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SETD4 as a marker of disease burden and treatment response in childhood acute lymphoblastic leukemia

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Abstract. Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy worldwide. Despite a good rate of treatment success, the poor prognosis underscores the urgent need for new prognostic markers and effective therapeutic strategies. The SET family of lysine methyltransferases (KMTs) has been implicated in several cancers. While the KMT *SMYD2* has been identified as a prognostic marker in ALL, *SETD4* remains poorly characterized. The present study analyzed the expression patterns of *SETD4* in 83 pediatric ALL patients at diagnosis and during treatment using reverse transcription-quantitative PCR. Kaplan-Meier analysis was employed to evaluate survival outcomes between the high and basal *SETD4* expression groups. It was found that *SETD4* expression is markedly upregulated in bone marrow (BM) samples derived from ALL patients compared with non-neoplastic BM (median fold-change of 5.14 $P=0.0095$) and *SETD4* expression is correlated with leukemic burden. Importantly, the levels of *SETD4* decreased in chemotherapy-responsive patients. The present study further investigated whether *SETD4* levels are associated with those of *SMYD2*. Notably, a positive correlation between both genes was observed at diagnosis (Spearman $\rho=0.759$, $P<0.0001$), with a substantial correlation persisting throughout treatment (Spearman $\rho=0.925$; $P<0.01$). Furthermore, patients classified in the high-risk category exhibited elevated *SETD4* expression, with those displaying high *SETD4* transcription exhibiting the poorest survival outcomes. The findings revealed the involvement of *SETD4* in leukemogenesis and highlighted its potential as a promising prognostic marker.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy, with an incidence of ~2-4 cases per 100,000 children under 15 years of age. Notably, the peak incidence occurs between 3 and 5 years of age (1). Despite favorable treatment outcomes, relapsed disease remains the leading cause of mortality in pediatric ALL patients. Consequently, ongoing research has focused on identifying improved prognostic markers and treatment enhancements for ALL management (1). ALL comprises two primary subtypes: B-cell ALL and T-cell ALL. B-ALL is markedly more prevalent, constituting ~85% of all cases (2). Cytogenetic aberrations are common findings in ALL, especially among pediatric patients and have long been recognized for their substantial impact on clinical outcomes (3,4). However, recent advances in sequencing and genomic analysis technologies have revealed novel alterations at the submicroscopic scale. These subtle changes play crucial roles in determining disease aggressiveness and resistance to chemotherapy. Collectively, these scientific breakthroughs enable the identification of new ALL subtypes and enhance the precision of patient prognosis, thereby facilitating more effective risk-adapted treatment strategies and supportive care (2).

Lysine methyltransferases (KMTs) are proteins that add methyl marks to lysine residues in both histones and non-histone proteins. These marks contribute to a wide range of epigenetic modifications, including the establishment and propagation of various gene expression patterns. Dysregulation of KMT activity can cause widespread epigenetic changes that contribute to cancer development and progression (5). Among these enzymes, SET and MYND domain-containing protein 2 (SMYD2) is known to play significant roles in cancer (6,7). In acute lymphoblastic leukemia (ALL), aberrant *SMYD2* expression has been associated with poor prognosis, associated with unfavorable clinical features such as advanced age and increased blast counts following chemotherapy (8,9).

While the role of SMYD2 in ALL has been previously documented, the contribution of other KMTs to leukemogenesis remains less well defined. The lysine methyltransferase SET domain-containing protein 4 (SETD4), has been implicated in the regulation of various cellular processes, including cell proliferation, cell cycle regulation, and maintenance of

cancer stem cell (CSC) quiescence (10-14). Although *SETD4* has been studied in the context of breast cancer, non-small cell lung cancer (NSCLC), and radiation-induced lymphomagenesis, its potential involvement and clinical significance in ALL have not yet been investigated.

The present study analyzed the expression pattern of *SETD4* among pediatric ALL patients and non-neoplastic bone marrow samples and investigated the correlation between *SETD4* transcription changes and the leukemic burden in ALL patients during chemotherapy and its association with *SMYD2* transcription.

Materials and methods

Patient sample collection. The present study was approved by the Ethical Committee of the Federal District Foundation for Teaching and Research in Health Sciences (approval no. CEP/FECPS 555/11), with written informed consent from patients and/or guardians. Bone marrow aspirates from 83 pediatric ALL patients (40 female and 43 male; mean age at diagnosis, 7.63 years; recruited between June 2008 and October 2011) were collected at José Alencar Children's Hospital of Brasilia, Federal District, Brazil, during initial disease presentation as part of routine diagnosis and genetic analysis of leukemia. B-ALL patients were treated according to the Brazilian Cooperative Group for Treatment of Childhood Acute Lymphocytic Leukemia protocol (15) and T-ALL patients were treated according to the ALL-Berlin-Frankfurt-Münster (BFM-95) protocol (8). Bone marrow samples from 15 patients were obtained on the 15th and 29th days of induction chemotherapy. Additionally, bone marrow samples from eight children with idiopathic thrombocytopenic purpura were used as non-neoplastic controls. Blast percentages were confirmed in Wright-Giemsa-stained smears, with all leukemic samples containing >40% blasts.

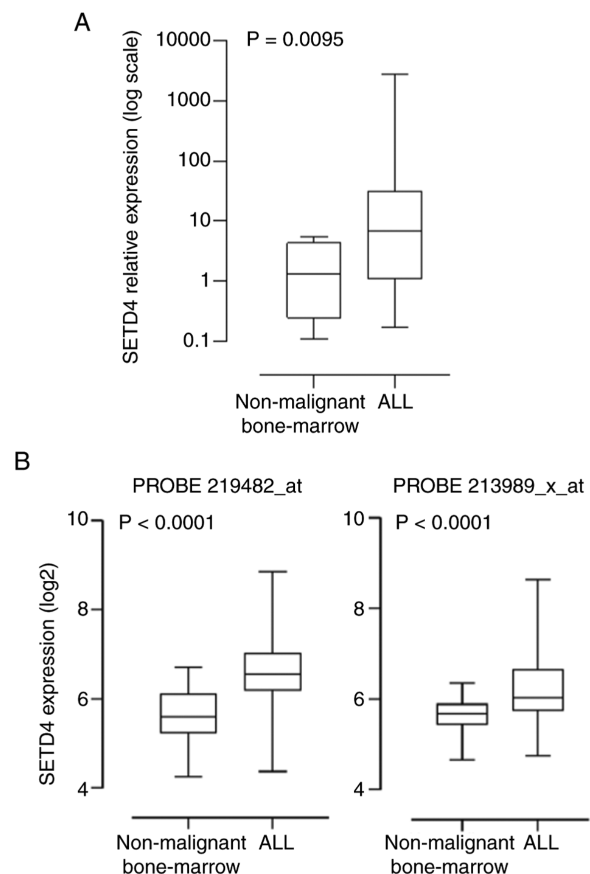

Figure 1. SETD4 mRNA expression in leukemic and non-neoplastic BM samples. (A) RT-qPCR of SETD4 expression in leukemic (n=83) and non-neoplastic (n=8) BM samples, normalized to ACTB (log scale). Group differences were assessed using the Mann-Whitney U test. (B) SETD4 mRNA expression in non-malignant and ALL BM samples from the BloodSpot dataset, showing increased expression in ALL, confirmed by two independent probes. BM, bone marrow; RT-qPCR, reverse transcription-quantitative PCR; ALL, acute lymphoblastic leukemia.

Clinical data collection. Clinical characteristics, including sex, age and white blood cell (WBC) count in peripheral blood at ALL diagnosis, immunophenotyping of bone marrow blasts, cytogenetic alterations and bone marrow status at the 15th and 29th days of chemotherapy, were obtained from medical records and described previously (9). High-risk patients included those who were <2 years old or >9 years old, and/or had peripheral blood WBC counts exceeding 50,000/mm^3, and/or showed infiltration in the central nervous system at the time of diagnosis and/or unfavorable cytogenetic findings.

RNA isolation, cDNA synthesis and reverse transcription-quantitative (RT-q) PCR. Bone marrow mononuclear cells were isolated by Ficoll density centrifugation at 400 x g for 30 min. Total RNA was extracted from each sample containing 5-10x10^6 cells using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Single-stranded complementary DNA was generated from total RNA with reverse transcriptase and random primers, using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using Taq-Man Gene Expression Assays according to the manufacturer's instructions (Hs00213731_m1; cat. no. 4331182 for *SETD4;* Hs00220210_m1, cat. no. 4331182 for *SMYD2*; and Hs99999903_m1, cat. no. 4331182 for *ACTB*; Thermo Fisher Scientific, Inc.). RT-qPCR assays were carried out in a final volume of 10 μl in 96-well plates. RT-qPCR was performed in technical triplicate under the following conditions: 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 40 sec. Quantitation cycle (Cq) values were obtained from this experiment and the $2^{-\Delta\Delta Cq}$ method was applied to these values using *ACTB* as a reference gene for input normalization and scaling all samples by the mean Cq values of non-neoplastic samples (16). The 3rd quartile of non-neoplastic samples relative quantification was used as a threshold to classify a sample as having high or basal *SETD4* mRNA expression.

Exploratory analysis of online data repositories. Microarray expression data for *SETD4* were downloaded from the BloodSpot 3.0 database (https://www.fobinf.com/) as $\log_2$-scaled intensity values for two Affymetrix probe sets, 219482_at and 213989_x_at (17) (Affymetrix; Thermo Fisher Scientific, Inc.). The dataset comprised non-neoplastic bone-marrow samples and ALL specimens stratified by cell lineage (B-ALL and T-ALL, n=47 and n=7) and by

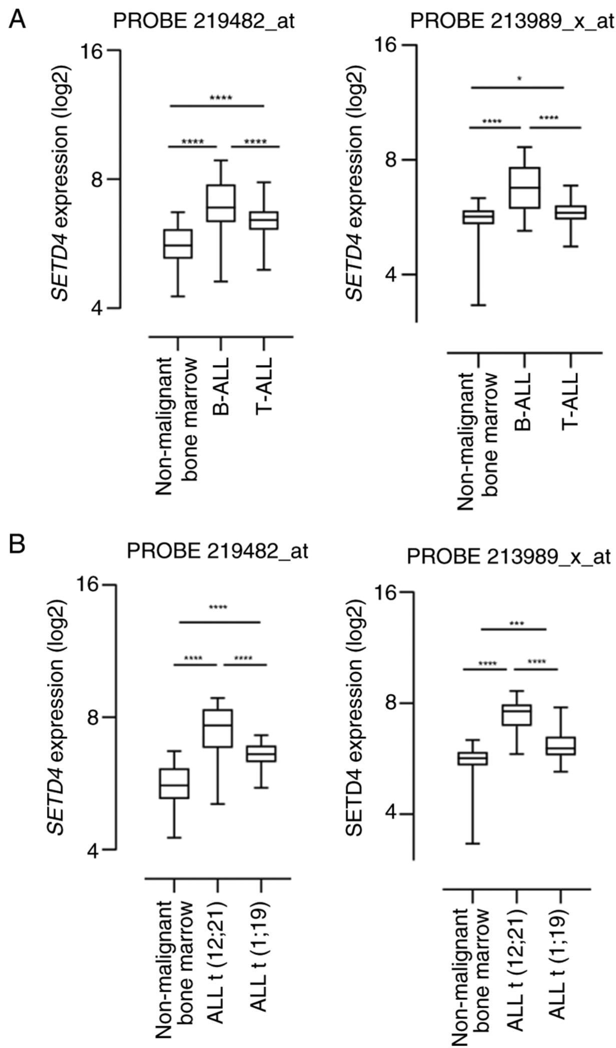

Figure 2. *SETD4* mRNA expression in healthy and ALL BM samples from the BloodSpot dataset. (A) SETD4 expression in non-malignant, B-ALL, and T-ALL samples assessed using probes 219482_at and 213989_x_at. The analyses both showed higher *SETD4* expression in B-ALL compared with non-malignant BM and T-ALL (P<0.0001). (B) Analysis of ALL molecular subtypes using the same probes revealed increased *SETD4* expression in t(12;21) compared with t(1;19). *P<0.05; ***P<0.001; ****P<0.0001. ALL, acute lymphoblastic leukemia; BM, bone marrow; B-ALL, B-cell ALL; T-ALL, T-cell ALL.

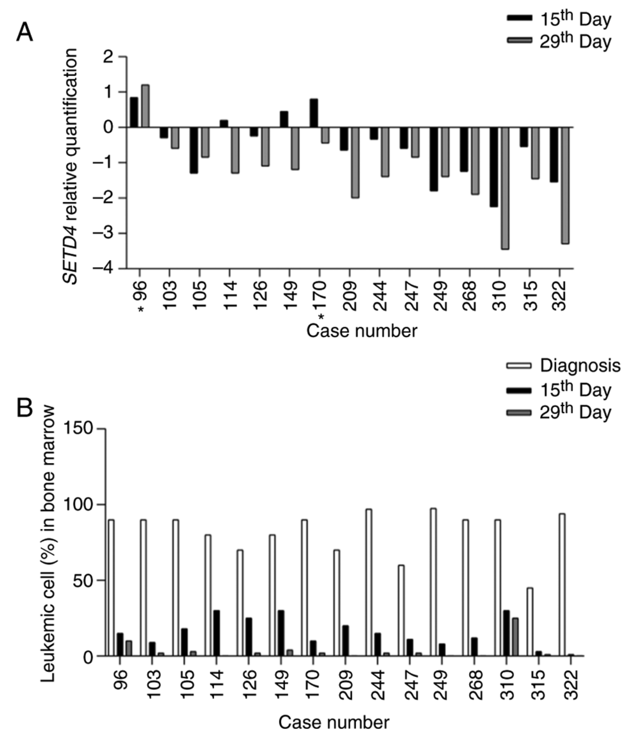

Figure 3. Correlation between BM leukemic burden and *SETD4* mRNA expression during therapy. (A) Log-scaled SETD4 RT-qPCR in 15 patients on days 15 and 29 of chemotherapy. Asterisks indicate patients without reduced SETD4 expression on day 15. (B) Percentage of leukemic BM cells at diagnosis and at days 15 and 29 of chemotherapy. BM, bone marrow; RT-qPCR, reverse transcription-quantitative PCR.

cytogenetic subtype [t(12;21) and t(1;19)]. Data normality was assessed prior to hypothesis testing: groups with normally distributed values were compared using one-way ANOVA, whereas non-normally distributed data were analyzed with the non-parametric Kruskal-Wallis test, with post hoc Tukey's multiple comparisons test.

Statistical analysis and survival curves. Descriptive statistics were used to summarize the data. P<0.05 was considered to indicate a statistically significant difference. The Mann-Whitney U test was used to compare *SETD4* expression between ALL and non-neoplastic bone marrow samples and between high-risk and low-risk groups; only samples with complete clinical information were included in risk stratification analyses.

Heatmaps were generated using z-scores calculated from ΔCt values processed in RStudio. Correlations between *SETD4* and *SMYD2* mRNA expression levels in 83 ALL samples were assessed using Spearman's correlation analysis.

Survival curves were estimated using the Kaplan-Meier method, and patients alive at the last follow-up were censored. The median follow-up time was 16.6 months (range: 0.3-45.2 months) for overall survival (OS) and 16.5 months (range: 0.1-45.2 months) for event-free survival (EFS). OS and EFS were both analyzed. Survival outcomes were compared using the log-rank test. Hazard ratios and 95% confidence intervals were calculated using univariate Cox proportional hazards regression.

Results

SETD4 transcription is upregulated in ALL patients. To verify whether *SETD4* was differentially expressed in ALL, RT-qPCR was performed on extracts from bone marrow (BM) aspirates of ALL patients and of non-malignant BM samples and evaluated the relative expression using the housekeeping gene *ACTB* for normalization. The expression of *SETD4* was significantly greater in malignant samples, with a median fold-change of 5.14 (95% CI=0.4539-23.74; P=0.0095; Mann-Whitney U test, Fig. 1A).

Similarly, two distinct microarray datasets with 318 and 317 ALL patients (Affymetrix Probes 219482_at and 23989_X_at, respectively) available in the BloodSpot database indicated that *SETD4* was highly expressed (P<0.0001) in the ALL samples compared with the non-leukemic samples (Fig. 1B). Further analysis of the mRNA levels using these same probes

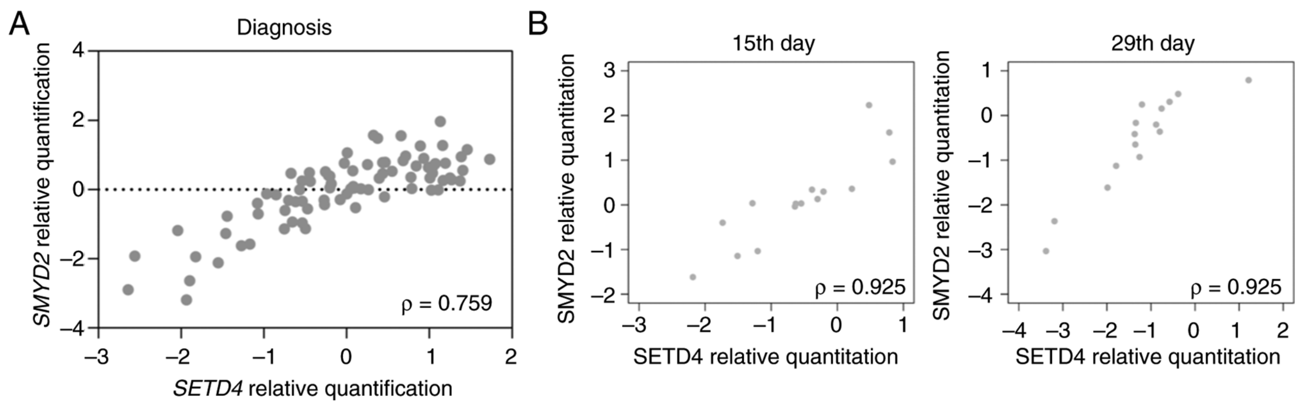

Figure 4. Correlation between *SETD4* and *SMYD2* expression profiles. (A) Samples from 83 patients, revealing a substantial correlation between both genes (Spearman ρ=0.759; P<0,0001). (B) Samples from 15 patients on days 15 and 29 of treatment and (Spearman ρ=0.925; P<0.01). Axes represent log-scaled relative quantitation values for each gene. Points illustrate the relationship between these values.

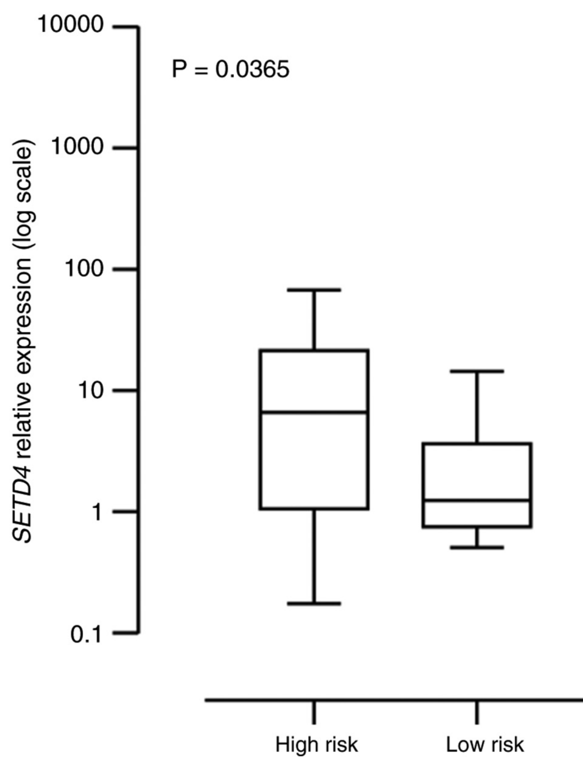

Figure 5. *SETD4* mRNA expression in BM samples from the high-risk and low-risk groups. Boxplot illustrating log-scaled RT-qPCR *SETD4* relative quantitation values normalized to *ACTB*. Out of 83 patients, 47 presented complete clinical data and were categorized as high-risk (n=38) or low risk (n=9). The Mann-Whitney U test was used to assess group differences. BM, bone marrow; RT-qPCR, reverse transcription-quantitative PCR;

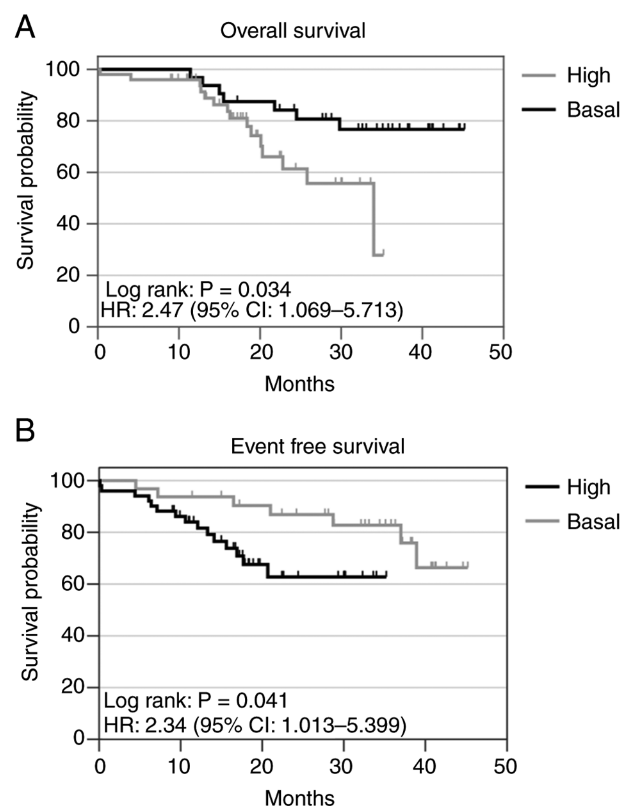

Figure 6. Overall survival and event-free survival of ALL patients according to *SETD4* expression levels. (A) High *SETD4* expression is associated with poor OS in childhood ALL patients (P=0.034) with an HR of 2.47 (95% CI: 1.069-5.713). The 3-year OS probability for patients with high SETD4 expression was 27.8 (B) High *SETD4* expression is also associated with poor EFS in childhood ALL patients (P=0.041), with an HR of 2.34 (95% CI: 1.013-5.399). The EFS probability was 82.78% for the basal group. ALL, acute lymphoblastic leukemia; OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval.

showed that *SETD4* expression was higher in LLA-B compared with LLA-T and also higher in ALL t(12;21) in comparison to ALL t(1;19) (Fig. 2A and B). Subtype analyses were not performed in the cohort due to an insufficient and unbalanced number of samples in each subgroup.

SETD4 transcription correlates with a decrease in leukemic burden and SMYD2 transcription during chemotherapy. To investigate whether *SETD4* is predominantly transcribed in leukemic cells compared with normal cells, RT- qPCR we conducted on samples from 15 patients on days 15 and 29 of chemotherapy and it was assessed whether *SETD4* expression decrease concomitantly with the reduction in leukemic burden. At the time of diagnosis, 13 patients exhibited high *SETD4* expression. Among these, 11 patients demonstrated reduced *SETD4* levels by day 15. Notably, the two patients with basal

SETD4 expression at diagnosis did not exhibit lower levels at this time point. However, except for patient 96 (from the basal expression group), all patients showed decreased *SETD4* levels by day 29 (Fig. 3A).

Additionally, the present study evaluated the leukemic burden in the bone marrow of these 15 patients at three time points: diagnosis, day 15 and 29 after initiating treatment (Fig. 3B). As expected, all patients experienced a decline in leukemic cells by days 15 and 29 post-diagnosis, with five patients achieving complete clearance of leukemic blasts by day 29.

We previously reported that *SMYD2* transcription levels are upregulated in ALL patients and is a poor prognostic factor (9). The present study investigated whether *SETD4* and *SMYD2* share any biological relationship in this context. First, it compared the transcription levels of *SETD4* and *SMYD2* among all patients at diagnosis (Fig. 4A). Next, the transcription levels of *SETD4* and *SMYD2* were examined on days 15 and 29 of chemotherapy. Surprisingly, a positive and strong correlation was detected between both genes at diagnosis (Spearman ρ=0.759; P<0,0001) and on either day of treatment (Spearman ρ=0.925; P<0.01) (Fig. 4B).

SETD4 transcription levels may be a marker for risk stratification. To investigate the relationship between *SETD4* expression levels and the risk stratification of ALL patients, samples from high-risk and low-risk groups were compared. Importantly, high-risk patients presented increased relative *SETD4* mRNA expression (Fig. 5).

SETD4 overexpression is associated with a lower overall survival in pediatric ALL patients and a worse outcome. Since *SETD4* is upregulated in most ALL patients, particularly those classified as high-risk, their survival was analyzed to determine whether this methyltransferase could markedly impact survival outcomes. It was observed that high expression levels of *SETD4* were associated with decreased OS and EFS in this subset of patients.

Kaplan-Meier analysis revealed that the 3-year OS probability for the group of patients with high *SETD4* expression was 27.8%, whereas it was 76.7% for the basal expression group (P<0.05). Additionally, the 3-year EFS probability for the basal *SETD4* expression group was 82.78%, which was markedly higher than that of the group with high *SETD4* expression (Fig. 6).

Discussion

Several KMTs have been identified as key players in leukemogenesis. In mixed lineage leukemia (MLL), the uncontrolled activities of the KMTs DOT1-like histone lysine methyltransferase and ASH1-like histone methyltransferase are crucial for abnormal cell proliferation (18). Concurrently, rearrangements in MLL, which is also a methyltransferase, are considered unfavorable prognostic factors (19-21). Another KMT, Wolf-Hirschhorn syndrome candidate 1 (WHSC1) (also known as MMSET or NSD2), is aberrantly highly expressed due to the t(4;14) chromosomal translocation in a myeloma subtype with a poor prognosis (22). Furthermore, the *WHSCH1* p.E1099K mutation is markedly prevalent among patients who experience relapsed ALL, suggesting its importance in clonal evolution and the development of drug resistance (23). Additionally, *SETD8* has been shown to regulate the interaction of p53 with Numb through methylation of the phosphotyrosine-binding domain of the latter (24). Also, the association between the rs16917496 polymorphism of the *SETD8* gene and the risk of ALL was significant (25). On the other hand, *SETD1A* has been implicated in regulating p53 and its target genes expression by binding to the Trp53 promoter and inducing specific miRNAs associated with it (26-28). Moreover, it has been linked to the process of progenitor B-cell maturation in mice (29).

The first study highlighting the oncogenic significance of *SETD4* was published by Faria *et al* in 2013 (10). These findings highlight the role of SETD4 in breast cancer. SETD4 was found in both the nucleus and cytosol in the breast cancer cell lines MDA-MB-231, MGSO-3, and MACL-1. Its upregulation was linked to an ER-negative and triple-negative phenotype. Knockdown of *SETD4* decreased cell proliferation in MDA-MB-231 cells by affecting the G1/S cell cycle transition, markedly reducing cyclin D1 levels.

Another study revealed that *SETD4* controls breast CSC quiescence (qCSC) through heterochromatin formation via trimethylation of H3K20, leading to chemoradiotherapy resistance and tumor relapse in mice. Moreover, the authors identified SETD4 qCSCs in several cancer types, such as gastric, lung, liver, ovarian and cervical cancers (11). In addition, *SETD4* upregulation has recently been identified in advanced-stage NSCLC tissues compared with early-stage tissues, particularly in the chemoresistant group. Furthermore, *SETD4* overexpression facilitated PTEN-mediated inhibition of the PI3K-mTOR pathway in activated qLCSCs, indicating that SETD4 plays a role in conferring chemoresistance, tumor progression, and poor prognosis in NSCLC by regulating the behavior of CSCs (13). Notably, another study revealed that the knockdown of *SETD4* in hepatocellular carcinoma cells resistant to sorafenib restored their sensitivity to the drug, leading to a decrease in cell viability. A reduction in *SETD4* expression combined with sorafenib treatment, downregulated AKT phosphorylation, thereby inducing the death of HCC cells (30). However, the involvement of *SETD4* in leukemia has not been previously explored.

Despite the limited cohort size, *SETD4* mRNA levels were consistently higher in diagnostic bone-marrow (BM) samples from ALL patients than in non-neoplastic BM controls, a finding validated across public datasets and by independent methods (RT-qPCR and microarray). Additionally, further investigation in public databases revealed that *SETD4* expression was markedly higher in B-ALL compared with T-ALL, as well as in ALL t(12;21) compared with ALL t(1;19).

SETD4 is located on chromosome 21, a region extensively implicated in leukemogenesis. Alterations involving chromosome 21, including trisomy 21 and intrachromosomal amplification of chromosome 21 (iAMP21), are well-established factors predisposing to leukemogenesis and markers of high-risk disease in pediatric ALL (31). Given the relevance of chromosome 21-encoded genes to leukemic transformation, several genes mapped to this region, such as *RUNX1*, *ERG*, *ETS2*, and *HMGN1*, have been extensively studied and shown to play important clinical and prognostic roles in ALL (32-35). In this genomic context, the enrichment of *SETD4* expression in B-ALL, particularly in the t(12;21) subtype observed in

public datasets, raises the possibility that *SETD4* may be integrated into transcriptional networks driven by B-cell-specific oncogenic programs.

Notably, SETD4 expression levels showed a marked decrease on treatment days 15 and 29, time points that may reflect early treatment response and leukemic blast clearance in pediatric ALL. This dynamic reduction is consistent with the association of S*ETD4* expression with leukemic burden states at diagnosis. However, the interpretation of these treatment-related dynamics is limited by the availability of paired longitudinal samples, as only 15 patients could be followed during therapy.

As with SETD4, SMYD2 trimethylates histone H3 lysine 4 (H3K4me3) and accelerates the G1/S transition (36-38). Our previous study showed that *SMYD2* is over-expressed in ALL, negatively associates with OS and EFS, and decreases during chemotherapy (9). SMYD2 is therefore considered a therapeutic target in hematological malignancies and a regulator of cancer-stem-cell quiescence; its depletion in acute myeloid leukemia reduces chemosensitivity (39). In the current cohort, *SETD4* and *SMYD2* transcript levels were strongly and positively correlated both at diagnosis and during therapy, suggesting shared upstream regulators or convergent epigenetic programs. Future functional studies are warranted to dissect this relationship and to test whether the combined inhibition of SETD4 and SMYD2 offers therapeutic benefit in ALL.

Additionally, the present study observed that patients in the high-risk group exhibited elevated *SETD4* mRNA expression. Moreover, individuals with high *SETD4* levels showed markedly worse overall survival and event-free survival compared with those with low expression. Although it remains unclear whether this upregulation represents a driver or passenger event in leukemogenesis, it is plausible that *SETD4* contributes to the progression of ALL. This hypothesis is supported by the broader oncogenic potential of SET domain-containing lysine methyltransferases, which regulate chromatin dynamics, transcriptional repression, and cellular differentiation, processes often disrupted in hematological malignancies (40). Given the putative role of SETD4 in maintaining stemness and quiescence in hematopoietic progenitors, its dysregulation may provide a survival advantage to leukemic stem-like cells, thereby promoting disease progression and therapeutic resistance.

Despite the likely oncogenic effect of *SETD4* on ALL leukemogenesis, its role in cancer development is ambiguous and appears to be context dependent. *SETD4* is mostly downregulated in prostate cancer cells and tissue samples. A decrease in the expression of *SETD4* is associated with inferior clinicopathological characteristics, such as pathological grade, clinical stage and Gleason score (11). Moreover, *SETD4* overexpression inhibited prostate cancer cell proliferation (11). By contrast, the expression of *SMYD2* was elevated in prostate cancer tissues compared with that in benign prostate tissues, and an increase in *SMYD2* expression was linked to a greater risk of biochemical recurrence following radical prostatectomy. Additionally, reducing *SMYD2* levels suppressed the proliferation of prostate cancer cells *in vivo* and *in vitro* (41).

Importantly, despite the limited sample size, the data of the present study supports a clinical association between SETD4 expression and leukemic burden, treatment response, and survival. One limitation of the present study was the restricted availability of paired longitudinal samples during therapy, which constrained further evaluation of SETD4 expression dynamics over the course of treatment. Future studies with larger longitudinal cohorts will be important to clarify the temporal role of SETD4 in ALL.

In addition, although *SETD4* and *SMYD2* transcript levels were strongly and positively correlated, the potential interaction between these methyltransferases was not explored in the present study. Further investigations addressing this relationship may help elucidate whether SETD4 and SMYD2 act within shared epigenetic programs and whether their combined targeting could be therapeutically relevant in ALL.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FP-S, ABM and LHTS were responsible for conceptualization. LHTS, DARR and FP-S were responsible for methodology. LHTS managed specimen collection and collected the data. LAMT performed experiments and contributed to data analysis. MBL contributed to the experiments presented in the present study. FP-S, LAMT and MBL wrote and finalized the manuscript. All authors have read and approved the final manuscript. FP-S, LHTS, LAMT and MBL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study protocol was approved by the Ethical Committee of the Federal District Foundation for Teaching and Research in Health Sciences, Brazil, with written informed consent from patients and/or guardians, under approval no. CEP/FEPECS 555/11. The study followed the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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