Zinc Finger Protein 384 (ZNF384) Impact on Childhood Mixed Phenotype Acute Leukemia and B-Cell Precursor Acute Lymphoblastic Leukemia

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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 clinic for risk calculation and treatment decision. ZNF384 rearrangements are one of the new oncogenic subtypes that have been identified in BCP-ALL by recent studies. Also patients with ZNF384 fusions have been reported in with mixed phenotypic acute leukemia (MPAL). In this study, we screened 133 pediatric patients with ALL for the most common ZNF384 fusions; ZNF384-TCF3, ZNF384-EP300 and ZNF384-TAF15 by using qPCR. The total frequency of ZNF384 gene rearrangements was 8.2% in the cohort. We identified ZNF384 fusions in 9.5% of mixed phenotypic leukemia and 7.6% of BCP-ALL groups. Moreover, a novel breakpoint was identified in ZNF384-TCF3 fusion. Patients with MPAL showed significantly higher ZNF384 expression than BCP-ALL and controls. Patients with ZNF384 rearrangements had intermediate survival rates based on other subtypes.

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 leukemias express both lymphoid and myeloid lineage specific antigens, which makes it difficult to determine lineage and it was grouped under acute leukemias 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 and *KMT2A* rearrangement, hyperdiploidy and hypodiploidy 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 *ETV6-RUNX1*- like gene expression*, DUX4, MEF2D* and *ZNF384* rearrangements, were identified by whole genome approaches [6-8].

ZNF384 gene rearrangements is observed approximately in 3-4% of pediatric and in about 7% of adult patients with BCP-ALL[8,9]. In fact, the *ZNF384* rearrangements are largely specific to ALL, which suggests that this zinc finger transcription factor has a potential role in leukemogenesis. Also, 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 the 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 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, SMARCA2, SYNRG, EP300, TAF15* and *TCF3* . The *EP300* gene encodes a histone acetyltransferase (HAT) 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. Furthermore, we evaluated expression and translocation results with clinical findings.

Material-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 were retrospectively enrolled 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 12900 ($x10^9$/L) (minimum 1030- maximum 702000), median hemoglobin (Hb) level was 8.05 (g/dL) (minimum 3.4-maximum 13.4), median platelet (PLT) count was 45500 ($x10^9$/L) (minimum 2020- maximum 924000). 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 were detected in twenty-six cases. Patients with BCP-ALL and MPAL were treated according to the ALL-BFM 95 (Berlin-Frankfurt-Munster) or ALLIC2009 protocols at diagnosis . All of the patients with MPAL were treated with ALL protocols according to the risk groups. Median follow-up time was 5 years (minimum:1, 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. In BFM protocol, BCP-ALL requires strong positivity in [?]2 of four antigens which are CD19, CD10, CD22, 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 as 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* (exon13)-*ZNF384* (exon3), *TCF3* (exon17)-*ZNF384* (exon7) and *TAF15-ZNF384* fusion gene regions were amplified by conventional polymerase chain reaction (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 degC for 2 min, denaturation at 94 degC for 30 s, annealing at 57degC-60degC for 30 s, extension 72degC for 1 min and a final extension step at 72 degC 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. 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

Mann Whitney U 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. 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 five different breakpoints for *ZNF384* in 133 primary BCP-ALL and MPAL samples by qPCR and Sanger Sequencing. In total, 8.2% (11 in 133) of patients with BCP-ALL and MPAL had*ZNF384* gene rearrangements. The most common partners of*ZNF384* for fusions were*TCF3-ZNF384* (n=6), *EP300-ZNF384* (n=3) and*TAF15-ZNF384* (n=5). *ZNF384* fusion frequency was 9.5% (n=4) in patients with MPAL and 7.6% (n=7) in patients with BCP-ALL.*TCF3-ZNF384* fusion was detected in six patients, five patients had *TAF15-ZNF384* fusion, and three patients carried*EP300-ZNF384* . *TCF3*- exon 13 breakpoint created a fusion with *ZNF384* -exon 3, *TCF3*- exon15 and 17 breakpoints fused to *ZNF384*- exon 7 **(Supplemental Fig. S2)** . *TCF3*(Ex15)-*ZNF384* (Ex7) fusion is a novel rearrangement and reported for the first time with this study **(Fig. 1)** . *TAF15* exon 6 and 7 breakpoints fused with *ZNF384*- exon 3 and *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.

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. All patients showed significantly higher *ZNF384* expression than the controls (*p=0.015*). On the other hand, BCP-ALL group had significantly decreased *ZNF384* expression compared to controls (*p=0.040* **, Fig. 2A).** Although the differences were not significant MPAL samples showed higher *ZNF384* expression compared to controls and patients with BCP-ALL (*p=0.156* and *p=0.318*respectively*,* **Fig. 2A).**

Relative gene expression levels of *ZNF384* were analyzed according to different ALL and MPAL subgroups and although there were few patients in the T-myeloid subgroup, the expression level of *ZNF384* was found significantly lower than in the B-T (MPAL) subgroup*(p=0.036).* Also, when the B-T (MPAL) subgroup was compared with BCP-ALL, the expression level of *ZNF384* was significantly higher in the B-T (MPAL) subgroup (*p=0.037*). Patients with B-T (MPAL) subgroup showed relatively an increased expression level then B-myeloid subgroup, but the difference was not significant (*p=0,134* **, Fig. 2B).**

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 (ΔCt=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 fusion positive

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* ,**Fig. 3).** There was no significant expression difference between the patients CD13 and CD33 expressions and *ZNF384* fusions. Four patients out of 11 with *ZNF384* related fusions (36%) showed higher expression of CD13 and five out of 11 patients (45%) showed higher expression of CD33, but the difference was 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 vs wild type patients in terms of age at diagnosis, gender, relapse, induction therapy response and immunophenotype.

Three patients (n=1 BCP-ALL and n=2 MPAL) were found positive for two other *ZNF384* fusions (partner genes). One patient (P#100) had a novel breakpoint in *TCF3* (Ex15)-*ZNF384* (Ex7) fusion who was also t(12;21) translocation carrier.

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) **(Fig. 4)** . We found significant survival differences between *ZNF384* fusion positive patients compared to wild type*ZNF384,* t(12;21) and t(9;22) positive patients. Patients with*ZNF384* fusions had shorter survival than wild type *ZNF384*and t(12;21) cases, whereas they had more favorable survival rate than t(9;22) positive cases, OS *(p=0.008)* **(Fig. 4A)** and EFS*(p=0.005)***(Fig. 4B).** Only *ZNF384* fusions in independent variables showed statistical significance upon the patient's life expectancy in the Cox regression analysis *(p=0.044)***(Supplemental Fig. S4).** Survival analyses were performed for patients with high or low*ZNF384* expression levels by using ROC cut off value (ΔCt=3.815). Although there was no statistical difference, higher expression levels seem to be related with reduced survival rates (for OS *p= 0.203,* **Fig. 4C** and for EFS *p value=0.182* **, Fig. 4D).** 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 BCP-ALL[24]. Among the most common fused genes, *EP300,* plays an important role as a cofactor for several transcription factors in hematopoiesis. Chromosomal translocations of*EP300* gene have been associated with certain types of leukemia[25,26]. Another fusion partner of *ZNF384* is*TCF3* gene, which encodes a member of the E protein family. E proteins are involved in lymphopoiesis, and they are essential for the development of B and T lymphocytes [27]. *TAF15, TLS, 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].

In this study, we detected *ZNF384* fusions (*TCF3, EP300, TAF15)* in 8.2% of 133 childhood cases with BCP-ALL and MPAL. The frequency of *ZNF384* gene fusions was reported as 1-6% in pediatric patients and 5-15% in adults with BCP-ALL[8,10,15]. Our results seem to be slightly higher in childhood cases than the literature, but our cohort also consists cases with MPAL that *ZNF384* fusions were detected in almost 50% of the patients. In line with the previous reports, *TCF3-ZNF384* was the most common fusion in our cohort [7]. *ZNF384*fusions were observed more frequently (9.5%) in patients with B/myeloid MPAL than the other subgroups. Limited number of reported cases related to *ZNF384* rearrangements in MPAL supports this finding. 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 harbored two different *ZNF384* fusions at the time of diagnosis. P#28 (*TCF3*- and *EP300* -) and P#127 (*TCF3* - and *TAF15* -) who had polyclonal 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 (124800 x10^9/L).

The survival rates of patients with *ZNF384* fusion positive were more unfavorable than wild-type *ZNF384* cases, and t(12:21) positive cases, whereas more favorable than t(9;22) positive cases. 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 high-risk group 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.

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]. 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, 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]. The patients showed high *ZNF384* expression who had *TCF3-ZNF384 or EP300- ZNF384* fusions but not *TAF15-ZNF384* . The fusion partners of*ZNF384* may play distinct roles in expression of *ZNF384* .*ZNF384* expression levels were significantly increased in patients with MPAL. Although there was no statistical difference between patients with high and low *ZNF384* gene expression levels, patients with high expression 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* rearrangement. Cells undergoing monocytic change are reported to remarkably reduce the expression of immature or lymphoid markers and upregulate myeloid markers [37]. In this study, *ZNF384* expression level were found significantly different between myeloid and lymphoid lineages in patients with MPAL. Patients with B-T MPAL showed higher expression levels than the patients with B-myeloid and T-myeloid MPALs and BCP-ALL showed lower *ZNF384* expression level than patients with MPAL. The difference in*ZNF384* expression levels between myeloid and lymphoid lineages might lead the monocytic transition.

In conclusion, *ZNF384* gene rearrangements were observed in a specific subtype of ALL. These patients were expressing the myeloid markers and had intermediate survival rates. Although patients with MPAL harbored more frequent *ZNF384* rearrangements, BCP-ALL group also showed fusion transcripts of *ZNF384* , and larger cohort studies were needed to validate these results. These information indicate 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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Legends

Figure 1. Novel breakpoint of *TCF3-ZNF384* fusion

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

Figure 2. *ZNF384* Gene Expression in ALL

Relative *ZNF384* expression in BCP-ALL, MPAL and controls. (*$p=0.040$ *by Mann Withney test, BCP vs controls),* The red dot indicates *ZNF384* fusion positive patients. :bone marrow samples. Data are shown as mean ± SEM

Relative expression level of *ZNF384* in different genetic groups of ALL patients. (**$p=0.037$ *by Mann Withney test BCP vs B/T ALL and* *$p=0.036$ *by Mann Withney test B/T vs T/myeloid ALL).* Data are shown as mean ± SEM

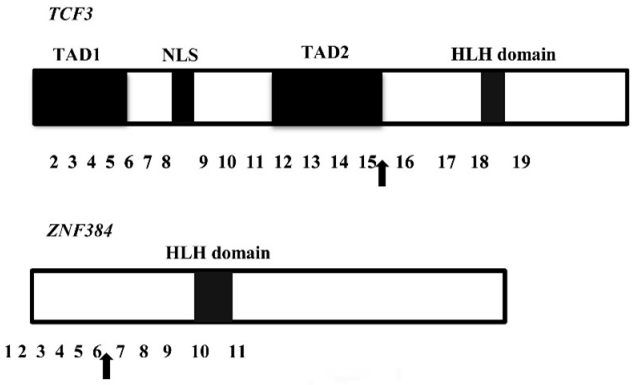
Figure 3. CD10 expression of patients in *ZNF384* rearranged.

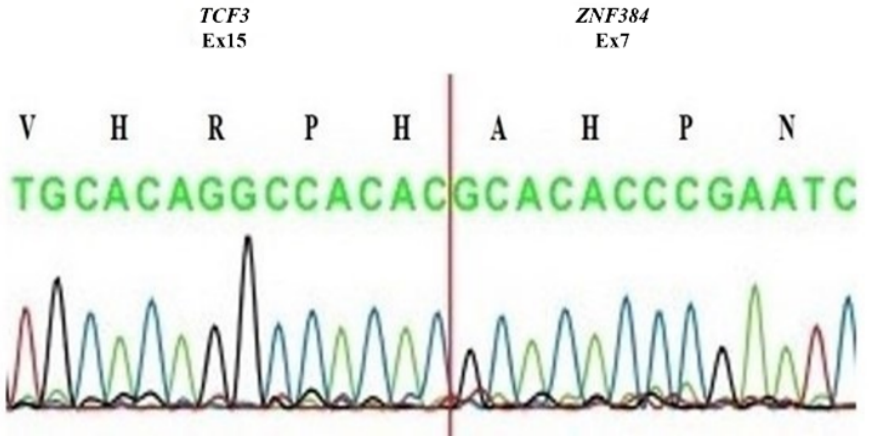
(**$p=0,0308$ *by* unpaired t -test)

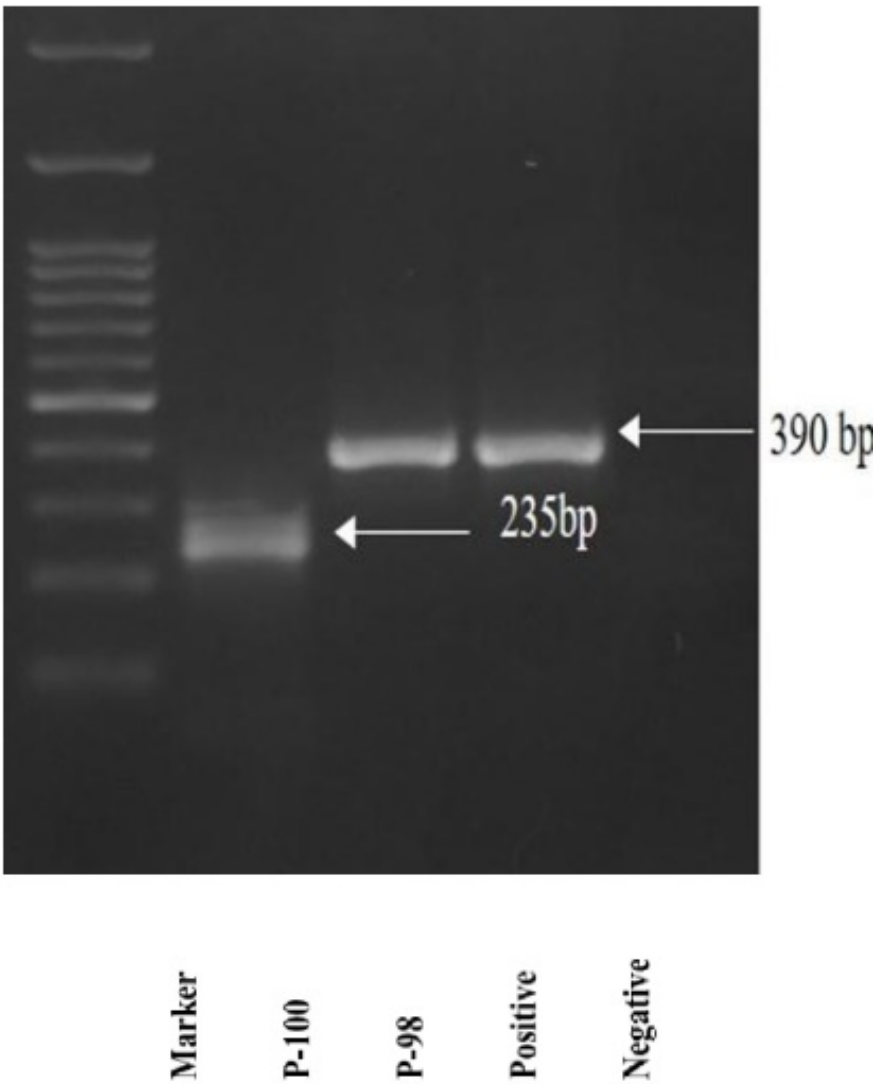
Data are shown as mean ± SEM

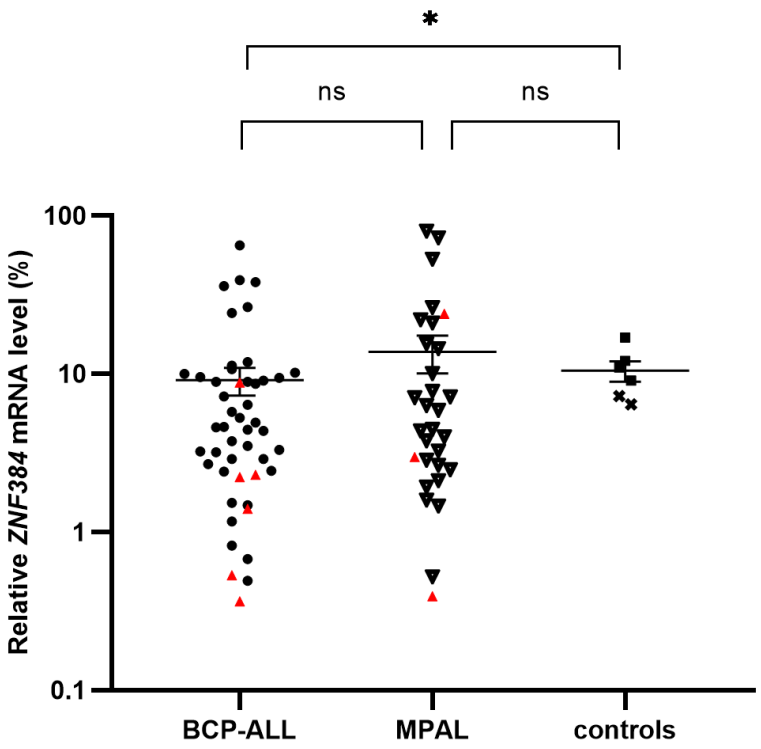
Figure 4. Survival analyses of ALL patients

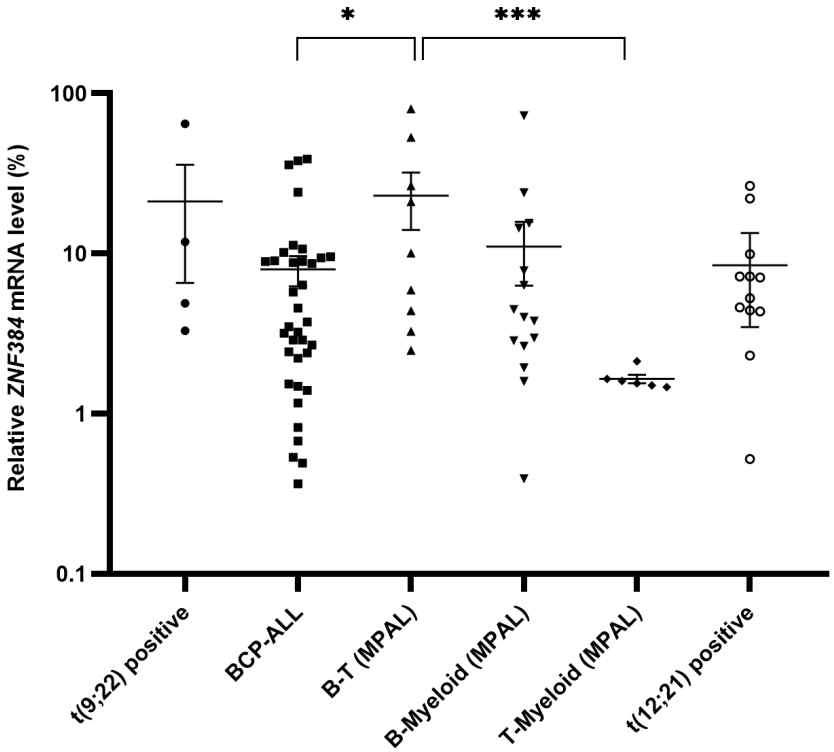
1. Overall survival curve of patients with non-*ZNF384* fusions (t12;21 and t(9;22) and *ZNF384* fusions (+),
2. Event free survival curve of patients with non-*ZNF384* fusions (t12;21 and t(9;22) and *ZNF384* fusions (+),
3. Overall survival curve of *ZNF384* expression levels of patients,
4. Event free survival curve of *ZNF384* expression levels of patients

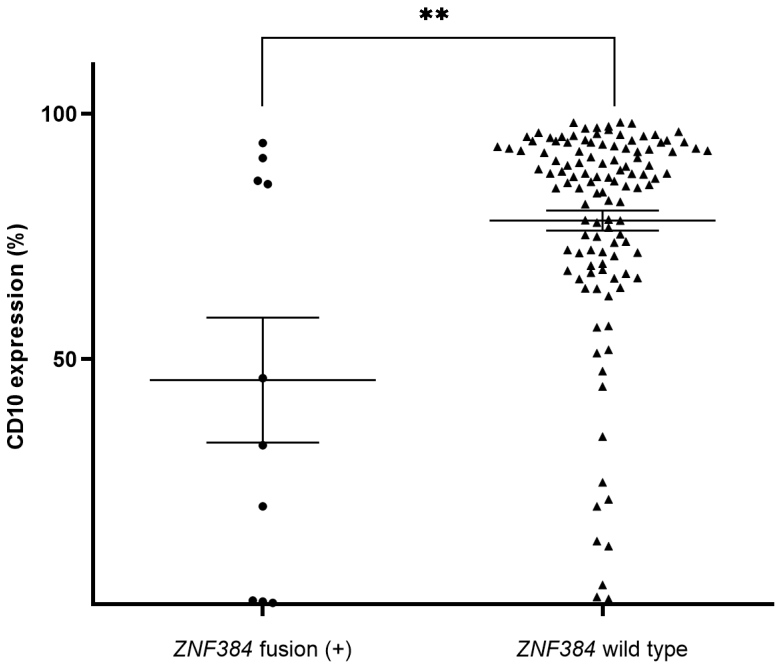


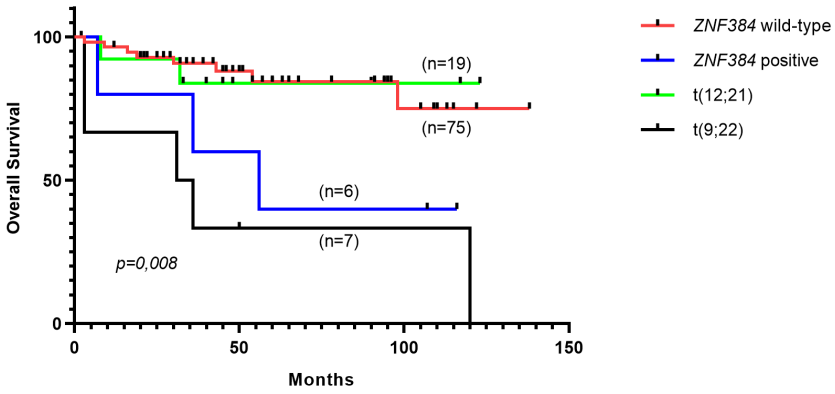


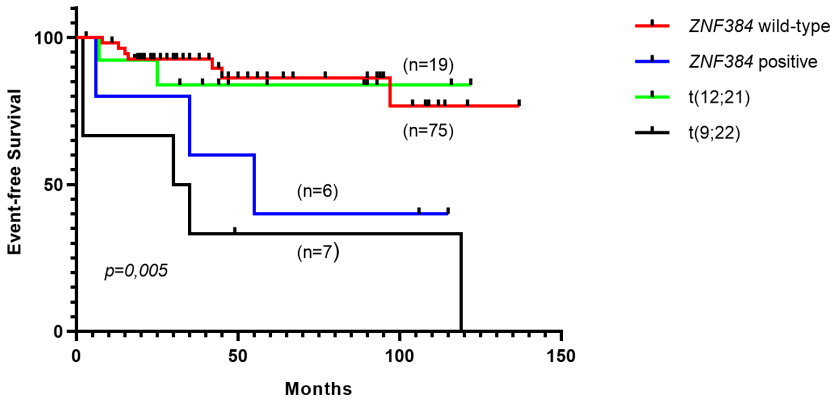


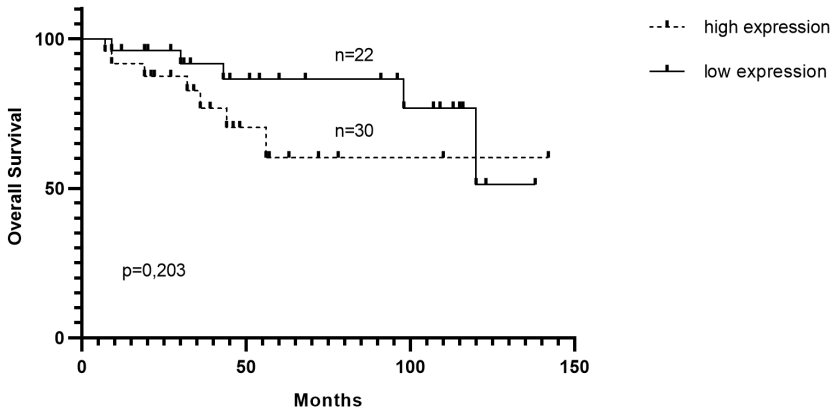












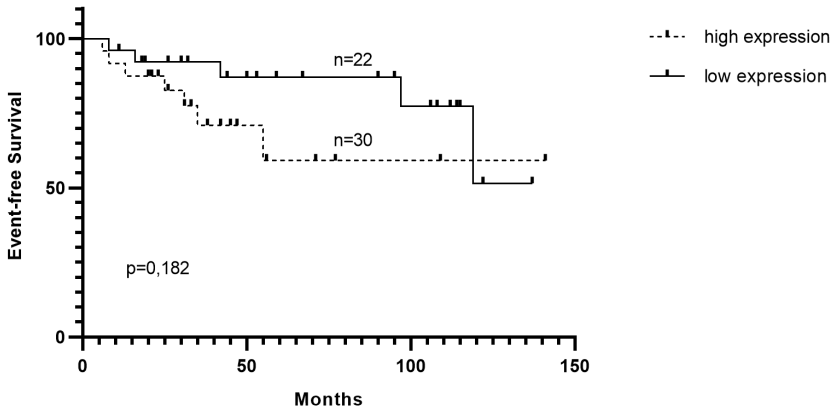


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TABLE 2.docx available at https://authorea.com/users/474314/articles/564220-zinc-finger-protein-384-znf384-impact-on-childhood-mixed-phenotype-acute-leukemia-and-b-cell-precursor-acute-lymphoblastic-leukemia