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ABSTRACT

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a heterogeneous malignancy and consists of several genetic abnormalities. Some of these abnormalities are used in clinics for risk calculation and treatment decisions. Patients with *ZNF384* rearrangements had a distinct expression profile regardless of their diagnosis, BCP-ALL or mixed phenotype acute leukemia (MPAL) and defined as a new subtype of ALL. In this study, we screened 42 MPAL and 91 BCP-ALL patients for the most common *ZNF384* fusions; *ZNF384::TCF3, ZNF384::EP300* and *ZNF384::TAF15* by using PCR. We identified *ZNF384* fusions in 9.5% of MPAL and 7.6% of BCP-ALL. A novel breakpoint was identified in *ZNF384::TCF3* fusion in one BCP-ALL patient. T-myeloid MPAL patients showed significantly lower *ZNF384* expression compared to lymphoid groups. Patients with *ZNF384*r had intermediate survival rates based on other subtypes. Prognostic and patient-specific treatment evaluation of *ZNF384* fusions in both ALL and MPAL might help to improve risk characterization of patients.

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KEYWORDS
B-cell precursor ALL; mixed phenotype acute leukemia; *ZNF384* fusion; *ZNF384* expression levels

Introduction

Acute lymphoblastic leukemia (ALL) is a group of diseases and characterized by uncontrolled proliferation of T-cell or B-cell progenitors. Mixed phenotype acute leukemia (MPAL) express both lymphoid and myeloid lineage specific antigens, which makes it difficult to determine lineage and it was grouped under acute leukemia of ambiguous lineage in the classification of myeloid neoplasms and acute leukemia by the World Health Organization (WHO) in 2016 [1–3].

More than 75% of ALL cases are B-cell lineage [4,5]. B-cell precursor (BCP)-ALL has been characterized by several different genetic subtypes including *ETV6::RUNX1, TCF3::PBX1,* and *BCR::ABL1* translocations that have an impact on prognosis and are used in risk classification in some treatment protocols. The other oncogenic subtypes of BCP-ALL such as *DUX4, MEF2D,* and *ZNF384* rearrangements were identified by whole genome approaches [6–8].

ZNF384 gene rearrangements are observed approximately in 3%–4% of pediatric patients with BCP-ALL [8,9]. The *ZNF384* rearrangements are largely specific to ALL, which suggests that this zinc finger transcription factor has a potential role in leukemogenesis. Leukemic cells that harbor fusion genes associated with *ZNF384* often express myeloid markers, suggesting an association with MPAL [10]. *ZNF384* gene rearrangements have distinct immunophenotype profile ranging from BCP-ALL to MPAL. Thus, they are defined as a new subtype of acute leukemia, which is clustered separately from other subtype [11].

ZNF384 gene encodes a C2H2-type protein which is involved in transcription and nucleocytoplasmic transport [12]. *ZNF384* is a conserved gene and expressed in various human tissues [13]. Transcription factors are

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important in cell growth regulation, development and differentiation in the hematopoietic system [14]. *ZNF384* gene has numbers of fusion partners such as *ARID1B, BMP2K, CREBBP, EWSR1, EP300, TAF15,* and *TCF3*. The *EP300* gene encodes a histone acetyltransferase and has tumor suppressor activity that affects transcription by chromatin remodeling [12,15]. The *TAF15* gene is a member of the human *TET (TLS, EWS,* and *TAF15*) protein family [16]. This protein family has been associated with early transcriptional initiation and elongation [17]. *TCF3* protein plays an important role in lymphoid and hematopoietic stem cell development [18].

The expression of *TCF3::ZNF384* was recently discovered to cause lineage disruption in human hematopoietic cells [19]. In this study, we determined the frequency of *ZNF384* rearrangements with the most common gene partners *TCF3::, EP300::, TAF15::ZNF384,* and measured *ZNF384* expression level in pediatric patients with BCP-ALL and MPAL. We evaluated expression and translocation results with clinical findings.

Material and Methods

Patients and controls

One hundred and thirty-three patients with BCP-ALL $n = 91$ and MPAL $n = 42$ who were diagnosed in pediatric hematology oncology clinics between 2005 and 2014 years were retrospectively enrolled in the study. MPAL cases were systematically selected; on the other hand, BCP-ALL cases were randomly included in the study. Bone marrow samples of pediatric patients at diagnosis with blast counts >80% were used in this study. Median age was 4 years old (minimum 1–maximum 18 years) and sex ratio was 80:53 (Male: Female). Median white blood cell (WBC) count at diagnosis was 10,200 ($\times 10^9$/L) (minimum 1030–maximum 702,000), median hemoglobin (Hb) level was 8.05 (g/dL) (minimum 3.4–maximum 13.4), median platelet (PLT) count was 45,500 ($\times 10^9$/L) (minimum 2020–maximum 924,000). In our study cohort, one patient was t(4;11) positive, seven patients were t(9;22) and twenty patients were t(12;21) fusions positive. Two patients showed central nervous system ($n = 2$), testis ($n = 1$), and mediastinum ($n = 2$) involvements and lymphadenopathy was detected in 26 cases. Patients with BCP-ALL and MPAL were treated according to the ALL-BFM 95 (Berlin-Frankfurt-Munster) or ALLIC2009 protocols at diagnosis based on patients' risk classification. Median follow-up time was 5 years (minimum: 1 and maximum: 11).

Patients were evaluated and classified according to Berlin, Frankfurt, Münster(BFM), WHO, and EGIL protocols. BCP-ALL samples were selected according to BFM protocol criteria, which require strong positivity in ≥2 of four antigens which are CD19, CD10, CD22, and CD79a [20]. MPAL samples were classified according to 2008/2016 WHO and EGIL classifications. Based on 2008/2016 WHO criteria, we used especially CD19 and CD10 markers for B lineage, Myeloperoxidase (MPO) for myeloid lineage and CD3 for T lineage. The WHO classification does not specify an expression threshold for these markers [21,22]. In this study, CD19, CD10, and CD20 expression levels were used for B lineage determination. CD33, CD13, and MPO markers were used for myeloid lineage, CD3, CD5, and CD7 markers were used for T lineage in EGIL classification. As defined in EGIL classification, the threshold for the positive values referred to is at least 20% expression levels for surface markers and minimum 10% expression levels for cytoplasmic markers. Based on EGIL classification, MPAL is classified when scores are >2 in two lineages [22]. Our patients with MPAL were further classified as T-myeloid ($n = 2$), B-myeloid ($n = 28$), or B/T ($n = 12$) phenotypes. Peripheral blood ($n = 4$) and bone marrow samples ($n = 2$) were available from healthy donors and used as controls.

Written informed consents were obtained from parents of the patients. The study was approved by the Istanbul University Istanbul Faculty of Medicine Clinical Research Ethics Committee (2018/705).

Detection of ZNF384 fusions by PCR

Total RNA was extracted from the bone marrow blast of >80% samples of the patients before treatment by using RNeasy Mini Kit (Qiagen). RNA quantification was determined by spectrophotometer. cDNA was synthesized from one microgram of total RNA by First-Strand cDNA Synthesis kit (Invitrogen) according to the instructor's manual. *EP300::ZNF384*, *TCF3* (exon 13)::*ZNF384* (exon 3), *TCF3* (exon 17)::*ZNF384* (exon 7), and *TAF15::ZNF384* fusion gene regions were amplified by conventional PCR using a T100 Thermal Cycler (Bio-Rad, USA) with specific primers (Supplemental Table S1). The PCR was performed the following conditions: an initial denaturation at 95 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 57 °C–60 °C for 30 s, extension 72 °C for 1 min and a final extension step at 72 °C for 10 min. PCR amplifications were checked on a 2% agarose gel. *CYPA* gene was used as internal control. The control plasmid samples were kindly provided by Kentaro Ohki (Department of Pediatric

Hematology and Oncology Research, National Research Institute for Child Health and Development, Tokyo, Japan). We used plasmid DNA as a positive control for each fusion transcript into which breakpoints were copied by means of pGEM-T Easy Vector. Each translocation positive samples' PCRs were repeated two times for confirmation. Samples with translocation were screened to determine exact fusion points by Sanger sequencing.

ZNF384 gene expression by QPCR

Expression levels of *ZNF384* were detected by using Quantitative Real-time PCR (qPCR) with the qPCR SYBR Green Master kit (Roche Diagnostics, Manheim, Germany) on the Light Cycler 480 Instrument (Roche Applied Sciences, Manheim, Germany) with specific primers. *GAPDH* was used for normalization with specific primers listed in the Supplemental Table S1. Primers (5 pmol) and cDNA (200 ng) were used in each run and all samples were studied in duplicates. Melting curve analysis was performed to evaluate the specific amplification, and ΔCt method was used for relative quantification [23]. The PCR program was settled as follows: initial denaturation at 95 °C for 7 min; amplification for 5 s at 95 °C, 10 s at 56–60 °C, and 10 s at 72 °C for 45 cycles; and melting curve for 15 s at 60 °C for one cycle.

Statistical methods

Unpaired t-test Welch's correction test was used to compare the relative mRNA levels of the samples and chi-square ($\chi 2$) test was used for categorical variables. Overall survival (OS) and event free survival (EFS) analysis were estimated by Kaplan–Meier, log-rank test, and survival regression models such as the Cox model. To analyze the patients CD10, CD13, and CD33 expressions, unpaired *t*-test was used. A ROC curve analysis was performed to evaluate the validity of gene expressions. Area under the ROC curve namely AUC value is between 0 and 1. The higher of AUC values of model means the greater the discrimination power of the test. The p value < 0.05 was considered statistically significant. All analyses were performed by using GraphPad Prism 9 and SPSS (IBM SPSS Data Editor Inc).

Results

ZNF384 rearrangements in patients with BCP-ALL and MPAL

Here we examined four different breakpoints for *ZNF384* in 133 primary BCP-ALL and MPAL samples by qPCR and results were validated by Sanger Sequencing. In total, 9.5% ($n = 4$) of MPAL and 7.6% ($n = 7$) of BCP-ALL patients had *ZNF384* gene rearrangements. *TCF3*::*ZNF384* fusion was detected in 4.7% ($n = 2$) of MPAL and 4.4% ($n = 4$) of BCP-ALL patients. *TCF3*- exon 13 breakpoint created a fusion with *ZNF384*-exon 3, *TCF3*-exon 15 and 17 breakpoints fused to *ZNF384*-exon 7 (Supplemental Fig. S2). *TCF3* (Ex15)::*ZNF384* (Ex7) fusion is reported for the first time with this study (Figure 1). P#100 had a novel breakpoint in *TCF3* (Ex15)::*ZNF384* (Ex7) fusion who was also t(12;21) translocation carrier.

TAF15::*ZNF384* fusion was observed in 7.1% ($n = 3$) of MPAL and less frequently in BCP-ALL (2.2%, $n = 2$). The frequency of *EP300*::*ZNF384* fusion was 2.3% ($n = 1$) in MPAL group and 2.2% ($n = 2$) in BCP-ALL patients. *TAF15* exon 6 and 7 breakpoints fused with *ZNF384*-exon 3, *EP300* from exon 6 breakpoint fused to exon 3 of *ZNF384*. One patient with BCP-ALL (P#28) and two patients with MPAL (P#126, P#127) harbored two different *ZNF384* fusions. P#28 (*TCF3*- and *EP300*-) and P#127 (*TCF3*- and *TAF15*-) who had biclonal or clonal background for *ZNF384* fusions were classified in medium risk group (MRG), both obtained induction therapy response (ITR), and they were relapse free for five years. On the other hand, a patient with B-myeloid MPAL (P#126) (*TCF3*- and *TAF15*-) was classified in high-risk group (HRG) with high WBC counts ($124{,}800 \times 10^9$/L). Patients with ZNF384r were listed in Table 1 together with basic clinical features (Table 1).

ZNF384 gene expression in BCP-ALL and MPAL

We examined mRNA levels of *ZNF384* gene from the available materials of patients with BCP-ALL ($n = 48$) and patients with MPAL ($n = 30$). Healthy bone marrow and peripheral blood samples were used as controls. No significant difference was observed between the BCP-ALL and control samples in regards to their *ZNF384* expression ($p = 0.572$, Figure 2(A)). Similarly, MPAL samples did not show any differences for*ZNF384* expression compared to controls and BCP-ALL patients ($p = 0.415$ and $p = 0.261$ respectively, Figure 2(A)).

Relative gene expression levels of *ZNF384* were analyzed according to different BCP-ALL and MPAL subgroups. Although there were few patients in the T-myeloid (MPAL) subgroup, they showed significantly lower *ZNF384* expression compared to B-T (MPAL) samples ($p = 0.044$) and BCP-ALL patients ($p = 0.0007$). B-T (MPAL) subgroup showed relatively increased expression levels according to B-myeloid subgroup,

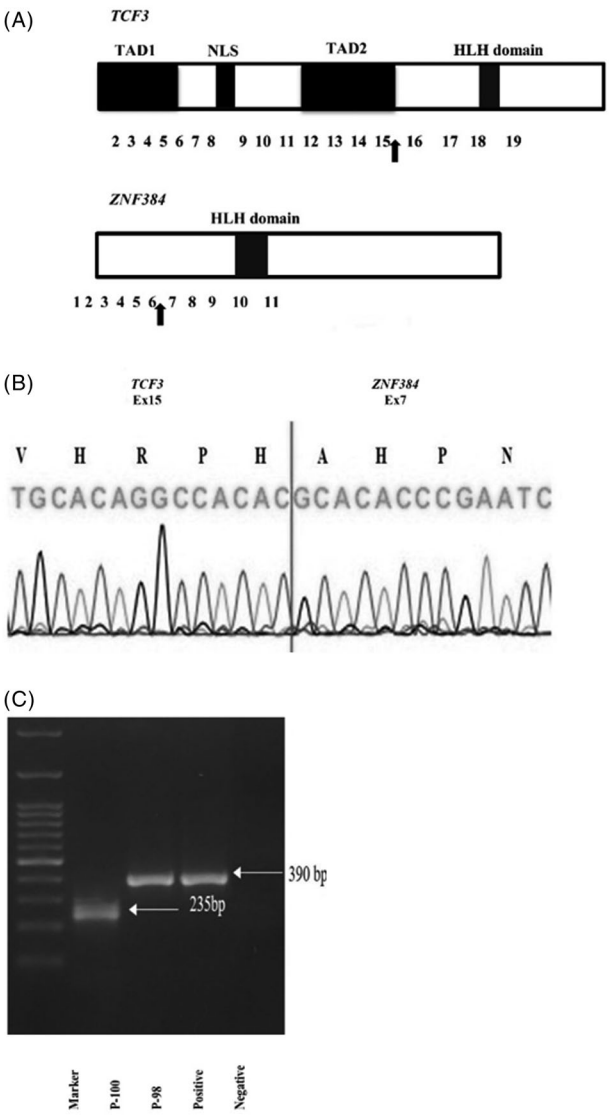


Figure 1. Novel breakpoint of *TCF3::ZNF384* fusion. (A) Fusion protein structure of *TCF3* (ex15) – *ZNF384* (ex7). HLH domain: helix loop helix domain; NLS: nuclear localization signal; TAD1: transcriptional activation domain 1; TAD2: transcriptional activation domain 2. (B) Sanger diagram of novel *TCF3::ZNF384* fusion breakpoint. (C) PCR product of novel *TCF3::ZNF384* fusion. Marker; 100bp DNA ladder, P-100; P-98; Positive control (plasmid); Negative.

but this finding did not reach statistical significance ($p = 0{,}066$, Figure 2(B)).

The ROC curve analysis was used to evaluate the validity of gene expression based on the sensitivity and specificity rate (AUC = 0.7545 and $p = 0.004$; Supplemental Fig. S3). The cut off value ($\Delta Ct = 3.815$ with 60.26% sensitivity and 91.67% specificity) was determined by using ROC curve analysis. The patients were divided into high and low expression groups by using cut off value determined by AUC analysis. Then, clinical and physiological features were compared based on *ZNF384* low versus high expression groups. *ZNF384* high and low expression groups did not show any significant differences for clinical features (Table 2).

Immunophenotypic characteristics of patients with ZNF384 fusions

We evaluated the CD10, CD13, and CD33 levels among the patients and found that *ZNF384* fusion carriers had lower CD10 expression than patients without *ZNF384* fusions ($p = 0.03$, Figure 3). Patients with *ZNF384* fusions did not show a significant difference for CD13 and CD33 expressions compared to patients who did not harbor any *ZNF384* fusion. Four out of 11 patients (36%) with *ZNF384* related fusions showed higher expression of CD13 and five out of 11 patients (45%) showed higher expression of CD33, but the differences were not statistically significant ($p = 0.70$ and $p = 0.20$, respectively).

Clinical and survival characteristics of patients with BCP-ALL and MPAL with ZNF384 rearrangements

Clinical features were compared between *ZNF384* fusion positive and negative patients (Table 2). Patients who carried *ZNF384* fusion, had significantly higher WBC counts compared to -*ZNF384* fusion negative group ($p = 0.016$). Moreover, the last status (dead or alive) of *ZNF384* fusion carriers were found significantly different ($p = 0.026$, Table 2). No statistically significant difference was found either for fusion positive versus wild type patients in terms of age at diagnosis, gender, relapse, ITR, and immunophenotype.

Survival data was available for a group of patients, non-*ZNF384* fusions ($n = 75$), t(12;21) ($n = 19$), t(9;22) ($n = 7$), *ZNF384* fusions ($n = 6$) (Figure 4). We found significant survival differences between *ZNF384* fusion positive patients compared to wild type *ZNF384,* and t(12;21) positive patients, OS ($p = 0.008$) (Figure 4(A)), and EFS ($p = 0.005$) (Figure 4(B)). Patients with *ZNF384* fusions had shorter survival than wild type *ZNF384* (for OS $p = 0.044$ and for EFS $p = 0.029$) and t(12;21) cases (for OS $p = 0.041$ and for EFS $p = 0.031$), whereas they had relatively more favorable survival rate than t(9;22) positive cases (for OS $p = 0.489$ and for EFS $p = 0.445$) (Figure 4). *ZNF384* fusions showed statistical significance upon the patient's life expectancy in the univariate Cox regression analysis ($p = 0.044$) (Supplemental Fig. S4). Kaplan-Meier survival analyses were performed for patients with high or low *ZNF384*

Table 1. Clinical features of *ZNF384* fusion positive acute leukemia patients.

ZNF384 Fusions of Pediatric ALL Patients

	Age (years)	Sex	Phenotype	Fusion Partner	Breakpoint region	WBC	Risk	Last Status	Other Genetic Variations	ITR
P11	7	M	BCP-ALL	*TAF15::ZNF384*	Exon 7-Exon 3	28500	MRG	Alive	–	Yes
P26	3	F	B/Myeloid	*EP300::ZNF384*	Exon 6-Exon 3	35620	NA	NA	–	NA
P28	12	M	BCP-ALL	*TCF3::ZNF384* *EP300::ZNF384*	Exon 13-Exon 3 Exon 6-Exon 3	43000	MRG	Alive	–	Yes
P30	5	M	BCP-ALL	*TAF15::ZNF384*	Exon 6-Exon 3	37700	NA	NA	–	NA
P75	8	F	BCP-ALL	*EP300::ZNF384*	Exon 6-Exon 3	55500	MRG	Dead	–	Yes
P88	15	F	BCP-ALL	*TCF3::ZNF384*	Exon 13-Exon 3	53900	MRG	Dead	–	Yes
P98	6	M	BCP-ALL	*TCF3::ZNF384*	Exon 17-Exon 7	217000	HRG	Dead	–	Yes
P100	3.5	F	BCP-ALL	*TCF3::ZNF384*	Exon 15-Exon 7 (novel)	120000	NA	NA	t(12;21)	NA
P126	2	M	B/Myeloid	*TCF3::ZNF384* *TAF15::ZNF384*	Exon 13-Exon 3 Exon 6-Exon 3	124800	HRG	NA	–	NA
P127	1	F	B/Myeloid	*TCF3::ZNF384* *TAF15::ZNF384*	Exon 13-Exon 3 Exon 6-Exon 3	44700	MRG	Alive	–	Yes
P133	11	M	B/Myeloid	*TAF15::ZNF384*	Exon 6-Exon 3	21000	MRG	Alive	46,XY/46,XY, del(9)(q21q22),t(12;17)(p13;q21)	Yes

HRG: high risk group; ITR: induction therapy response; MRG: medium risk group; NA: not available; WBC: white blood cell count at diagnosis.

expression levels by using ROC cut off value (ΔCt = 3.815). Neither MPAL nor BCP-ALL patients did not show any survival difference between the high versus low *ZNF384* expression groups (BCP-ALL, OS $p = 0.465$, and EFS $p = 0.613$; MPAL, OS $p = 0.666$, EFS $p = 0.676$). When we combined the patients, no significant difference in the OS rate and EFS rate was observed according to their *ZNF384* expression in our cohort (OS $p = 0.203$, Figure 4(C) and EFS $p = 0.182$, Figure 4(D)). In the Cox regression of this survival analysis, no statistical significance was found for clinical assessments such as WBC, relapse, therapy response at day 33 (Supplemental Fig. S5).

Discussion

Molecular studies have identified various ALL subtypes and developed risk-adopted multi-agent treatment regimens that have greatly improved pediatric BCP-ALL survival [8]. *ZNF384* rearrangements are one of the new subtypes defined as a distinct subgroup of ALL [24]. Among the most common fused genes, *EP300,* plays an important role as a cofactor for several transcription factors in hematopoiesis [25,26]. Another fusion partner of *ZNF384* is the *TCF3* gene, which encodes a member of the E protein family which is involved in lymphopoiesis, and they are essential for the development of B and T lymphocytes [27]. *TAF15*, *TLS*, and *EWS* are distinct subsets of ribonucleoproteins, collectively known as *TET* genes. The fusion of the amino transactivation domain of TET proteins with the DNA binding domain of a transcription factor creates abnormal transcription factors that are thought to be common markers of cancer [17].

The frequency of *ZNF384* gene fusions was reported as 1%–6% in pediatric patients and 5%–15% in adult BCP-ALL [8,10,15]. There are limited numbers of reported cases related to *ZNF384* rearrangements in MPAL. To better understand the molecular changes in this rare subtype, we selectively enriched our study cohort for MPAL cases. In the cohort, 9.5% of MPALs carried *ZNF384* fusions (*TCF3*, *EP300*, and *TAF15)* and less frequently 7.6% of BCP-ALL cases harbored *ZNF384* fusions. In line with the previous reports, *TCF3-ZNF384* was the most common fusion in our cohort [7]. Alexander et al. performed whole genome analysis for pediatric ambiguous lineage acute leukemia and they found *ZNF384* rearrangements in 42% of B/MPALs [11]. Herein, we observed less frequent *ZNF384* fusions in B/MPALs (14%). We used RT-PCR method for selected gene fusion partners but we may not have been able to amplified small clones that harbor *ZNF384* fusions or missed fusions with other partner genes in B/MPAL. Therefore, technical differences between these two studies could be one of the reasons of the *ZNF384* fusion frequency differences in B/MPAL cases.

The main feature of MPAL is that in addition to myeloid lineage markers, the cells show T and B cell markers as well which makes it even harder to develop an efficient treatment protocol with a consensus [11]. Therefore, it is important to elucidate the genetic heterogeneity of MPAL in terms of therapy [28–30]. One of the most important findings of this study was the polyclonal background of *ZNF384* fusions. Patients showed heterogeneous characteristics at the cellular level and the existence of different leukemia clones with different fusions partners of the same gene. Three patients, who classified in MRG or HRG, harbored two different *ZNF384* fusions at the time of diagnosis. Unfortunately, patient's fresh tissues were not available for FISH analysis to confirm this unlikely occasion.

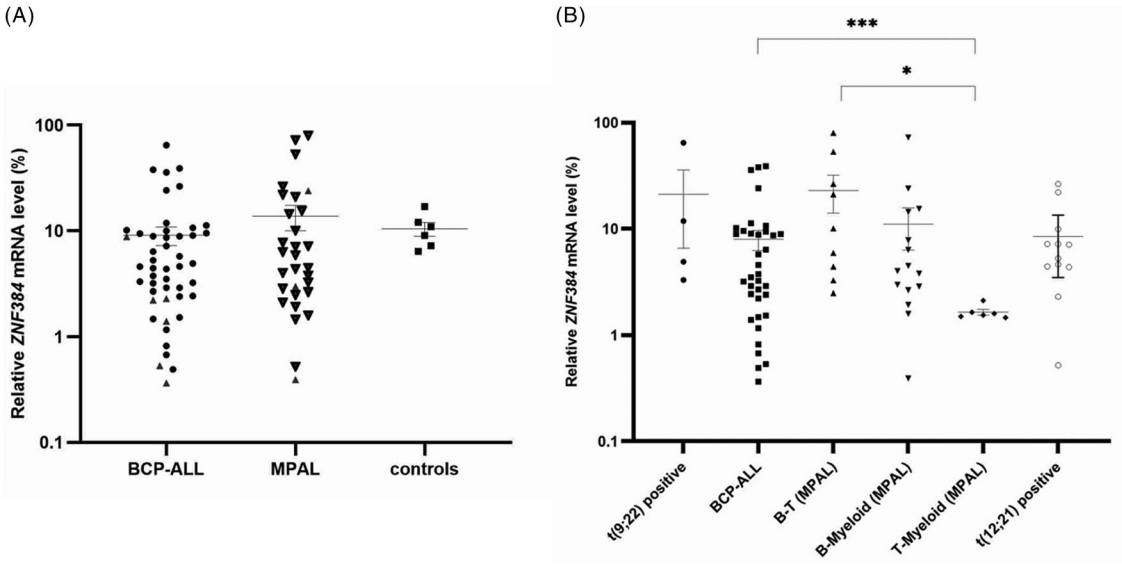


Figure 2. ZNF384 gene expression. Relative ZNF384 expression in BCP-ALL, MPAL and control samples. (A) BCP-ALL and MPAL samples did not show any differential expression for ZNF384 expression compared to control samples (BCP vs. controls, $p = 0.572$; MPAL vs. controls, $p = 0.415$; Unpaired t test with Welch's correction. Data are shown as mean ± SEM. Gray triangles represent the ZNF384r patients. (B) Relative expression level of ZNF384 in different genetic subgroups of ALL and MPAL patients (T/myeloid MPAL vs. B/T MPAL $^{*}p = 0.044$, BCP-ALL versus T/myeloid MPAL, $^{***}p = 0.0007$, Unpaired t test with Welch's correction was performed). Data are shown as mean ± SEM.

Table 2. Clinical correlation of *ZNF384* fusions and expression level.

Clinical features	*ZNF384* fusion (+) ($n = 11$)	*ZNF384* fusion (–) ($n = 122$)	*p* value	*ZNF384* expression		
				Low ($n = 31$)	High ($n = 47$)	*p* value
Sex						
Ratio (male:female)	6:5	74:48	0.6	19:12	25:22	0.4
Median age						
Years (min–max)	6 (1–15)	4 (1–18)	0.7	6 (1–14)	4 (1–16)	0.8
WBC ($\times 10^9$/L)						
Median (min–max)	54,700 (28,500–217,000)	10,200 (1030–702,000)	***0.01***	15,050 (1400–226,000)	17,200 (1700–702,000)	0.6
Last Status, *n*(%)			***0.02***			0.4
Alive	3 (50)	64 (85)		18 (81.82)	22 (73.33)	
Dead	3 (50)	11 (14)		4 (18.18)	8 (26.67)	
ITR, *n* (%)			0.5			0.8
Yes	6 (100)	57 (93.44)		17 (94.44)	22 (95.65)	
No	0 (0)	4 (6.56)		1 (5.56)	1 (4.35)	
Relapse, *n* (%)			0.3			0.3
Yes	1 (16.67)	13 (37.14)		3 (21.43)	6 (37.5)	
No	5 (83.33)	22 (62.86)		11 (78.57)	10 (62.5)	
Immunophenotype *n* (%)			0.7			0.8
BCP-ALL	7 (63.6)	84 (69)		20 (64.5)	28 (59.5)	
MPAL	4 (36.4)	38 (31)		11 (35.5)	19 (40.5)	

BCP-ALL: B-cell precursor acute lymphoblastic leukemia; MPAL: mixed phenotype acute leukemia; WBC: white blood cell count at diagnosis.
Bold and italic values indicate *p* values <0.05. ZNF384 expressions were determined as high and low by cut off value = 3.815.

ZNF384 fusion carriers had moderate survival rate. Wild-type *ZNF384* cases and t(12;21) carrier showed longer survival rate than *ZNF384* carriers. On the other hand, t(9;22) positive patients, had relatively worst survival rates. Hirabayashi et al. presented the comparative survival analysis of *TCF3::ZNF384* fusion positive, *EP300::ZNF384* fusion positive and wild type *ZNF384* cases and showed that although there was no statistically significant difference, the survival times were shorter in patients with BCP-ALL with *TCF3::ZNF384* fusion [7]. In a study demonstrating the clinical significance of *ZNF384* fusions in B-ALL, 28% of patients with the *EP300::ZNF384* fusion were assigned to the HRG due to advanced age, while 46% of patients with *TCF3::ZNF384* fusion had elevated leukocyte counts and poor initial response [31].

The literature has controversial results in which some reports suggest relations between *TAF15::ZNF384* fusion and overall good prognosis and response to induction therapy [32] and some others associate the fusion with a poor clinical outcome in ALL [33,34]. These findings might indicate that the patients with

different *ZNF384* rearrangements might show different prognosis regarding to type of the fusion protein.

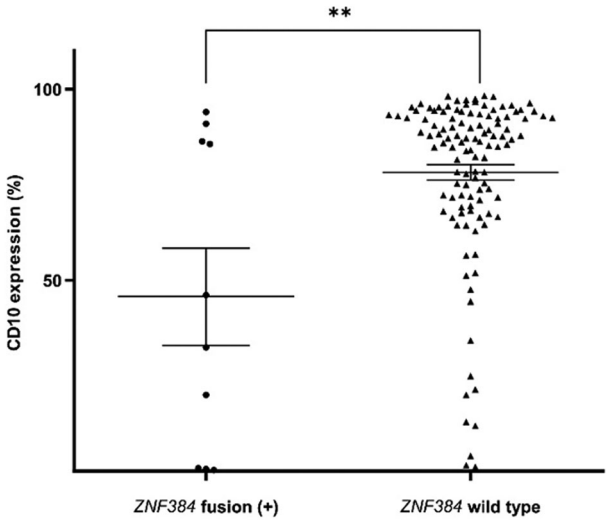


Figure 3. CD10 expression of patients in ZNF384 rearranged. ZNF384 fusion carrier had lower CD10 expression (**$p = 0.0308$ by unpaired t-test, ZNF384 fusion positive vs. ZNF384 wild type). Data are shown as mean ± SEM.

In this study, patients with *ZNF384* fusions had significantly elevated WBC counts compared to wild type cases. Furthermore, in the Cox regression analysis, *ZNF384* fusions were found to be an independent risk factor for survival. When the immunophenotype characteristics of patients were evaluated with and without *ZNF384* fusion, we found that CD10 expression was significantly decreased in patients with *ZNF384* fusion. Besides, one of the four patients with high CD10 expression was a patient with the novel fusion. Previously, *ZNF384* rearrangements were characterized by low CD10 expression and high CD13 and CD33 expressions [7]. The lack of CD10 expression has been associated with poor clinical outcomes due to a decrease in the tendency of cells to undergo apoptosis [35]. Unfortunately, in our cohort, the number of patients with *ZNF384* fusion positive is not sufficient to make significant statistical analysis of patient survival for expression of CD10, CD13, and CD33.

A novel breakpoint was determined for *TCF3 (exon15)::ZNF384 (exon7)* fusion gene in a BCP-ALL patient. Unlike other *ZNF384* fusion positive patients,

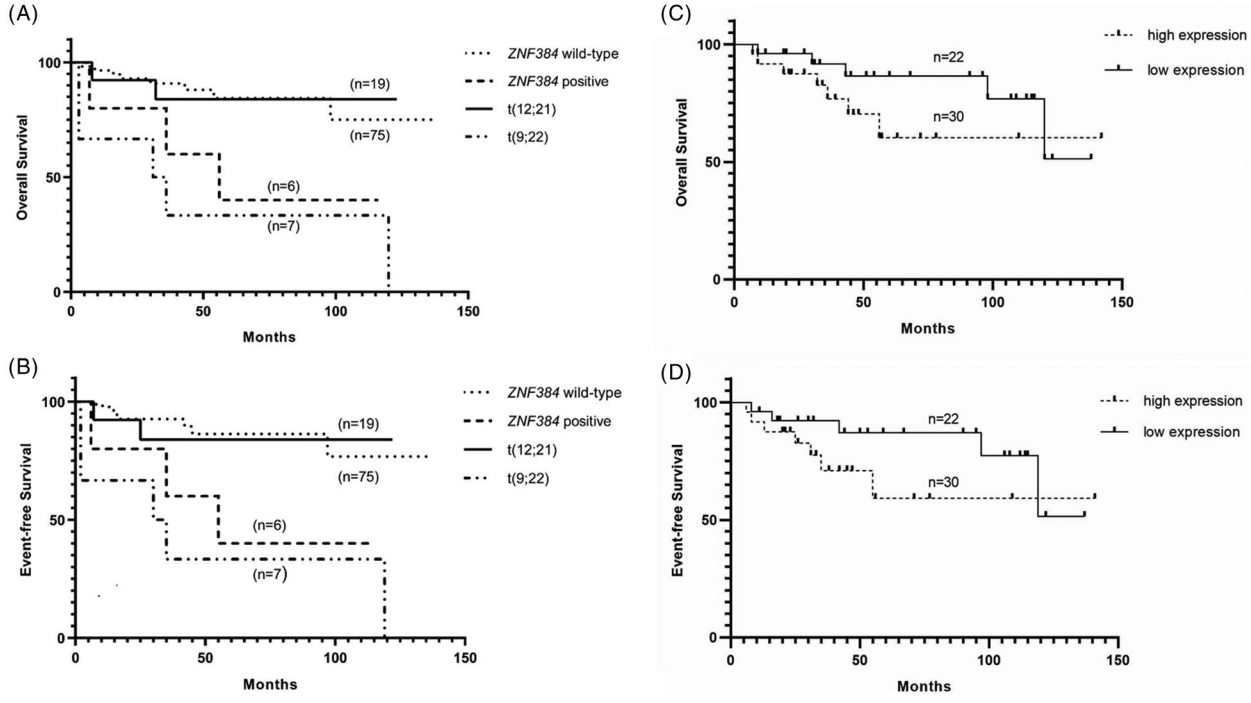


Figure 4. Survival analyses of acute leukemia patients. (A) OS curve of patients with non-*ZNF384* fusions t(12;21) and t(9;22) and *ZNF384* fusions (+), *ZNF384* fusion carriers had longer survival rate compared to t(9;22) carriers ($p = 0.008$). Patients with *ZNF384* fusions had shorter survival than wild type *ZNF384* (for OS $p = 0.044$) and t(12;21) cases (for OS $p = 0.041$), whereas they had relatively more favorable survival rate than t(9;22) positive cases (for OS $p = 0.489$). (B) EFS curve of patients with non-*ZNF384* fusions t(12;21) and t(9;22) and *ZNF384* fusions (+), *ZNF384* fusion carriers had longer EFS rate compared to t(9;22) carriers ($p = 0.005$). Patients with *ZNF384* fusions had shorter survival than wild type *ZNF384* (for EFS $p = 0.029$) and t(12;21) cases (for EFS $p = 0.031$), whereas they had relatively more favorable survival rate than t(9;22) positive cases (for EFS $p = 0.445$). (C,D) Overall and EFS curves were not significantly different between high *ZNF384* expressed group versus low expressed group (OS $p = 0.203$; EFS $p = 0.183$). MPAL and BCP-ALL patients did not show any survival difference between the high versus low *ZNF384* expression groups separately (BCP-ALL, OS $p = 0.465$ and EFS $p = 0.613$; MPAL, OS $p = 0.666$, and EFS $p = 0.676$).

this patient also had additional t(12;21) translocation. t(12;21) translocation has been associated with good prognosis in BCP-ALL [36]. Unfortunately, the patient had a very short follow up and no further clinical data could be obtained to discuss the coexistence of the *ZNF384* fusion and t(12;21).

The role of *ZNF384* gene expression in acute leukemia is unclear and there is no previous data. Among the different hematopoietic compartments, *ZNF384* expression is downregulated in hematopoietic stem cells. It also remains largely constant during cell differentiation from ProB, PreB to mature B cell [10]. *ZNF384* expression levels did not found different in MPAL patients compared to controls. Although there was no statistical difference between patients with high and low *ZNF384* gene expression levels, patients with high expression tend to had relatively lower survival. It would be clinically valuable to evaluate the differences in expression levels in larger cohorts among these patient groups.

Monocytic transition is frequently observed in patients with BCP-ALL with *ZNF384* rearrangements. Cells undergoing monocytic change are reported to remarkably reduce the expression of immature or lymphoid markers and upregulated myeloid markers [37]. In this study, *ZNF384* expression levels were found significantly different between myeloid and lymphoid lineages in patients with MPAL. Interestingly, T-Myeloid (MPAL) patients had lower expression of *ZNF384* compared to B-T (MPAL) and BCP-ALL subtypes. The difference in *ZNF384* expression levels between myeloid and lymphoid lineages might lead to the monocytic transition.

In conclusion, *ZNF384* gene rearrangements were observed as a distinct subtype in MPAL and BCP-ALL patients. These patients were expressing the myeloid markers and had intermediate survival rates. Both MPAL and BCP-ALL patients were harbored *ZNF384* rearrangements; BCP-ALL group also showed fusion transcripts of *ZNF384*, and larger cohort studies were needed to validate these results. This information indicates that prognostic and patient-specific treatment evaluation of *ZNF384* fusions in both ALL and MPAL might help to improve risk characterization of patients.

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The authors declare that there are no competing interests to declare.

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