of rs4132601 and rs11978267 may be related to differential expression of *IKZF1* and contribute to leukemic transformation in a similar way of deregulation that is promoted by *IKZF1* deletions (as summarized in Supplementary Fig. S2).

The *IKZF1* transcripts are highly expressed in hematopoietic progenitor cells and during lymphopoiesis. Investigation of Ikaros function in early B-cell development suggested that it regulates its targets in a stage-specific manner (29). Our study demonstrated that *IKZF1* expression is more pronounced in earlier stages of B-cell differentiation (CD10-negative B cells), where it could dictate lymphoid gene expression.

The analysis whether the risk alleles were associated to any specific combination of genes abnormalities showed that the presence of variant alleles of both *IKZF1* SNPs increases the risk of BCP-ALL with: *IKZF1*+9p, "*IKZF1* plus" and *IKZF1*+*BTG1* deletions. One of the most interesting findings from this study was the risk association for individuals harboring both *IKZF1* and *BTG1* deletions; after analyzing an increased number of patients, we could confirm this result. According to Scheijen and colleagues, *BTG1* deletions strongly enhance the risk of relapse in patients with *IKZF1* deletions and augment the glucocorticoid resistance phenotype mediated by loss of *IKZF1* function, while combined loss of *IKZF1* and other deletions was also significantly associated but had no impact on prognosis (9, 12). The biological mechanism underlying such association is uncertain. Nevertheless, recent studies demonstrated that the acquisition of *IKZF1* and *BTG1* deletions is mediated by RAG (3, 30). As *IKZF1* encodes a transcription factor that regulates many downstream targets in the lymphoid differentiation pathway (e.g., RAG and TdT), we hypothesize that reduced levels of *IKZF1* transcripts may disrupt RAG expression and, thus, promote the concurrent occurrence of somatic deletions.

Some limitations in this analysis should be mentioned. First, the small number of cases after some subsets stratification raises concern with regard to statistical power. Second, the presence of bias in the selection of controls cannot be ruled out, once the samples were obtained from hospitalized children and not from the overall population. Third, we had missing biological sample in some cases that precluded us to have all samples screened for mRNA expression. Finally, the findings obtained in this study have not been validated by independent replication. Therefore, data presented here must be carefully interpreted, and future studies are needed to confirm these results.

We can also mention some study strengths. This study included children from varied geographical regions in Brazil, being the risk associations identified very consistent and concordant with previously published data indicating, therefore, good reliability and sensitivity of our data. Another important contribution of the current work is the replication of genome-wide association studies in a population different from the American and European ones, where most of the studies were so far concentrated. Moreover, studies involving gene–gene interactions are highly desirable in the context of the etiology of childhood ALL, and innovative results have been obtained in this field.

In summary, our findings demonstrate that germline genetic variation can contribute not only to the risk of childhood BCP-ALL in general, but also specifically contribute to the risk of BCP-ALL subtypes, in this case patients with *IKZF1* deletions only or with additional somatic alterations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Emerenciano
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.A. Lopes, T.C. Barbosa, C.P. Poubel, M. Emerenciano
Writing, review, and/or revision of the manuscript: B.A. Lopes, T.C. Barbosa, M. Emerenciano

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