



CD44 Expression Profile Varies According to Maturational Subtypes and Molecular Profiles of Pediatric T-Cell Lymphoblastic Leukemia

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CD44 is a glycoprotein expressed in leucocytes and a marker of leukemia-initiating cells, being shown to be important in the pathogenesis of T cell acute lymphoblastic leukemia (T-ALL). In this study, we have (i) identified the aberrant antigenic pattern of CD44 and its isoform CD44v6 in T-ALL; (ii) tested the association with different T-cell subtypes and genomic alterations; (iii) identified the impact of CD44 status in T-ALL outcome. Samples from 184 patients (123 T-ALL and 61 AML; <19 years) were analyzed throughout multiparametric flow cytometry. Mutations in *N/KRAS*, *NOTCH1*, *FBXW7* as well as *STIL-TAL1* and *TLX3* rearrangements were detected using standard molecular techniques. CD44 expression was characterized in all T-ALL and AML cases. Compared with AML samples in which the median fluorescence intensity (MFI) was 79.1 (1–1272), T-ALL was relatively low, with MFI 43.2 (1.9–1239); CD44v6 expression was rarely found, MFI 1 (0.3–3.7). T-ALL immature subtypes (mCD3/CD1a^{neg}) had a lower CD44 expression, MFI 57.5 (2.7–866.3), whereas mCD3/TCRγδ^{pos} cases had higher expressions, MFI 99.9 (16.4–866.3). *NOTCH1*^{mut} and *STIL-TAL1* were associated with low CD44 expression, whereas *N/KRAS*^{mut} and *FBXW7*^{mut} cases had intermediate expression. In relation to clinical features, CD44 expression was associated with tumor infiltrations ($p = 0.065$). However, no association was found with initial treatment responses and overall survival prediction. Our results indicate that CD44 is aberrantly expressed in T-ALL being influenced by different genomic alterations. Unraveling this intricate mechanism is required to place CD44 as a therapeutic target in T-ALL.

Keywords: CD44, CD44/CD44v6, T-cell leukemia, acute myeloid leukemia, *NOTCH1*

INTRODUCTION

Childhood T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplasm characterized by increased lymphoblast count, mediastinal enlargement, central nervous system infiltration and poor outcome. Despite the great success obtained in ALL treatment along these past three decades, about 30% of T-ALL present high relapse rate with decreased chances of cure (1). Thus, with the better understanding of the T-cell development prognostic indicators, the identification of therapeutic targets in T-ALL are being urged to improve prognostic rates (2).

In this context, CD44 is an adhesion glycoprotein widely expressed in hematopoietic cells, binding to hyaluronic acid and other extracellular matrix components, assisting in the homing and anchorage of HSC to their niche (3, 4). In T-cell development, CD44 has been shown to guide precursors from the bone marrow to the thymus in mouse models (5). In addition to its role as an adhesion and homing molecule, CD44 can also transduce anti-apoptotic and proliferation signaling (6). CD44 is expressed in several malignant hematopoietic disorders, including both T-ALL and acute myeloid leukemia (AML) (7). Although clinically and genetically heterogeneous diseases, T-ALL and AML present overlaps in genetic alterations that lead to pathogenic pathways, such as mutations in genes of the RAS signaling pathway (8), and cellular expression of lineage development markers, such as CD7 expression in AML and CD13 or CD33 in T-ALL (9).

Recently CD44 was also associated with chemotherapy resistance in T-ALL, where its expression was related with the efflux of drugs from the cell *in vitro* (10). The authors also demonstrated that treatment with CD44 blocking antibodies increased the sensitivity of leukemic cells to chemotherapeutics. In addition, CD44 was identified as a direct transcriptional target of NOTCH1 in murine model of T-ALL, where NOTCH1 binding sites were found in the CD44 promoter (11). In the same model, the occupation of leukemic cells in bone marrow, spleen and thymus during disease progression was shown to be dependent on CD44, and the inhibition of CD44 by anti-CD44 antibodies led to the eradication of established T-ALL *in vitro* (11).

Based on this scenario, the aim of this study was to investigate CD44 and its isoform CD44v6 in a cohort of T-ALL cases, in order to determine its aberrant antigenic patterns and its association with different cell maturational subtypes, genetic alterations and clinical characteristics, thus contributing to a better understanding of the role of CD44 in the pathogenesis of T-ALL.

MATERIALS AND METHODS

Subjects

Samples from T-ALL and AML patients (<19 years) as well as 8 umbilical cord blood (UCB) samples were included in this analysis. Bone marrow (BM) aspirates and/or peripheral blood (PB) samples were sent to the Pediatric Hematology-Oncology Research Program, Instituto Nacional de Câncer, Rio de Janeiro, Brazil. Morphology, immunophenotyping by multiparametric flow cytometry (M-FCM), and molecular tests were performed as previously described (12–14). The diagnosis of acute leukemia subtypes followed the World Health Organization (WHO) criteria (15). T-ALL was grouped in immature or mature phenotypes. Immature phenotypes included ETP-ALL, identified according to Coustan-Smith et al. (16) and Inukai et al. (17) criteria and pre-T-ALL, which does not express CD1a and membrane CD3, but expresses other T-cell markers such as CD2, CD5, CD8. Mature phenotype included cortical T-ALL, characterized by cellular expression of CD1a and mature T-ALL characterized by the absence of CD1a and expression of membrane CD3 (15, 18). AML were grouped according to

morphological differentiation as minimally differentiated AML (M0, M1), AML with granulocytic differentiation (M2, M3), myelomonoblast AML (M4, M5), and megakaryoblastic AML (M7). Minimally differentiated AML with CD7 positivity were selected, to compare with immature T-ALL cases, as these diseases have many similar pathophysiological features.

The referring physicians provided clinical and demographic data for each case at initial diagnosis, including sex, age, presence of mediastinal mass, central nervous system (CNS) involvement, organomegaly, white blood cell count (WBC) and blast cell count in the PB on day 8 (D8) after 7 days exposure to prednisone. A PB blast count cut-off of 1,000/ μ L was used to assign patients into two groups; good responders to prednisone (<1,000/ μ L) and poor responders (>1,000/ μ L) (19, 20). Patients were not formally enrolled in treatment protocols, but were treated according to either the Brazilian Group for Treatment of Childhood Leukemia (GBTLI-ALL 99 and 2009, $n = 56$), or the Berlin-Frankfurt-Munster ALL (BFM 95 and 2002; $n = 67$) backbone strategies (21, 22).

Ethics

This study was carried out in accordance with the recommendations of Instituto Nacional de Câncer Research and Ethics Committee with written informed consent from the parents or legal guardians of all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Instituto Nacional de Câncer Research and Ethics Committee under the registry number CEP/INCA#117/12; CONEP: PB #888.277.

CD44 and CD44v6 Cellular Expression

To evaluate CD44 cellular expression, monoclonal antibodies (mAbs) anti-CD44 (G44-26 clone) conjugated to allophycocyanin (APC) fluorochrome were tested on cellular membrane T-ALL and AML blasts and UCB as comparative groups, with follow combinations of mAbs, according to subtype: CD4FITC/CD7PE/CD45PercPCy5.5/CD8PE-Cy7/CD44APC (T-ALL), CD36FITC/CD117PE/CD45PercPCy5.5/HLA-DR PE-Cy7/CD44APC (AML) and CD4FITC/CD34PE/CD45PercPCy5.5/CD8PE-Cy7/CD44APC in control UCB. The monoclonal antibodies were added to 50 μ L of BM or 1×10^6 cells, then the staining was performed according to EuroFlow protocol (23). One hundred thousand events were acquired by the FACS Canto-II Flow cytometer (Becton, Dickinson and Company, CA, USA). Data analyses were performed by Infinicyt™ version 1.8 (Cytognos, Salamanca, Spain) Software. CD44 expression was evaluated by median fluorescence intensity (MFI) in blast cells, identified by low/intermediate expression of CD45. CD44v6 FITC (R&D Systems, IgG₁ Clone #2F10) was also tested in 60 T-ALL samples.

CD44 and CD44v6 MFI normalization was performed in all cases through the ratio between MFI value on leukemic blasts and mononuclear total cells stained with IgG of the same isotype conjugated with APC for CD44 and FITC for CD44v6. The values of CD44 MFI in the median and 75th percentile were used as cutoffs to discriminate between high, intermediary or low CD44 cellular expression.

Molecular Tests

Mutational screening of *NOTCH1* (exons 26, 27 and 34, referent to HD and PEST/TAD domains), *FBXW7* (exons 9 and 10) and *NRAS*, *KRAS* (codons 12/13) were performed with conditions previously described (12). The presence of *STIL-TAL1* and *TLX3* transcripts were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) using conditions previously described (24). For all sequencing analyses, the PCR products were purified using the GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare, Amersham, UK) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) in a 3500 Genetic Analyzer (Applied Biosystems). The analyses were performed with BioEdit 7.0.9 software, comparing electropherograms with the reference sequences accessed from the National Center for Biotechnology Information (NCBI); *FBXW7* (NM_1013415.1; NG_029466.1), *NOTCH1* (NG_007458.1; NM_017617.3), *KRAS* (NG_7524.1; NM_004985.4) and *NRAS* (NM_002524.4; NG_007572.1).

Statistical Analysis

To compare the distribution of categorical variables, the Chi-square test was used, and Fisher's exact test (2-tailed) was used when the expected count in at least one cell of the table was <5. Mann-Whitney U test (comparing two Groups) and Kruskal-Wallis test (comparing more than two groups) were used to test for differences in CD44 MFI. The probability of overall survival (OS) was measured from the date of diagnosis to the date of the last follow-up or death from any cause. Relapse-free survival (RFS) was measured from the date of diagnosis to the date of relapse. Patients who did not experience an event or patients lost to follow-up were censored at their date of last known contact. The Kaplan-Meier survival analysis method was used to calculate the OS, and estimated survival values were compared using the log-rank test in order to verify the differences in outcome between CD44 with high, intermediate and low expression. Confidence Intervals (CI) of 95% were used, and *P*-values of <0.05 were considered statistically significant. Analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA, 2004) and PRISM (PRISM, GraphPad, La Jolla, CA, USA) software.

RESULTS

One hundred twenty-three pediatric T-ALL and 61 AML cases were included in this study with demographic and clinical features shown in **Table 1**. Patients with T-ALL and AML had a median age of 8 and 9 years-old, respectively; T-ALL were predominant in males with a ratio of 3.2 males: females (M: F), while in AML patients there was a 1.2 M: F ratio. The higher WBC initial count ($\geq 50 \times 10^9/L$) was predominant in T-ALL ($p < 0.001$), as well as organ infiltration presence ($p < 0.001$). Eight UCB samples were analyzed to evaluate the physiological CD44 expression in precursor cells, as shown in **Figure 1**. The CD45 and CD34 positive precursor cells population was CD44 positive, with a MFI of 194.6 (ranging from 10.7 to 619.1).

The immunophenotypic profile is shown in **Table 1**. Forty-two (34.7%) immature T-ALL (ETP-ALL and pre-T-ALL) and 79 (65.3%) mature T-ALL (cortical T and mature T-ALL) cases were

TABLE 1 | Demographic, clinical features and subtype of patients with T-cell acute lymphoblastic and acute myeloid Leukemia.

	Total	T-ALL	AML	<i>p</i>
AGE (YEARS)				
<10	98 (53.3%)	67 (54.5%)	31 (50.8%)	0.75
10–21	86 (46.7%)	56 (45.5%)	30 (49.2%)	
SEX				
Males	127 (69%)	94 (76.4%)	33 (54.1%)	0.004
Females	57 (31%)	29 (23.6%)	28 (45.9%)	
WBC ($\times 10^9 /L$)				
<50	55 (30.4%)	25 (20.3%)	30 (49.2%)	<0.0001
≥ 50	129 (69.6%)	98 (79.7%)	31 (50.8%)	
ORGAN INFILTRATIONS				
Yes	148 (80.1%)	110 (90.2%)	38 (62.3%)	<0.0001
No	35 (19.9%)	12 (9.8%)	23 (37.7%)	
SUBTYPE				
T-ALL				
ETP-ALL		16 (13.2%)	–	
pre-T		26 (21.5%)	–	
Cortical T		42 (34.7%)	–	
Mature T		37 (30.6%)	–	
AML				
With minimal differentiation		–	8 (13.1%)	
Without maturation		–	4 (6.6%)	
With maturation		–	7 (11.5%)	
Promyelocytic		–	10 (16.4%)	
Myelomonocytic		–	19 (31.1%)	
Monoblastic		–	8 (13.1%)	
Megakaryoblastic		–	4 (6.6%)	
Total	184	123 (66.8%)	61 (33.2%)	

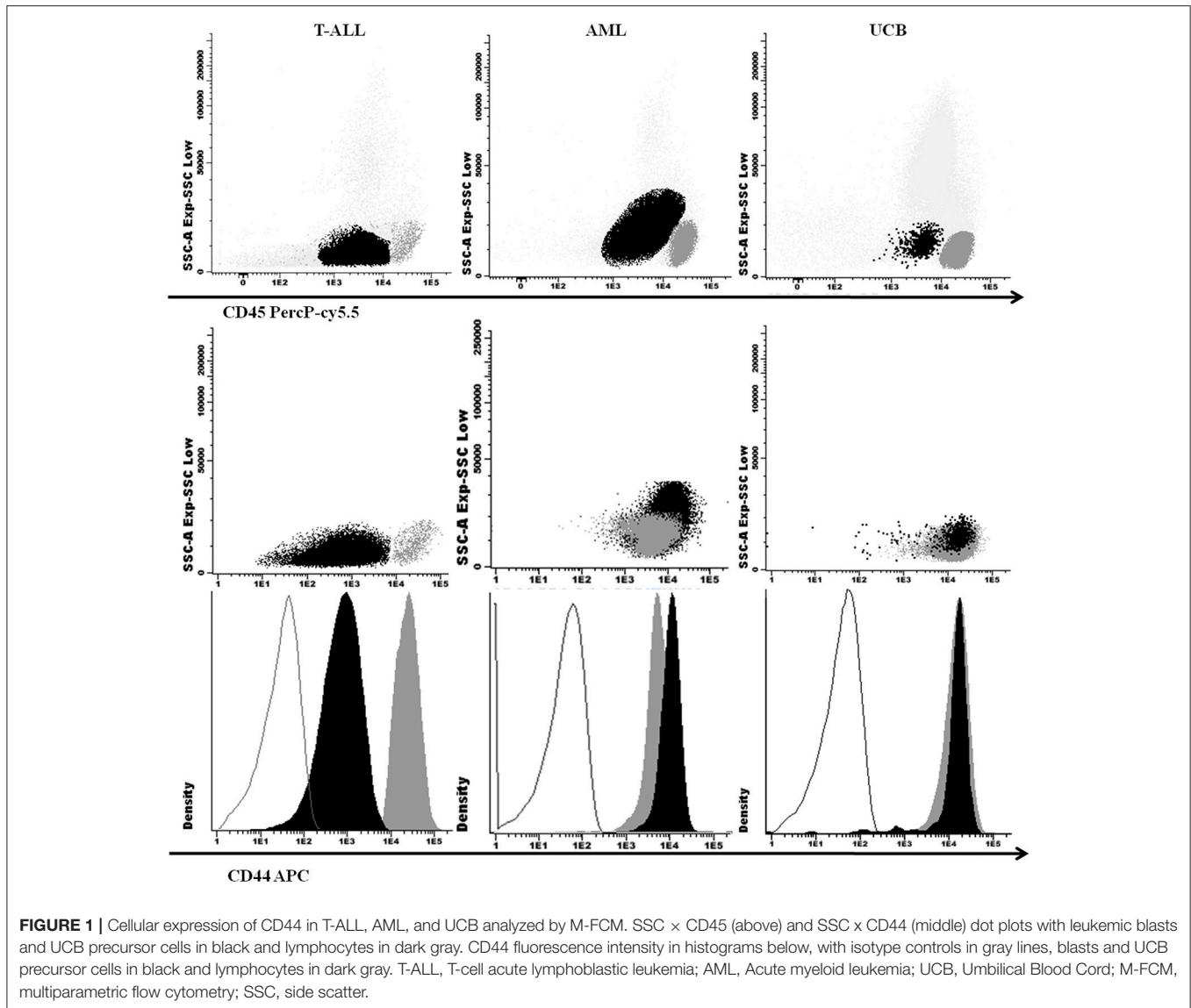
T-ALL, T-cell acute lymphoblastic leukemia; ETP, early T-cell precursor; AML, Acute myeloid leukemia; WBC, White blood cell count.

identified; amid AML subtypes, 12 (20.0%) cases were minimally differentiated AML, 17 (28.3%) with granulocytic differentiation, 27 (45.0%) myelomonocyte and 6 (6.7%) were megakaryoblastic. Aberrant phenotype with CD7 antigen was observed in 25.9% of AML samples, mainly M0 subtype.

We compared the physiological CD44 expression in UCB precursor cells, and CD44 expression in AML blasts, with T-ALL, and observed a lower CD44 expression in T-ALL ($p = 0.04$; **Figures 1, 2A**).

CD44 was also analyzed in AML samples, as a comparative group, and a MFI of 79.1 (1–1272) was observed, with a higher expression in cases of myelomonocytic AML [M4-M5; MFI 175.5 (10.2–649)] compared to other AML subgroups [AML with minimal differentiation [M0-M1], MFI 31.95 [7.2–545.7], with granulocytic differentiation [M2-M3], MFI 30.0 [7.9–191], and megakaryoblastic AML [M7], MFI 35.5 [1–1272]; $p = 0.034$; **Figure 2B**. Analyzing CD7 positivity in AML samples with minimally differentiated blast cells, a lower CD44 expression [median 23.0 (7.2–98.3)] was found, compared to CD7 negative AML [median 409.7 (311.1–545.7)] ($p = 0.004$; **Figure 2C**).

Of the 123 T-ALL cases analyzed for CD44 cellular expression, only one presented <20% positive blasts, with a percentage



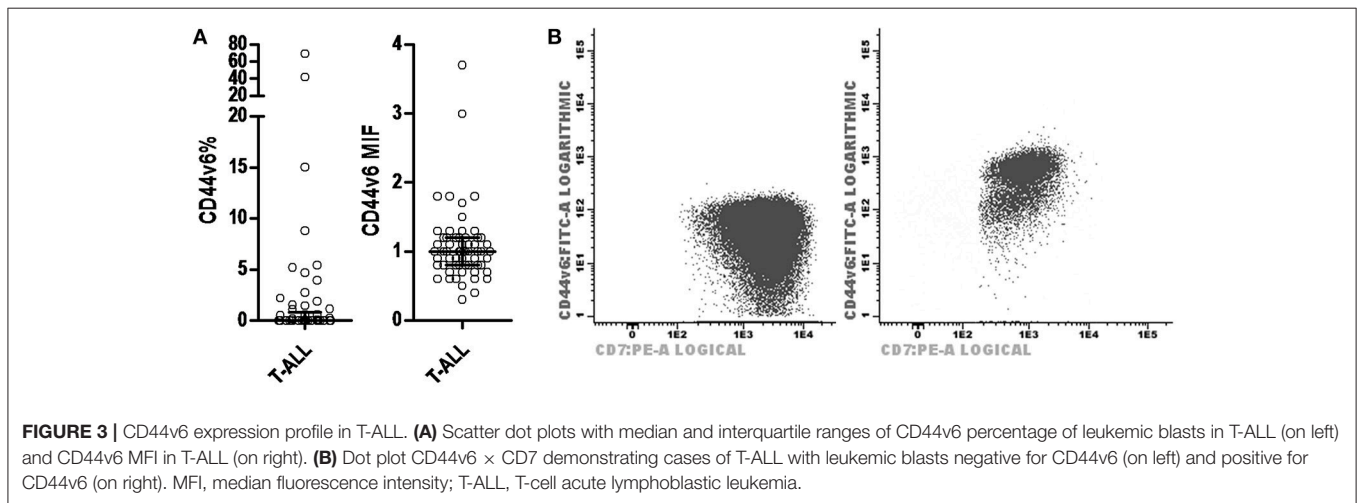
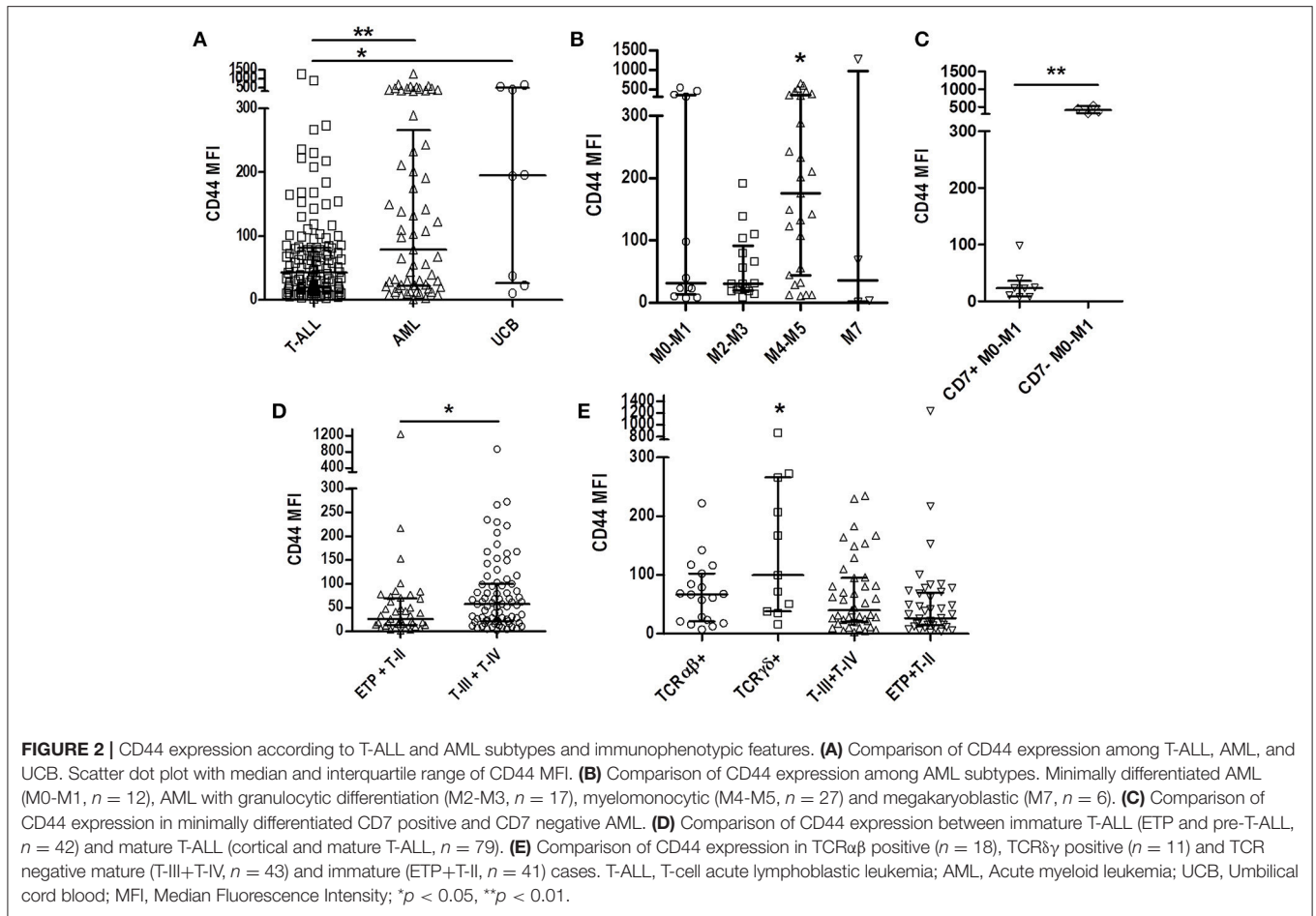
median of 95.7% (ranging from 15.6% to 100%) and median MFI of all cases of 43.2 (1.9–1239). To assess CD44 relation with T-ALL blasts' maturational profile, we compared immature T-ALL cases with mature T-ALL cases, and observed a higher CD44 expression in mature cases (MFI 57.5 [2.7–866.3], compared to MFI 26.2 [1.9–1239] ; $p = 0.013$; **Figure 2D**). Stratifying the mature cases between TCR $\gamma\delta$ or TCR $\alpha\beta$ positive cases, very high levels of CD44 expression was observed in TCR $\gamma\delta$ positive cases MFI 99.9 [16.4–866.3] compared to MFI 66.7 [7.7–222.1] ($p = 0.014$; **Figure 2E**).

Regarding CD44v6 expression in T-ALL, of the 61 cases analyzed for this marker the majority did not present expression, with a median of 0% positive cells (0–69.3%) and MFI of 1 (0.3–3.7; **Figure 3**). The description of cases with CD44v6 expression is shown in **Table 2**.

The CD44 expression in T-ALL and AML patients was analyzed according to major clinical signs, such as white

blood cell count (WBC), the presence of organ infiltration and prednisone response at day 8 of T-ALL treatment. CD44 MFI was not associated with WBC count in both T-ALL and AML (**Figure 4A**), while T-ALL cases with organ infiltration presented CD44 MFI 49.4 (1.9–1239) compared to MFI 23.7 [6.7–84.7], ($p = 0.0646$). In AML cases with organ infiltration had CD44 MFI 127.8 (3.1–649), and without infiltration MFI 32.3 (1–1272; $p = 0.214$; **Figure 4B**). Poor corticoid response was observed in 39 out of 67 (58.2%) and was not significantly associated with CD44 median expression in T-ALL (**Figure 4C**). T-ALL relapse occurred in 16 cases out of 62 (25.8%) and was also not significantly associated with CD44 median expression (**Figure 4D**). CD44 expression was not associated with patient's age and sex (**Table 3**).

T-ALL most common genetic alterations such as *STIL-TALI* rearrangement, *TLX3* rearrangement, *NOTCH1* and *FBXW7* mutations were assessed, to evaluate CD44 expression levels



in different molecular subgroups; 22 cases (21.0%) were *STIL-TAL1* positive and 5.3% *TLX3*; 50 cases (48.5%) had *NOTCH1* mutations, and 19 *FBXW7* mutations. *N/KRAS* mutations and CD44 cellular expression were evaluated in 121 T-ALL and the frequency of *N/KRAS* mutations was 10.7%. In T-ALL, *N/KRAS* and *FBXW7* mutations were associated with an intermediate

CD44 expression ($p = 0.029$ and $p = 0.032$, respectively; **Table 3**). T-ALL cases harboring *NOTCH1* mutations and *STIL-TAL1* positive cases had lower CD44 expression than wild type cases (*NOTCH1* mutated MFI 39.4 [5.8–866.3], *STIL-TAL1* positive MFI 19.3 [3.9–234.7], *NOTCH1* mutated and *STIL-TAL1* positive MFI 20.9 [6.7–84.7], *STIL-TAL1* negative and

TABLE 2 | Description of CD44v6 positive T-ALL cases.

Patient	CD44v6 MFI	CD44v6%	CD44 MFI	Age (yrs)/Sex	WBC	Infiltration*	Subtype	Molecular alteration [#]
#1	1.3	0.6	Low	10/M	117,000/ μ L	Yes	T-IV, TCR $\gamma\delta$	-
#2	1.3	4.0	Interm.	16/M	200,120/ μ L	No	T-III	-
#3	1.3	2.7	Low	6/M	56,000/ μ L	Yes	T-III	<i>NOTCH1</i> mut; <i>N/KRAS</i> mut
#4	1.5	4.7	Low	11/F	250,000/ μ L	Yes	T-IV, TCR $\alpha\beta$	<i>NOTCH1</i> mut; <i>STIL-TAL1</i> pos
#5	1.7	5.4	Interm.	0.3/M	399,000/ μ L	Yes	ETP	-
#6	1.8	8.8	Interm.	17/M	800,000/ μ L	No	T-IV, TCR $\alpha\beta$	<i>STIL-TAL1</i> pos
#7	1.8	15	Interm.	11/M	451,000/ μ L	Yes	ETP	-
#8	1.8	5.2	Low	13/M	145,600/ μ L	Yes	T-III	<i>FBXW7</i> mut
#9	3.0	41.7	Interm.	7/M	198,000/ μ L	Yes	T-II	<i>NOTCH1</i> mut; <i>N/KRAS</i> mut
#10	3.7	69.3	Low	8/M	12,300/ μ L	Yes	T-III	<i>STIL-TAL1</i> pos

*Mediastinum enlargement, cases #3, #4 and #10; Central nervous system infiltration, case #5. Interm., intermediate; mut, mutation; pos, positive.

NOTCH1 WT MFI 68.75 [3.3–272.3], $p = 0.017$, $p = 0.031$ and $p = 0.034$; **Figure 5**. *TLX3* had no association with CD44 expression (**Table 3**).

To assess the value of CD44 in T-ALL as a prognosis marker, we compared overall survival rates between CD44 low, intermediate and high expression groups. Overall, T-ALL cases presented a survival of 56.9%. CD44 levels were not associated with survival in T-ALL ($p = 0.436$; **Figure 4E**).

DISCUSSION

Here we explored CD44 cellular expression in a series of childhood T-ALL. Previously, CD44 phenotype in 30 T-ALL cases was positively associated with increased CNS and tumor infiltrations (25). Since then, the mutational landscape of T-ALL was successfully investigated bringing insight into the T-ALL pathogenesis mechanisms, while the CD44 molecule role was unveiled in animal models and in human cell-lines (6, 8, 26, 27). Following the first clinical report on CD44 expression in T-ALL, very few clinical-translational approaches were performed to test the value of CD44 as a marker either in tumor expansion or its relevance in the role of T-ALL outcome. Therefore, the challenge of the present study was to bring more information regarding the relevance of CD44 expression in T-ALL pathogenesis.

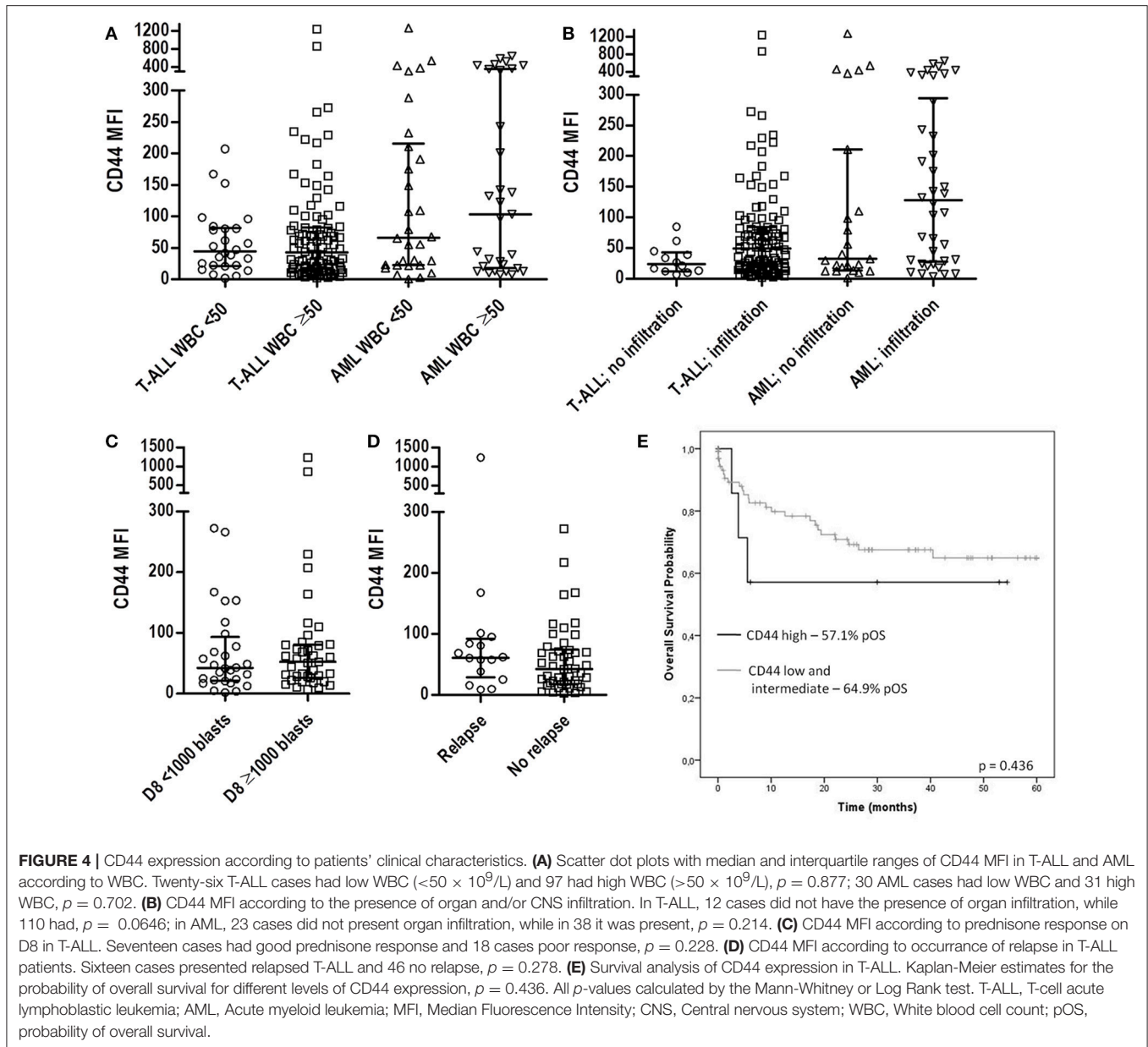
Taking into account the normal thymocyte differentiation, CD44 expression is quickly lost in cells in the CD4 CD8 double negative stage and later regained in the double positive stage in T-cells (2). The fact that CD44 positive expression was found in all T-ALL subtypes indicates that CD44 represents an aberrant phenotype in T-ALL and must be considered in the constellation of leukemic markers. In the initial analysis to establish the physiological pattern of CD44 in precursor cells, CD44 expression was screened on CD34 positive UCB precursor cells and also compared to the expression in AML where it was observed that the aberrant expression pattern of CD44 in T-ALL varies from low to intermediate, since normal CD34 positive precursor cells have high expression. AML blasts also had lower expression of CD44 compared to precursor cells of UCB. In the physiological context, the magnitude of CD44 expression is crucial for the differentiation of CD34 positive precursors,

where high levels of CD44 lead to differentiation of precursors into monocytes and dendritic cells, and decrease in CD44 expression, differentiation into thymocytes (28). Evaluating CD44 expression in AML maturational subtypes, we found that myelomonocytic AML had the highest CD44 expression among other subtypes, which mirrors the CD44 expression in normal myelomonocytic differentiation, where monocytic precursors show the highest expression of this marker (29). On the other hand, we observed that minimally differentiated AML with CD7 expression and immature T-ALL (ETP and pre-T ALL) had low CD44 expression. Low CD44 expression could then indicate multilineage potential in these diseases.

The highest expression of CD44 in mature subtypes of T-ALL compared to the immature subtypes is similar to the increase in expression that occurs later in differentiation of T cells (2). TCR $\gamma\delta$ + T-ALL had the highest levels of CD44 expression, compared to TCR $\alpha\beta$ + and other T-ALL subgroups. These mature T-ALL with different TCR expressions represent phenotypically and biologically distinct subgroups that differ primarily in relation to specific molecular alterations for each TCR lineage, such as *STIL-TAL1* alteration in $\alpha\beta$ -specific lineage, and *TLX3* in $\gamma\delta$ -specific lineage (30). These divergent characteristics between TCR $\alpha\beta$ or $\gamma\delta$ positive T-ALL are also reflected in the different expression of CD44 for each subtype, which makes us consider that CD44 is crucial in the fate of these cells.

Regarding the CD44v6 variant molecule expression, only two cases of T-ALL exhibited more than 20% positive leukemic blasts. CD44v6 was previously associated with the prognosis of patients with AML (29). The almost homogeneous absence of CD44v6 expression in this series of T-ALL cases may be indicative that in the cellular context of T-ALL, the absence of CD44v6 may not play a significant role in the pathogenesis of the disease, but may be interpreted as phenotype aberration. In a study with patients with B-ALL (31), CD44v6 was also rarely expressed, and not associated with survival or relapse of patients, demonstrating that CD44v6 expression does not provide additional information to aid in the designation of patients for specific treatment protocols.

The leukemic transformation of immature thymocytes is the result of a multi-step process involving several different gene



alterations that cooperate toward pathogenesis. It is essential to understand the possible interaction between CD44 cell expression and molecular alterations in the T-ALL, resulting in possible therapeutic interventions. With this motivation, we analyzed CD44 expression according to T-ALL genomic alterations, and observed that alterations in *NOTCH1*, *STIL-TAL1*, *FBXW7*, and *N/KRAS*, are associated with different phenotypic expression levels of CD44, suggesting that these genes may participate in regulation of CD44.

The low expression of CD44 was associated with *NOTCH1* mutations and the presence of *STIL-TAL1*. CD44 has previously been described as *NOTCH1* transcription target (11), and may explain this association. However, in relation to the *TAL1* transcription factor, there are still no studies to date that

demonstrate this association with CD44. Thus, these alterations may be interacting with each other and influencing the low expression of CD44. Unraveling this intricate mechanism represents yet another step toward the introduction of new therapeutic approaches in T-ALL.

On the other hand, higher CD44 expression was associated with mutations in *N/KRAS* and *FBXW7*, where the majority of cases with these mutations more frequently had an intermediate CD44 expression. The positive feedback loop between CD44 and the RAS signaling pathway (32) may explain this increase in CD44 expression in *N/KRAS* mutation cases. It is interesting to note that of only two cases with high expression of CD44v6, one of them presented mutation in *N/KRAS*, which corroborates the data described by Cheng

TABLE 3 | Demographic and clinical characteristics and genomic alterations of T-ALL cases, according to CD44 expression levels.

	Total (%)	CD44 expression			p
		Low	Intermediate	High	
Age (years)					
<10	67 (54.5%)	35 (56.5%)	14 (46.7%)	18 (58.1%)	0.608
10–18	56 (45.5%)	27 (43.5%)	16 (53.3%)	13 (41.9%)	
Sex					
Male	94 (76.4%)	44 (71.0%)	25 (83.3%)	25 (80.6%)	0.345
Female	29 (23.6%)	18 (29.0%)	5 (16.7%)	6 (19.4%)	
WBC					
<50000/ μ L	25 (20.3%)	13 (21.0%)	6 (20.0%)	6 (19.4%)	0.982
\geq 50000/ μ L	98 (79.7%)	49 (79.0%)	24 (80.0%)	25 (80.6%)	
Organ infiltrations*					
Mediastinum enlargement					
Yes	45 (36.9%)	23 (37.1%)	9 (30.0%)	13 (43.3%)	0.563
No	77 (63.1%)	39 (62.9%)	21 (70.0%)	17 (56.7%)	
Hepatomegaly					
Yes	84 (68.9%)	42 (67.7%)	21 (70.0%)	21 (70.0%)	0.964
No	38 (31.1%)	20 (32.3%)	9 (30.0%)	9 (30.0%)	
Splenomegaly					
Yes	91 (74.6%)	43 (69.4%)	23 (76.7%)	25 (83.3%)	0.337
No	31 (25.4%)	19 (30.6%)	7 (23.3%)	5 (16.7%)	
Lymph node enlargement					
Yes	84 (68.9%)	42 (67.7%)	21 (70.0%)	21 (70.0%)	0.964
No	38 (31.1%)	20 (32.3%)	9 (30.0%)	9 (30.0%)	
CNS Infiltration					
Yes	5 (4.3%)	2 (3.3%)	1 (3.4%)	2 (7.4%)	0.656
No	112 (95.7%)	59 (96.7%)	28 (96.6%)	25 (92.6%)	
NOTCH1					
Mut.	49 (47.6%)	29 (56.9%)	11 (44.0%)	9 (33.3%)	0.13
WT	54 (52.4%)	22 (43.1%)	14 (56.0%)	18 (66.7%)	
FBXW7					
Mut.	19 (18.4%)	6 (11.8%)	9 (36.0%)	4 (14.8%)	0.032
WT	84 (81.6%)	45 (88.2%)	16 (64.0%)	23 (85.2%)	
STIL-TAL1					
Positive	33 (27.7%)	21 (35.6%)	4 (13.3%)	8 (26.7%)	0.085
Negative	86 (72.3%)	38 (64.4%)	26 (86.7%)	22 (73.3%)	
TLX3					
Positive	6 (5.3%)	4 (7.0%)	1 (3.4%)	1 (3.6%)	0.703
Negative	108 (94.7%)	53 (93.0%)	28 (96.6%)	27 (96.4%)	
N/KRAS					
Mut.	13 (10.7%)	5 (8.2%)	7 (23.3%)	1 (3.3%)	0.029
WT	108 (89.3%)	56 (91.8%)	23 (76.7%)	29 (96.7%)	
Total	123	62 (50.4%)	30 (24.4%)	31 (25.2%)	

*One T-ALL case did not have further information of organ infiltrations. T-ALL, T-cell acute lymphoblastic leukemia; WBC, White blood cell count; CNS, Central nervous system; Mut, mutation detected; WT, wild type. Bold and italic values represent $p < 0.05$.

et al. (32). As for *FBXW7* mutations, it was previously reported by Babaei-Jadidi et al. (33) an increase in CD44 expression in *FBXW7* deleted intestinal cells in murine models.

In the present study it was observed that the higher expression of CD44 was associated with infiltrations in the patient's organs,

which has already been described previously (25), and can be explained by the role of adhesion molecule and homing of CD44. High expressions may also have the future role of predicting hidden infiltrations in patient organs. Although CD44 gene expression was previously described as a predictor of relapse in T-ALL (34), and its associated cell expression and chemoresistance

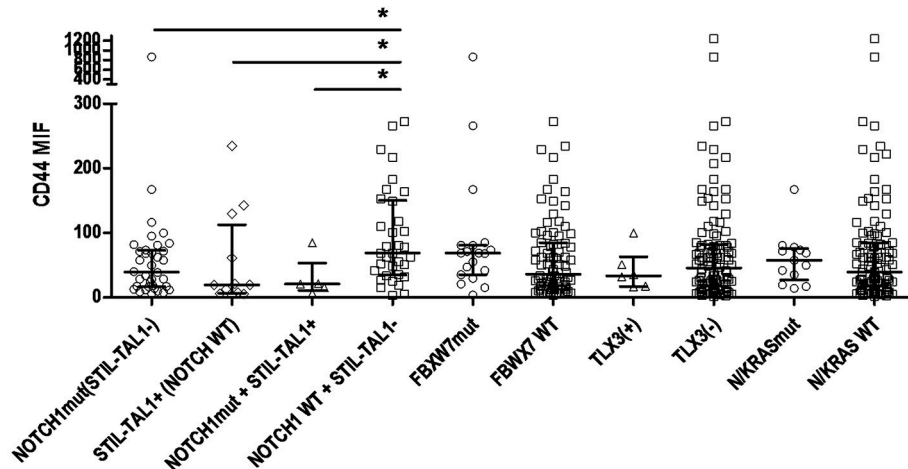


FIGURE 5 | CD44 expression according to status of genomic alterations in T-ALL. Scatter dot plots with median and interquartile ranges of CD44 MFI in T-ALL. Cases with *NOTCH1* mutations and *STIL-TAL1* (-), $n = 36$; *NOTCH1* WT, *STIL-TAL1*(+) cases, $n = 12$; *STIL-TAL1*(+) and *NOTCH1* mutated cases, $n = 5$; *NOTCH1* WT and *STIL-TAL1*(-) cases, $n = 34$; *FBXW7* mutated, $n = 19$; *FBXW7* WT, $n = 84$; *TLX3* positive, $n = 6$; *TLX3* negative, $n = 108$; *NIKRAS* mutated, $n = 13$; WT, $n = 108$. MFI, median fluorescence intensity; mut, mutation; WT, wild type; (+), positive cases; (-), negative cases; * $p < 0.05$.

(10), the present study did not observe association between CD44 cellular expression and prednisone response, as well as overall survival of the patient. CD44, then, has not been shown to be useful as a marker of prognosis or response to treatment.

In conclusion, CD44 was highly expressed in pediatric T-ALL whereas the isoform CD44v6 was rarely expressed. CD44 expression was associated with different immunophenotypic profiles and genomic alterations. CD44 was correlated with presence of patients' organ infiltrations, although no association was found with initial response to glucocorticoid, treatment response, and overall survival prediction. These results suggest that CD44 is aberrantly expressed in T-ALL blasts, in different genomic alteration subtypes. Further studies are needed to test the CD44 aspect in homing of leukemic blasts.

AUTHOR CONTRIBUTIONS

LVCN conducted and analyzed all the experiments and wrote the paper. LVCN, ETG, and EPN performed the immunophenotypic analyses. LVCN, FGA, FVS-B, MBM, and EPN performed the molecular assays. EPN supervised the study, critically analyzed the data, and reviewed the writings. MSPO designed and supervised the study, critically analyzed the data, and reviewed the writings. All authors critically analyzed the data, reviewed the writing, and approved the final draft of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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