Identifying novel genetic alterations in pediatric acute lymphoblastic leukemia based on copy number analysis

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Abstract
Copy number variations (CNVs) analysis may reveal molecular biomarkers and provide information on the pathogenesis of acute lymphoblastic leukemia (ALL). We investigated the gene copy number in childhood ALL by microarray and select three new recurrent CNVs to evaluate by real-time PCR assay: 

- DMBT1
- KIAA0125
- PRDM16

were selected due to high frequency of CNVs in ALL samples and based on their potential biological functions in carcinogenesis described in the literature. DMBT1 deletion was associated with patients with chromosomal translocations and is a potential tumor suppressor; KIAA0125 and PRDM16 may act as an oncogene despite having a paradoxical behavior in carcinogenesis. This study reinforces that microarrays/aCGH is it is a powerful tool for detection of genomic aberrations, which may be used in the risk stratification.

Keywords: ALL (acute lymphoblastic leukemia) childhood leukemia, DMBT1, KIAA0125, PRDM16, Copy number variations

Introduction
Acute lymphoblastic leukemia (ALL) is the most common cancer in children [1]. Leukemia represents the ninth most common cancer in Brazil and the fifth most frequent in the north region [2]. Advances in cytogenetics and molecular cytogenetics has allowed the identification of genetic aberrations in more than 80% of ALL cases [3]. Establishing genetic background in ALL patients is important for the diagnosis, risk classification and therapeutic interventions [3]. However, some patients do not have an established chromosomal aberration, which complicates the risk classification.

Recent analysis has shown that copy number variations (CNVs) are common in ALL and leukemia in general, especially in genes involved in transcription, cell cycle regulation and B-cell differentiation, (e.g., CDKN2A/B, IKZF1, ETV6, EBF1, PAX5, BTG1 and PAR1) [4]. Additional CNVs could be helpful to refine ALL prognostic. The prognostic effect of CNVs depends on the other factors, such as the presence of additional molecular or cytogenetic aberrations; this situation reinforces the need to analyze these combined alterations [5].

The aim of this report is to assess and evaluate CNVs identified by aCGH from a cohort of Brazilian children with ALL. Three new recurrent CNVs were further evaluated by qPCR. We highlight that DMBT1, KIAA0125 and PRDM16 were chosen due to high frequency of aberrations in ALL samples and based on their biological functions as well the data present in the literature.

Methods
Patients
A total of 16 ALL pediatric patients (5 ± 3 years) treated at Octávio Lobo Children’s Cancer Hospital were selected for aCGH analysis. Additional 84 ALL pediatric
samples were used as validation group in copy number qPCR assay. These patients were classified by immunophenotyping and morphology. Gene fusions were investigated by reverse transcription polymerase chain reaction (RTq-PCR) (Tables 1 and 2). The samples were collected before cancer treatment between 2017 and 2019.

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, WBC count greater than \(5 \times 10^9\) cells/\(\mu L\), age 1 year or less, or age 10 years or more; and 2) standard risk, WBC count \(5 \times 10^9\) cells/\(\mu L\) or less, or between 1 and 10 years of age. The patients with BCR-ABL1 or MLL-AF4 also were assigned to the NCI high risk group. Written consent forms were obtained from all parents of patients. This study was approved by hospital ethics committee (CAAE: 00905812.1.0000.00.18).

Array comparative genomic hybridization
Genomic DNA was extracted from peripheral blood by Pure Link Genomic DNA Mini Kit (Invitrogen, California, USA). aCGH was performed using Agilent 4x180k CGH + SNP microarray (Santa Clara, USA). After DNA extraction, a restriction enzyme digestion step and labeling with fluorochrome cyanine 5 were performed using random primers and exo-Klenow fragment DNA polymerase. DNA control was labeled with fluorochrome cyanine 3. DNA samples from the patient and control were combined and hybridized on the microarray. Data were analyzed using the software Agilent’s CytoGenomics v5.0.

Real-time quantitative PCR
TaqMan Copy Number Assay (Applied Biosystems, California, USA) was used to assess copy number for DMBT1, KIAA0125 and PRDM16. Briefly, 1 µL of 10 ng DNA was added to 5 µL of TaqMan Universal Master Mix no UNG, with 0.5 µL of each probe and 3 µL of ultra-pure water. RNase P was used as a control. The amplification protocol consisted of: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative quantification was determined using the 7500 Real-time PCR system and all samples were analyzed in quadruplicate. After amplification, we imported the experiment results containing threshold-cycle values for the copy number and reference assay into the Copy Caller Software v2.0 for post-PCR data analysis as previously described [6].

Statistical analysis
Fisher’s exact test was used to compare the distribution of aberrations between subgroups (high or standard risk; positive or negative for chromosomal translocation) and pathological features of the patients; Odds ratio (OR) with a 95% confidence interval (CI) were also calculated through the statistical program BioEstat® v5.0 [7]. \(p\)-values less than 0.05 were considered significant.

Results
aCGH profiling identifies recurrent alterations
aCGH date were available for all 16 cases, the average of copy number variations (CNVs) was 8.3 per sample. Gains were the most frequent event, the most frequently gained regions were on chromosomes 14 (q32.33) and 10 (q26.13), these regions include KIAA0125 (an lncRNA) and DMBT1 genes, respectively. Frequent losses were identified on chromosome 7 (p12.2) and 9 (p21.3), which includes IKZF1 and CDKN2A/B genes, respectively.

Recurrent CNVs included gains of cytobands 14q (93.75%), 2p (68.75%), 17q (62.5%), 9q (56.25%), 10q

Table 1 Characteristics of pediatric patients with ALL included in the study

| Characteristic         | aCGH (n = 16) | qPCR (n = 84) |
|------------------------|--------------|---------------|
| Male: female           | 08:08        | 49:35         |
| Median age (y)         | 6.5          | 7.4           |
| Median WBC count (x 10^9/L) | 73          | 69.4         |
| Immunophenotype        |              |               |
| B                      | 15           | 80            |
| T                      | 1            | 4             |
| Chromosomal alteration |              |               |
| TCF3-PBX1 (n)          | 6            | 15            |
| BCR-ABL1 (n)           | 1            | 9             |
| MLL-AF4 (n)            | 5            | 7             |
| ETV6-RUNX1 (n)         | 3            | 3             |
| SIL-TAL1(n)            |              |               |
| NCI risk               |              |               |
| High (n)               | 7            | 30            |
| Standard (n)           | 9            | 54            |

Table 2 Nucleotide sequence of RTq-PCR primers

| Genes | Primers (5′-3′) | Size (bp) | Position | Exons |
|-------|-----------------|-----------|----------|-------|
| TCF3  | CTACTCCCCGGATCACTCAAA | 20 | 1086–1105 | 13     |
| PBX1  | AGGGCTTCTATCTGCGAGCAGT | 20 | 3893–3912 | 2     |
| MLL   | CGCCCAAGTATCCCTGTGTA | 20 | 4071–4090 | 8     |
| AF4   | GAGCATGGATGAGCTCTCTCTT | 20 | 1546–1565 | 8     |
| BCR   | TCGCAGAACTCGCAACAGT | 19 | 1707–1725 | 1     |
| ABL   | ACACCATTCCTCCCATGTGAT | 20 | 284–303 | 3     |
| ETV6  | TTCTCTCATGGGAAAGACCTG | 20 | 1191–1210 | 5     |
| RUNX1 | TGGCTAGATTTTCTCAACG | 19 | 619–637 | 5     |
| SIL   | TCTACCTGGCACAACAGACCC | 20 | 73–92 | 1     |
| TAL1  | AGGGCGAGGATCTCATTCCTT | 20 | 1250–1269 | 4     |
(56.25%), 19q (56.25%), 22q (56.25%), 1p (50%), 7q (50%), 8p (50%) and 21q (50%); losses involving 7p (62.5%), 9p (56.25%), 15q (47.75%), 4q (37.5%) and 12q (31.25%). The list of recurrent CNVs found in at least two samples is provided in Table 3.

All patients have alteration in at least one of the main genes associated with ALL; ETV6, RUNXI, IKZF1, KMT2A (MLL) and BTG1 (Table 3). The median of alterations in standard (SR) and high risk (HR) group were 56.6 (±15.4) and 52.2 (±14.2), respectively. We confirmed the association of CDKN2A/B losses with positive cases for TCF3-PBX1 or BCR-ABL1 (p < 0.05).

There was no statistically significant difference in the number of CNVs between patients with (CT+) or without (CT-) chromosomal translocation.

### CNV evaluation by real-time qPCR

To validate aCGH results DMBT1, KIAA0125 and PRDM16 genes were analyzed by qPCR. Genes were chosen due to the high frequency of aberrations found in samples and based on their biological function (mainly transcriptional regulation) described in literature. It is noted that the CNV found in these genes are described here for the first time in leukemia, especially in ALL. The aberrations of the three selected genes identified from aCGH and qPCR were illustrated in Fig. 1.

The results of qPCR were compared between positive (CT+) or negative (CT-) for gene fusions subgroups. DMBT1 deletion was observed in 74% of patients (n = 62; 97.4% of CT+ and 53% of CT-); KIAA0125 amplification was detected in 59% of cases (n = 50; 95% of CT+ and 29% of CT-), these amplifications were more frequent in cases CT+; while PRDM16 was deleted in 50% of patients (n = 42; 87% CT- and 8% CT+), amplifications were observed in 42% of samples, which only correspond to CT+ cases (Fig. 2).

Curiously, 50% (25/50) and 69% (24/35) of KIAA0125 and PRDM16 amplifications, respectively, were high-level amplifications (> 10 copies), however, classification of cases according to the level of amplification did not result in any significant association.

The frequency of aberrations in DMBT1, KIAA0125 and PRDM16 according to NCI risk group, gender, age and cytogenetic findings are show in Tables 4 and 5. Statistical analysis showed that DMBT1 deletion was more common in patients with > 1 to ≤ 10 years (OR = 3.38; 95% IC = 1.15–9.89) and more common in NCI-SR cases (OR = 0.198; 95% IC = 0.07–0.56). DMBT1 deletion also was associated with CT+ samples (OR = 33.2; 95% IC = 4.19–263.55) (Tables 4 and 5).

KIAA0125 amplification was associated with CT+ cases (OR = 45.5; 95% IC = 9.54–217.16). PRDM16 gene deletion was associated with NCI-HR patients (OR = 91.4; 95% IC = 11.32–738.6) and CT- cases (OR = 0.01; 95% IC = 0.00–0.05) (Tables 4 and 5), while amplification was related to CT+ samples (p-value = < 0.001), data not shown in Tables 4 and 5).

### Table 3 The most frequent copy number variations found in pediatric ALL by aCGH

| Frequency % (n = 16) | Chromosome | Reference region | Variant type | Genes involved |
|----------------------|------------|------------------|--------------|----------------|
| 94                   | 14         | q32.33           | Amp          | KIAA0125<sup>a</sup> |
| 75                   | 14         | q11.22           | Del          | Several genes  |
| 62.5                 | 7          | p12.2            | Del          | IKZF1          |
| 56.25                | 9          | p21.3            | Del          | CDKN2A/BMTAP   |
| 56.25                | 10         | q26.13           | Amp          | DMBT1<sup>a</sup> |
| 56.25                | 22         | q11.22           | Amp          | MIR650, IGLL5  |
| 50                   | 15         | q11.1            | Del          | HERC2P3        |
| 50                   | 1          | p36.32           | Amp          | PRDM16<sup>a</sup> |
| 50                   | 19         | q13.32           | Amp          | KLC3, ERCC2   |
| 37.5                 | 4          | q13.2-q13.3      | Del          | UGT2B4         |
| 31.25                | 12         | q21.33-q22       | Del          | BTG1           |
| 25                   | 13         | q14.2            | Del          | RB1            |
| 19                   | 7          | p14.1            | Del          | TRGC2, TARP    |
| 19                   | 11         | q23.3            | Del          | KMT2A          |
| 19                   | 12         | q2 p13           | Del          | ETV6           |
| 12.5                 | 12         | p21.3-p15.2      | Del          | Several genes  |
| 12.5                 | 3          | q29              | Del          | DLG1           |
| 6.25                 | 21         | iAMP21           | Amp          | RUNX1          |

<sup>a</sup>Alterations have never been described in literature for ALL. Amp amplification. Del deletion
Discussion

All patients analyzed by aCGH showed a heterogeneous copy-number pattern. We identified 133 CNVs, 18 them involved the most frequent changes already known or not yet related to ALL (Table 3). Unlike previous studies, here, amplifications were more frequent than deletions, possibly due the small sample number and the presence of hyperdiploid cases. On the other hand, similar to antecedent studies [4, 8, 9], the more frequently altered genes were related to cell cycle regulation (ETV6), tumor suppression (CDKN2A/B), apoptosis regulation (BTG1) and others (Table 3).

In agreement with the literature, in our study deletions of CDKN2A/B were associated with positive cases for TCF3-PBX1 or BCR-ABL1. CDKN2A/B are tumor suppressor genes acting in cell growth regulation and apoptosis [10]. The deletion of these genes are associated with poor prognosis, high white blood cell count and older age at diagnosis and BCR-ABL1 or TCF3-PBX1 translocations [11–13]; all characteristics found in our study group.

The aCGH study also identified for the first-time recurrent alterations of DMBT1, KIAA0125 and PRDM16 in ALL (Table 3). These genes were verified by qPCR in a larger sample number.

High amplification frequencies observed in aCGH was confirmed by qPCR just for KIAA0125. For the DMBT1 and PRDM16 deletions were prevalent in qPCR assays. This divergence is probably due to differences in sample size and by the presence of trisomy of chromosomes 1 and 10 in cases with copy number variation in PRDM16.
Table 4 Frequency of alterations according to characteristics of patients

|                | DMBT1 deletion |          | KIAA0125 amplification |          | PRDM16 deletion |          |
|----------------|----------------|----------|------------------------|----------|----------------|----------|
|                | Present | Absent | Present | Absent | Present | Absent |
| NCI-HR         | 16      | 14     | 19      | 11     | 41      | 13      |
| NCI-SR         | 46      | 8      | 31      | 23     | 1       | 29      |
| p-value        | 0.0036* |        | 0.6483  |        | < 0.001* |        |
| ≤1 years       | 5       | 2      | 4       | 3      | 3       | 4       |
| >1 to ≤10 years| 44      | 10     | 34      | 20     | 26      | 28      |
| >10 years      | 13      | 10     | 12      | 11     | 13      | 10      |
| p-value 1^1    | 0.6131  |        | 0.9994  |        | 1       |        |
| p-value 2^2    | 0.6693  |        | 1       |        | 0.6746  |        |
| p-value 3^3    | 0.0433* |        | 0.4497  |        | 0.6200  |        |
| WBC > 50       | 16      | 5      | 16      | 5      | 8       | 13      |
| WBC ≤ 50       | 46      | 17     | 34      | 29     | 34      | 29      |
| p-value        | 1       |        | 0.0802  |        | 0.4568  |        |
| Leucopenia     | 5       | 0      | 5       | 0      | 0       | 5       |
| Leucocytosis   | 54      | 18     | 40      | 32     | 37      | 35      |
| p-value        | 0.3336  |        | 0.0720  |        | 0.0554  |        |
| Male           | 38      | 11     | 31      | 18     | 21      | 28      |
| Female         | 24      | 11     | 19      | 16     | 21      | 14      |
| p-value        | 0.4515  |        | 0.5002  |        | 0.1839  |        |

NCI-HR NCI-High risk; NCI-SR NCI Standard risk; WBC White blood count. ^1 <1 years versus >1 to ≤10 years; ^2 ≤1 years versus >10 years; ^3 >1 to ≤10 years versus >10 years. *Significant difference between groups with and without aberrations, p ≤ 0.05, Fisher’s exact test

Table 5 Frequency of alterations according to cytogenetic subgroups

|                | DMBT1 deletion |          | KIAA0125 amplification |          | PRDM16 deletion |          |
|----------------|----------------|----------|------------------------|----------|----------------|----------|
|                | Present | Absent | Present | Absent | Present | Absent |
| BCR-ABL1       | 9       | 0      | 9       | 0      | 1       | 8       |
| Absence        | 53      | 22     | 41      | 34     | 41      | 34      |
| p-value        | 0.1036  |        | 0.0032* |        | 0.0294* |        |
| ETV6-RUNX1     | 7       | 0      | 7       | 0      | 0       | 7       |
| Absence        | 55      | 22     | 43      | 34     | 42      | 35      |
| p-value        | 0.1817  |        | 0.0381* |        | 0.0119* |        |
| MLL-AF4        | 5       | 0      | 4       | 1      | 1       | 4       |
| Absence        | 57      | 22     | 46      | 33     | 41      | 38      |
| p-value        | 0.3195  |        | 0.6438  |        | 0.3597  |        |
| TCF3-PBX1      | 14      | 1      | 14      | 1      | 1       | 14      |
| Absence        | 48      | 21     | 36      | 33     | 41      | 28      |
| p-value        | 0.0625  |        | 0.00031*|        | 0.0003* |        |
| SIL-TAL1       | 3       | 0      | 3       | 0      | 0       | 3       |
| Absence        | 59      | 22     | 47      | 34     | 42      | 39      |
| p-value        | 0.5634  |        | 0.2685  |        | 0.2410  |        |
| CT+            | 38      | 1      | 37      | 2      | 3       | 36      |
| Ct-            | 24      | 21     | 13      | 32     | 39      | 6       |
| p-value        | p < 0.001* |        | p < 0.001* |        | p < 0.001* |        |

CT+ chromosome translocation positive; CT chromosome translocation negative. *Significant difference between groups with and without aberrations, p ≤ 0.05, Fisher’s exact test
DMBT1, respectively. But new significant associations were observed for the three genes.

The high frequency of DMBT1 deletions observed here support aCGH results. DMBT1 encoding protein belongs to the scavenger receptor cysteine rich (SRCR) super family involved in mucosal immune defense, epithelial differentiation and tumor suppression [14, 15]. Many studies have showed that DMBT1 deletion or inactivation lead to tumorigenesis by regulating infiltration and metastasis of tumor cells [16]. Altered expression in certain stages of carcinogenesis was identified in different tumor types [17–19]. We found DMBT1 deletion associated with standard risk and CT+ cases. It is possible that DMBT1 deletion have a more specific function in development of ALL cases without a high risk chromosomal abnormality (which are mostly classified as standard risk), since only 14% of CT+ cases have high risk biomarker (BCR-ABL1 or MLL-AF4). Thus, DMBT1 loss collaborate as a secondary event in the progression of disease in CT+ patients, since it is know that chromosomal translocations are primary aberrations [13]. Although DMBT1 absence is considered a malignancy marker in many epithelial cancers, we reported for the first time DMBT1 deletion in ALL and we suggest that DMBT1 may be also involved in hematologic malignancies development.

LncRNAs are involved in gene expression at epigenetic, transcriptional and post-transcriptional level and are considered a strong promise as a biomarker and therapeutic target [20]. In this study, we found that KIAA0125 amplifications were more common in CT+ patients while in CT- cases, deletions were more prevalent. Recurrent KIAA0125 amplifications were statistically associated with CT+ cases. CNV or abnormal expression of KIAA0125 were observed in many tumor types [21–26]. Several recent studies in LncRNAs have shown that they have a critical role in different cancers acting as an oncogene or suppressor, in this sense, the role of KIAA0125 in carcinogenesis may be cell-type dependent [27]. In colon cancer development, KIAA0125 may contribute via the regulation of BCL2 expression by sponging hsa-miR-29b-3p or regulating PI3K-Akt signaling [28]. In addition, Forero-Castro et al. [4] identified losses on 14q32.33 (where KIAA0125 is located) related to overall survival of children ALL with leukocytosis. In 14q32 there are miRNA clusters that may influence the genes expression levels involved in lymphoid B-cell transformation and differentiation, suggesting that 14q32 losses could be used as a diagnostic marker [4, 29]. Hornung R. et al. (2018) have recently showed that KIAA0125 could play a mediating role in the influence RUNX1 gene fusions have on survival of LMA [30].

It is also probable that KIAA0125 may act as miRNA sponges regulates mRNAs expression levels also in ALL, however the exact mechanism of action and possible target genes need to be further investigated. These findings along with our data leading to the assumption that KIAA0125 plays important role in development of leukemia and reinforce previous studies that suggested that LncRNAs may be utilized as diagnostic and prognostic markers in leukemia [20].

PRDM16 is characterized by the combination of a conserved N-terminal PR domain and a variable number of zinc fingers [31], it encodes a SMAD binding protein that may repress SMAD-mediated transcription, also functions as a modulator of TGF-beta signaling and exhibit methyltransferase activity [32, 33]. PRDM16 is involved in various biological processes including maintenance of brown adipocytes and hematopoiesis [34, 35]. Two main PRDM16 isoforms are the full-length and the PR-lacking generated by alternative splicing or alternative use of different promoters [36, 37]. Notably, PRDM proteins sometimes exert opposing effects on tumor development [38, 39].

In the present study most cases have PRMD16 deletions (50%), however in 90% of CT+ patients this gene is highly amplified (21 samples with > 10 copies) and significantly related to presence of gene fusions. Overexpression of PRDM16 in AML is associated with worse overall survival [39, 40] and is considered a risk factor for primary induction failure [41]. In addition it is associated with other gene fusions not investigated here [42]. Hu et al. [43] reported that PRDM16 transforming megakaryocyte-erythroid progenitors into myeloid leukemia stem cells. In another study, PRDM16 knockdown induced cell proliferation in rhabdoid tumor cells [44], suggesting that PRDM16 may be an oncogene in leukemia development, although in other tumor types PRDM16 has a controversial role [45, 46]. Thus, the role of PRDM16 in cancer biology has been poorly studied and remains to be fully elucidated.

A limitation of this study was the small sample size. However, this is one of the few studies from the northern region of Brazil with genomic analysis in leukemia. This region has a large territorial extension, which makes the diagnosis of cancer a challenge due to its financial viability and the difficult access to geographically isolated regions of cancer treatment centers [47].

In conclusion, this study reinforces that aCGH it is a powerful tool for to identify regions of copy number variations in childhood ALL patients and to identify new genes associated to leukemia. Through this technique, we identified recurrent alterations in genes DMBT1, KIAA0125 and PRDM16; these alterations were verified by qPCR and confirmed the possible involvement of these genes in the development of leukemia, especially in ALL. DMBT1 probably is also a tumor suppressor in leukemia and is associated with standard risk and cases with gene fusions. Although both have a paradoxical behavior in tumorigenesis our data indicates that KIAA0125 and PRDM16 may act as oncogene, once
amplifications in these two genes were related to gene fusions and leukemotysis, respectively. The combination of two molecular cytogenetics techniques has identified three genes that may be targets for further biological analysis of acute lymphoblastic leukemia.

**Abbreviations**
aCGH: Array Comparative Genomic Hybridization; ALL: Acute lymphoblastic leukemia; CNV: Copy Number Variation; CT+: Chromosome translocation positive; CT-: Chromosome translocation negative; HR: High Risk; NCi: National Cancer Institute; qPCR: quantitative Polymerase Chain Reaction; RT-PCR: Reverse Transcription Polymerase Chain Reaction; SR: Standard Risk; WBC: White Blood Count

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**Authors’ contributions**
JG, FM and MS performed the experiments. AK, EO, NS and BK participated in coordination and helped to draft the manuscript. AW and LP were responsible for sample collection. All authors read and approved the final manuscript.

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**Availability of data and materials**
The data will not be shared because some analyses of another genes study by aCGH are still being analyzed together with other data and have not yet been published.

**Ethics approval and consent to participate**
Octávio Lobo Children’s Cancer Hospital ethics committee approved this study (CAAE: 00905812.1.0000.00.18). Written consent forms were obtained from all parents of patients and enabled the collection of biological samples and publication of results.

**Consent for publication**
Consent forms from the parents of patients were obtained to publish and to report individual data.

**Competing interests**
The authors declared that they have no conflict of interest.

**Contributions**
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