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Molecular Analysis of More Than 140 Gene Fusion Variants and Aberrant Activation of EVI1 and TLX1 in Hematological Malignancies

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Corresponding Author:	Ebrahim Sakhinia, Ph.D Tabriz University of Medical Sciences Tabriz, IRAN, ISLAMIC REPUBLIC OF
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Tabriz University of Medical Sciences
Corresponding Author's Secondary Institution:	
First Author:	Faramarz Ghasemian Sorbeni, MS.c
First Author Secondary Information:	
Order of Authors:	Faramarz Ghasemian Sorbeni, MS.c
	Soheila Montazersaheb, Ph.D
	Atefeh Ansarin, MS.c
	Ali Esfahani, Ph.D
	Azim Rezamand, Ph.D
	Ebrahim Sakhinia, Ph.D
Order of Authors Secondary Information:	
Funding Information:	•Hematology and Oncology Research Center, Tabriz University of Medical Sciences Tabriz, Iran — Mr. Faramarz Ghasemian Sorbeni
	•Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences Tabriz, Iran — Dr. Ebrahim Sakhinia
Abstract:	Gene fusions are observed in abnormal chromosomal rearrangements such as translocations in hematopoietic malignancies, especially leukemia subtypes. Hence, it is critical to obtain correct information about these rearrangements in order to apply proper treatment techniques. To identify abnormal molecular changes in patients with leukemia, we developed a multiplex reverse transcriptase polymerase chain reaction (MRT-PCR) protocol and investigated more than 140 gene fusions resulting from variations of 29 prevalent chromosomal rearrangements along with EVI1 and TLX1 oncogenic expression in the presence of optimized primers. The potential of the MRT-PCR method was approved by evaluating the available cell lines as positive control and confirmed by sequencing. Samples from 53 patients afflicted with hematopoiesis malignancies were analyzed. Results revealed at least one chromosomal rearrangement in 69% of acute myeloid leukemia subjects, 64% of acute lymphoblastic leukemia subjects and 81% of chronic myeloid leukemia subjects, as well as a subject with hypereosinophilic syndrome. Also, five novel fusion variants were detected. Results of this study also showed that, chromosomal rearrangements, both alone and in conjunction with other rearrangements, are involved in leukemogenesis. Moreover, it was found that EVI1 is a suitable hallmark for hematopoietic malignancies.

Title:

Molecular Analysis of More Than 140 Gene Fusion Variants and Aberrant Activation of *EVI1* and *TLX1* in Hematological Malignancies

Faramarz Ghasemian Sorbeni[1], Soheila Montazersaheb[2], Atefeh Ansarin[3], Ali Esfahani[1], Azim Rezamand[4], Ebrahim Sakhinia[5]

[1]Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, [2]Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, [3]Tabriz Genetic Analysis Center (TGAC), Tabriz University of Medical Sciences, Tabriz, Iran, [4]Department of Pediatrics, Children Hospital, Tabriz University of Medical Sciences, Tabriz, Iran, [5]Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Corresponding author:
Dr. Ebrahim Sakhinia
Molecular Medicine Division, Connective Tissue Diseases Research Center
Fourth Floor, Emam Reza Hospital, Golgasht St, Azadi Blvd, Tabriz, Iran
E-mail: ebrahim.sakhinia@yahoo.com
Phone: +984133370684

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Abstract

Gene fusions are observed in abnormal chromosomal rearrangements such as translocations in hematopoietic malignancies, especially leukemia subtypes. Hence, it is critical to obtain correct information about these rearrangements in order to apply proper treatment techniques. To identify abnormal molecular changes in patients with leukemia, we developed a multiplex reverse transcriptase polymerase chain reaction (MRT-PCR) protocol and investigated more than 140 gene fusions resulting from variations of 29 prevalent chromosomal rearrangements along with *EVI1* and *TLX1* oncogenic expression in the presence of optimized primers. The potential of the MRT-PCR method was approved by evaluating the available cell lines as positive control and confirmed by sequencing. Samples from 53 patients afflicted with hematopoiesis malignancies were analyzed. Results revealed at least one chromosomal rearrangement in 69% of acute myeloid leukemia subjects, 64% of acute lymphoblastic leukemia subjects and 81% of chronic myeloid leukemia subjects, as well as a subject with hypereosinophilic syndrome. Also, five novel fusion variants were detected. Results of this study also showed that, chromosomal rearrangements, both alone and in conjunction with other rearrangements, are involved in leukemogenesis. Moreover, it was found that *EVI1* is a suitable hallmark for hematopoietic malignancies.

Keywords:

Leukemia; Gene Fusion; Chromosomal Translocation; EVI1 Gene; Multiplex RT-PCR.

Introduction

Abnormal chromosomal rearrangements (such as translocations, deletions, insertions and duplications) are among genomic alterations [1] that have different effects on proto-oncogenes [2, 3]. Development of a double-strand break in DNA is a precursor for the formation of such rearrangement [1-3]. DNA double-strand breaks are generated in gene rearrangements of immunoglobulins and T-cell receptors which defect in postcleavage repair complexes promotes the generation of aberrant gene fusion [2, 3]. Gene fusions, for example t(15;17)(q22;q21) and inv(16)(p13.11;q22.1) can be developed in exonic or intronic breakpoints [4, 5]. In addition, a gene can have several different fusion partners, such as mixed lineage leukemia (*MLL*) gene on the 11q23 locus [6].

In the leukemia subtypes, chromosomal translocations are a major portion of detected molecular rearrangements, which involved in the leukemogenesis depending on the participant genes [2, 6]. In the other hand, in many cases, resistance to therapy or disease relapse is observed during treatment, that is sometimes associated with molecular rearrangements different from the initial detection [7-9]. Hence, an accurate diagnosis of causative agent and obtaining comprehensive information about the spread of the disease are of utmost importance for therapeutic measures. In the present study, we developed a MRT-PCR method proposed by pallisgaard et al. in order to simultaneously analyze multiple common abnormal chromosomal changes related to various leukemia subtypes, the sensitivity and specificity of which has been well described previously [3, 10, 11].

Materials and method

Patient samples and cell lines

This study was conducted according to the recommendations by the Local Ethics Committee. For the study purpose, 54 patients with hematopoietic malignancies were introduced for sampling from the Department of Hematology and Oncology, the Shahid Qazi Hospital and Children's Hospital of Tabriz University of Medical Sciences. To carry out experiments, 5 mL of peripheral blood were taken from each patient and stored in cryovials containing EDTA until use.

In order to verify the performance of primers, as well as having an external positive control for PCR reactions, we made use of RNAs extracted from the patients with known chromosomal rearrangements and available cell lines (Table 1). The cell line NB-4, KG-1 and RPMI8402 were obtained from the Pasteur Institute of Iran, the cell line K562, THP-1, REH and NALM-6 were obtained from the Stem Cell Research Center and the cell line HL-60 was obtained from the Biotechnology Research Center of Tabriz University of Medical Sciences. All the cell lines were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin antibiotic. The medium for the cell line THP-1 was supplemented with 50μM of 2-Mercaptoethanol [12].

RNA preparation

Total RNA was extracted from the cell lines and patients sample using the QIAamp RNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instructions and stored at -

80°C. Quantity and quality of RNA was assessed in two ways: first, two microliters of the final RNA mixture were assessed using an Nanodrop Epoch Microplate Spectrophotometer (BioTek, USA); second, a GAP-PCR protocol was used for examining the presence of DNA contamination in the RNA mixture. In this regard, the RT-PCR reaction was carried out using the REVERTA-L RT Reagents Kit (AmpliSens, Russia) based on random hexamer primers according to the manufacturer's instructions. Then, the GAP-PCR reaction mixture was performed with a final volume of 10µL including 2µL of the cDNA mixture, 6µL of Taq DNA Polymerase 1.1x Master Mix Red (Ampliqon, Denmark), 2µL of the primer mixture (5Pmol of each reverse and forward primer). The primers are shown in Table 2. The PCR program included an initial denaturation at 95ºC for 2 minutes, followed by 35 cycles of PCR amplification (annealing at 60ºC for 30 seconds, elongation at 72ºC for 30 seconds and denaturation at 95ºC for 30 seconds) and a final 5-minute extension at 72ºC. The PCR reaction amplifies a 219bp segment of the GAPDH cDNA. The presence of another band is indicative of DNA contamination (Figure 1C). Five microliters of each PCR product were electrophoresed on 1.5% agarose gel stained with the DNA Safe Stain (CinnaGen Co, Iran) at 100V for 60 minutes. Negative controls without the cDNA sample were considered for all the GAP-PCR reactions. Finally, in case of any DNA contamination, DNAse digestion of the RNA mixture and following that re-purification of the total RNA was performed using the RNase-Free DNase Set and RNA Cleanup protocol (Qiagen, USA), respectively.

Multiplex RT-PCR

In order to perform the MRT-PCR reaction with high sensitivity, a series of specific reverse primers for a group of fusion transcripts and gene rearrangements were used [3]. All tubes and pipette tips treated in DEPC water. One microgram of total RNA was incubated at 65°C for 5 minutes with a mixture of specific primers (3pmol of each) and then reverse transcribed by incubation at 37°C for 45 minutes in a total volume of 25µL containing 20U RNase inhibitor (Invitrogen, UK), 1mM of each dNTP, 10mM dithiothreitol, 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$, and 200U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, UK). After each MRT-PCR reaction, two microliters of the final product were withdrawn for quantification using a Nanodrop Epoch Microplate Spectrophotometer (BioTek, USA).

Nested multiplex-PCR

MRT-PCR amplification was carried out as 8 nested multiplex reactions in two steps by Eppendorf Mastercycler (Eppendorf, Germany), in the presence of two primer sets proposed by Pallisgaard et al. [3]. Each reaction mixture contained a pair of primers that amplifies a 690bp segment of ubiquitously expressed transcription factor E2A as internal positive control. The first step of the Nested Multiplex-PCR (NM-PCR) performed as 8 parallel reactions in a total volume of 20µL containing 1µL of cDNA mixture, 8µL of ddH_2O, 10µL of Taq DNA Polymerase 1.1x Master Mix Red (Ampliqon, Denmark), and 1µL of the primer mix (5pmol of each primer). The PCR program comprised of an initial denaturation at 95ºC for 15 minutes, followed by 25 cycles of PCR amplification (annealing at 58ºC for 30 seconds, elongation at 72ºC for 1 minute, and denaturation at 95ºC for 30 seconds). Two microliters aliquots from the first 8 PCR products were transferred to the second 8 PCR

reaction mixtures with a total volume of 23µL containing 8µL of ddH_2O, 12.5µL of Taq DNA Polymerase 1.1x Master Mix Red (Ampliqon, Denmark), and 2.5µL of the primer mix (5pmol of each primer). The PCR program comprised of an initial denaturation at 95ºC for 15 minutes, followed by 20 cycles of PCR amplification (annealing at 58ºC for 30 seconds, elongation at 72ºC for 1 minute, and denaturation at 95ºC for 30 seconds), and finally by 10 minutes of extension at 72ºC. Ten microliters of each PCR reaction electrophoresed in a 1.5% agarose gel stained with DNA Safe Stain (CinnaGen Co, Iran) for 60 minutes at 100V. Negative controls without cDNA template were included for all the PCR reactions. Also, the cDNA prepared from the HL-60 cell line as a normal control was used for all the PCR reactions.

Evaluation of primers

All the primers proposed for MRT-PCR and NM-PCR were evaluated using the OLIGO Primer Analysis Software Version 7.5 (Molecular Biology Insights Inc, USA) and by the published transcript sequence data in the ENSEMBL database. In this regard, the sequences of breakpoint associated with each fusion partner as donor (3' site) and acceptor (5' site) elements were placed next to each other, and PCR simulation was performed in the presence of primers specific to each fusion transcript. In most cases, the responses were appropriate, and only in two cases, the primers related to *AFF1* and *SET* were changed (Table 2). Moreover, the primers performance was confirmed when the cell lines and subjects with known abnormality were examined (Figure 1A). Thus, the materials from cell line RPMI8402 was used for act(3q26), act(10q24) and del(1p32), THP-1 for t(9;11)(p22;q23), K562 for t(9;22)(q34;q11) variant b3a2, REH for t(12;21)(p13;q22.3) and NB-4 for t(15;17)(q22;q21), as well as, positive control obtained from the subjects materials used for inv(16)(p13;q22.1), t(4;11)(q21;q23), t(6;11)(q27;q23), t(8;21)(q22;q22.3), t(9;22)(q34;q11) variant b2a2 and t(11;19)(q23;p13.1).

Split-out PCR reaction

Since each multiplex-PCR mixture consists of a combination of primer pairs for detection of several fusion transcripts, PCR products may have similar or closely similar sizes. Thus, when one or more fusion transcripts were detected in one or more PCR mixtures, multiplex PCR were separated into individual PCRs to accurately detect type of fusion transcript and also, to confirm the result of the NM-PCR (Figure 1B). In this regard, we performed the Split-Out PCR with individual primer pairs for each rearrangement belong to the second step of NM-PCR and 2µL from the first step product as a template. Moreover, the split-out PCR reaction was performed under the conditions similar to PCR program from the first step and reaction mixture from the second step of NM-PCR. Normal control of the NM-PCR product of the cell line HL-60, as well as the external positive control prepared from the cell lines and subjects with known abnormalities were considered for all the split-out reactions.

Sequencing analysis

Products from the split-out reaction were confirmed by sequencing of the amplified segment. In this regard, the DNA segments were purified from the agarose gel by the Keith Expin Gel SV Kit (GeneAll, Korea) according to the manufacturer's instructions. Purification product

delivered for automated standard sequencing by 3730xl DNA analyzer (Macrogen Inc, Korea). Results of the sequencing were evaluated using the chromatogram analysis software, Chromas 2.5.1 (Technelysium Pty Ltd, Australia).

Results and discussion

Of the 54 samples collected from the patients with hematopoietic malignancies, 53 subjects were investigated, including 26 patients with acute myeloid leukemia (AML), 14 patients with acute lymphoblastic leukemia (ALL), 11 patients with chronic myeloid leukemia (CML), one patient with lymphoblastic lymphoma and one patient with hypereosinophilic syndrome (HES) with active bone marrow. One patient with ALL was excluded due to very low count of white blood cells (Subject No. 51 in the remission status). The results in this study demonstrated at least one chromosomal rearrangement in 18/26 (69%) of the AML subjects, 9/14 (64%) of the ALL subjects and 9/11 (84%) of the CML subjects, and also an HES subject (Table 3). Multiple anomalies (MA) were significantly observed in majority of the positive subjects (Figure 2A), as a rearrangement was identified in conjunction with one or more various rearrangements rather than with different variants of a rearrangement.

The abnormal rearrangements observed as MA include the following: t(16;21)(p11.2;q22.3), t(12;21)(p13;q22.3), t(1;19)(q23;p13), t(4;11)(q21;q23) and inappropriate activation of the *TLX1* gene in the 10q24 locus (named as act(10q24) in this study) in the ALL subjects; t(9;11)(p22;q23), t(8;21)(q22;q22), t(10;11)(p12;q23), t(15;17)(q22;q21) and dup(11q23) in the AML subjects; t(9;22)(q34;q11) in the CML subjects; and dup(11q23) in the only HES subject. It is noteworthy that the t(4;11)(q21;q23), t(8;21)(q22;q22), t(15;17)(q22;q21) and t(9;22)(q34;q11) translocations were even individually detected. Moreover, inv(16)(p13.11;q22.1) was individually detected in the two AML-M4Eo subjects, and was not identified in form of MA in any subjects. However, the t(11;19)(q23;p13.1) and t(6;11)(q27;q23) translocations were detected as MA in the both groups of AML and ALL patients. Furthermore, aberrant activation of Ecotropic Viral Integration Site-1 (*EVI1*) proto-oncogene in 3q26 locus (named as act(3q26) in this study) was observed as individual in the AML, ALL and CML subjects. Act(3q26) was also detected as MA in the AML, ALL, CML and HES subjects (Figure 2B).

The results in this study revealed a number of novel fusion variants in the patient materials (Figure 3) in the form of MA: (a) subject No.3, the MRT-PCR analysis showed that MA included a novel variant of *KMT2A*ex11/*AFDN*ex2 from t(6;11)(q27;q23) and two novel variants of *KMT2A*ex11/*MLLT3*ex9 and *KMT2A*ex11/*MLLT3*ex6 from t(9;11)(p22;q23), t(11;19)(q23;p13.1), t(15;17)(q22;q21) and act(3q26); (b) subject No.11, the MRT-PCR analysis showed that MA included the act(10q24) as a predicted isoform in NCBI Reference Sequence, and act(3q26); (c) subject No.41, the MRT-PCR analysis also demonstrated that MA consisted of t(11;19)(q23;p13.1) which was previously reported only in an AML subject with French-American-British (FAB) classification M5 [70] (a novel disease observation), t(4;11)(q21;q23) as *KMT2A*ex10/*AFF1*ex6 and two novel variants *KMT2A*ex11/*AFF1*ex5 and *KMT2A*ex11/*AFF1*ex6, as well as act(3q26). More details such as cell blood counting (CBC) test results were indexed in Table 4.

Discussion

In a review study on researches published from 1998 to 2016, based on similar methodology with the one used in the current study and/or case reports, 29 novel fusion variants with considerable prognostic and therapeutic impact were reported, which are detectable with the primers used in this study, even a three-way translocation between the genes *PML*, *ADAMTS17* and *RARA* which has been recently reported in a patient with AML-M3 [76]. Summary of the data on the novel translocations and variants is given in Table 1. Hence, we developed the MRT-PCR method for the simultaneous detection of more than 140 gene fusion variants containing 29 abnormal chromosomal rearrangements. Moreover, a series of cell lines and novel disease observation are listed in Table 1.

The greatest frequency of detection belonged to act(3q26) (29.36=69%) in the positive leukemia subjects, followed by the translocations t(4;11)(q21;q23), t(15;17)(q22;q21) and t(9;22)(q34;q11) in the ALL, AML and CML subjects, respectively. Anomalies such as del(1p32), t(X;11)(q13.1;q23), t(1;11)(q21;q23), t(1;11)(p32;q23), t(2;5)(p23;q35.1), t(3;5)(q25.1;q35.1), t(3;21)(q26.2;q22.3), t(5;12)(q33;p13), t(5;17)(q35.1;q22), t(6;9)(p22.3;q34.1), t(9;9)(q34.11;q34.13), t(9;12)(q34.1;p13), t(11;17)(q23.1;q21), t(11;17)(q23;q21), t(11;19)(q23;p13.3) and t(17;19)(q22;p13.3) were not observed in our study. This is while such anomalies could be detected in case the MRT-PCR method is used. This is because of proper performance of the primers in the PCR simulation and also, due to the fact that these anomalies were detected in previous studies with similar methodology [3,10,11]. This result could be attributed to low frequency of these anomalies. For example, the incidence of less than 1% for t(17;19)(q22;p13.3) and t(6;9)(p22.3;q34.1) was respectively reported in ALL and AML [89]. Also, the absence of these translocations in our results could be due to the small sample size. However, we had the positive control materials for t(3;21)(q26.2;q22.3), t(5;12)(q33;p13), t(9;12)(q34.1;p13) and t(11;19)(q23;p13.3), which were obtained from the subjects who were not enrolled in the study and were confirmed by the sequencing. In addition, t(9;22) could be detected in more than 95% of the CML subjects [62]; however, t(9;22) was not detected in two of the study CML subjects (the subjects 37 and 50). These patients underwent targeted treatment by first-generation tyrosine kinase inhibitor and were in the remission status. Furthermore, many uncommon translocations related to immunoglobulin and T-cell receptor genes or tyrosine kinase and nucleoporin groups were not investigated in the MRT-PCR method. Thus, the presence of negative outcomes could be partly attributed to these anomalies. However, cytogenetic anomalies could not be ignored.

Importantly, anomalies were in form of MA in 21/37 positive subjects (≈57%), which were detected in all the Present, Remission and Relapse situations (respectively in the subjects 16, 28 and 48). This could be due to the presence of clones with several abnormalities or spread of several clones with different abnormalities compared to one another, which justifies the detection of the MA status in the new cases. As well as, detection of the rearrangements among the subjects in remission status could be attributed to the presence of residual leukemic cells (the subjects 10, 28 and 29). Also, the presence of cryptic clones with different anomalies that were not detected in the initial diagnosis and thus not received any treatment can be a cause for disease relapse (the subject 31). Such similar cases have been previously reported both in APL and CML patients [7,8]. Thus, the MA status can be attributed to a

cause for resistance to therapy or disease relapse, which commonly occurs during treatment. Moreover, several anomalies, including t(16;21)(p11.2;q22.3), t(12;21)(p13;q22.3), t(1;19)(q23;p13), act(10q24), t(9;11)(p22;q23), t(10;11)(p12;q23) and dup(11q23) were detected only in the MA status (Table 3). The question arises whether these anomalies are only involved in the aggressive form of the disease. This argument is very important for prognosis and minimal residual disease (MRD), and requires comprehensive genomic studies with greater sample sizes.

The *EVI1* gene is located downstream of the *MDS1* gene and encodes a nuclear DNA-binding transcription factor with two zinc finger domains [90]. Previous studies have shown that act(3q26) is involved in the pathogenesis of ALL, AML and myelodysplastic syndrome (MDS) [91-93]. These reports are in line with the results obtained in our study, as act(3q26) was detected in the positive AML (61%) and ALL (89%) subjects. In addition, the results in the current study revealed act(3q26) in 67% of the positive CML subjects, as well as in one HES subject.

In our study sample, there was no abnormality in the chromosome 3, especially the translocation t(3;21)(q26.2;q22.3) which involves the *MDS1-EVI1* complex locus (*MECOM*). This suggests that act(3q26) can be developed independently of 3q26 disorders. In addition, act(3q26) was identified both in the presence and absence of several abnormalities including *KMT2A* rearrangements, t(12;21)(p13;q22.3), t(9;22)(q34;q11) , and occasions where it observed alone (Figure 2B). These results are in line with those of previous studies [14,92,94]. Hence, these results suggest further pathways for creation of the act(3q26).

Aberrant activation of *EVI1* was observed in patients with a wide range of 2 to 92 years of age (Figure 2C). As a result, the relationship between age and act(3q26) could not be further discussed. Elsewhere, *EVI1* gene expression patterns were evaluated and observed to have a negative correlation with increasing age in AML patients [17].

This approved that increased or decreased *EVI1* expression is respectively associated with inhibition and incitement of cell differentiation [90,93]. On the other hand, immature blast cells were detected in CBC results of subjects with act(3q26) ranging from 4 to 36%. Accordingly, detection of act(3q26) in peripheral blood samples could be due to the presence of immature blast cells. In a summary of the discussion on *EVI1*, all the results show that *EVI1* is a suitable hallmark for hematopoietic malignancies and is considerable in terms of diagnostics.

TLX1, also known as *HOX11*, encodes a DNA binding protein that has a homeodomain and is involved in cell growth and differentiation as a transcription regulator of genes [95]. According to previous studies, aberrant activation of *TLX1* occurs during the translocations t(10;14)(q24;q11) and together with the variant t(7;10)(q35;q24) [18,96]. In this pathway, regulatory sequence of genes *TRA/D* (14q11) and *TRB* (7q35) is placed upstream of the *TLX1* and forces it to exhibit irregular expression, as an oncogenic approach to extend T-cell ALL described previously [97,98]. Act(10q24) was also detected in the subjects 1, 11 and 39. In the subject No.11, a *TLX1* transcript with 36bp additional sequence from the 3’ end of *TLX1* intron 1 fused to the 5’ end of exon 2 was detected as a splice variant known as Transcript Variant X1 (Table 1, Figure 3). This transcript also contains a homeodomain that can have performance similar to that of other variants. Spread of T-cell ALL in this patient may be due to the presence of this transcript as a result of the mentioned translocations. On the other

hand, regardless of RPMI8402 that is a T-cell ALL cell line, act(10q24) was detected during the MRT-PCR analysis of the cell lines KG-1 (AML), K562 (CML-BC), NALM-6 (Pre B-cell ALL) and REH (non B/T-cell ALL). This was a paradoxical result, since the translocations t(10;14)(q24;q11) and t(7;10)(q35;q24) have not been reported in these cell lines, and even in patients without acute T-cell leukemia. It is obvious that act(10q24) plays an important role in the development of leukemia; however, our results suggest that, in addition to the involvement of the aforementioned translocations, other mechanisms are also involved in creation of act(10q24). For example, it is shown that a complex containing PBX2 regulatory element contributes to act(10q24) in cell line K562 [99].

In conclusion, it became clear that chromosomal rearrangements in most cases are involved as MA in the development and spread of hematopoietic malignancies and may be correlated with disease relapse and resistance to therapy. Therefore, diagnosis of MA status in patients with hematopoietic malignancy is of paramount importance and helps in selecting the appropriate treatment. Moreover, act(3q26) was clearly observed to have an influential role in the development of hematopoietic malignancies, and is not merely associated with a specific leukemic subtype or molecular chromosomal abnormality. It also seems that, in addition to amplification of gene expression by the translocation, many other pathways are involved in the development of act(10q26).

Authorship contributions

FGS and ES, data collection and design of study; ES, standardization and management of research procedures; FGS, perform the molecular investigations on patient samples and cell lines; FGS, responsibility for the first draft of the manuscript; SM, implementation of process related to the preparation of cell lines; FGS and AA, RNA preparation; AE and AR, pathological assessment of patients and introduce them for sampling.

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Figure caption

Fig.1 Examples on results. **(A)** Electrophoresis of NM-PCR product of cell lines and two subject with MA status. R1 to R8; 8 parallel multiplex reactions. L; 50-1500bp DNA molecular weight marker (CinnaGen Co, Iran). A 690-bp segment of the ubiquitously expressed E2A mRNA is amplified in all the NM-PCR and Split-Out reaction mixtures as an PCR internal positive control. The band related to each rearrangement were indicated by numbers 1 to 6 near the band. In patient no.3, 1&2 indicate the tow rearrangements with closely similar sizes that separated by Split-Out reaction. **(B)** Split-Out result of THP-1 cell line. Multiplex mixture R5 was separated to five individuals PCR for t(4;11)(q21;q23) (R5A), t(11;19)(q23;p13.3) (R5B), t(9;11)(p22;q23) (R5C and R5D) and t(1;11)(q21;q23) (R5E). **(C)** checkup for DNA contamination in RNA mixtures by GAP-PCR protocol. An example of contamination was shown.

Fig.2 (A) MA status were significantly detected in majority of the positive cases. **(B)** Relationship of act(3q26) with other rearrangements in positive subjects. **(C)** Age-associated *EVI1* activation in positive subjects.

Fig.3 Nucleotide sequence of novel fusion transcript variant identified in the present study. A; transcript variant of *TLX1* has been predicted in NCBI Reference Sequence as transcript variant X1 observed in case No.11. B and C; two novel fusion variant of t(4;11)(q21;q23) in case No.41. D, E and F; three novel fusion variant of t(6;11)(q27;q23) and t(9;11)(p22;q23) in case No.3. Breakpoint sequence related to the t(11;19)(q23;p13.1) has been previously reported [70], which observed in a novel disease in this study (Case No.41).

Table 1

Table 1: Aberrant rearrangements investigated by MRT-PCR

Chromosomal Rearrangement	Genes Involved	HGNC Gene Symbols	ENSEMBL Transcript ID	Gene Fusion Region*	PCR Mix No. ‡	PCR Product Size ¥	Positive Control‴	Observations‴
act(3q26.2)	*EVI1*(3q26.2)	*MECOM*	ENST00000464456		R7C	262	RPMI8402[14]¶	MDS[15],AML[16],ALL[17],CML[18]
			ENST00000494292		R7C	289	RPMI8402[14]¶	MDS[15],AML[16],ALL[17],CML[18]
act(10q24)	*HOX11*(10q24)	*TLX1*	ENST00000370196		R4D	212	RPMI8402[19]¶	T-ALL[20]
			XM_011539744.2†		R4D	248		T-ALL§
del(1p32)	*SIL*(1p32)	*STIL*	ENST00000360380	*SIL*ex1/*TAL1*ex1b	R3D	184	RPMI8402[20,21]¶	T-ALL[20,21]
	TAL1(1p32)	*TAL1*	ENST00000294339					
dup(11q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex7/*MLL*ex2	R2F	184		ALL[3]
	MLL(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*MLL*ex2	R2F	258		AML[22],ALL[23]
				*MLL*ex9/*MLL*ex2	R2F	145(390)	PC	AML[22]
				*MLL*ex10/*MLL*ex2	R2F	259(504)		AML[22]
				*MLL*ex11/*MLL*ex2	R2F	406(651)		AML[22]
				*MLL*ex11[nt51]/*MLL*ex2	R2F	310(555)		AML[22]
inv(16)(p13;q22.1)	*CBF*(16q22.1)	*CBFβ*	ENST00000290858	*CBF*ex5/*MYH11*ex34 (A)	R1A	270	PC,ME-1[24]	AML[24]
	MYH11(16p13)	*MYH11*	ENST00000616439	*CBF*ex5/*MYH11*ex33 (B)	R1A	483		AML[24]
				*CBF*ex5/*MYH11*ex32 (C)	R1A	663		AML[24]
				*CBF*ex5/*MYH11*ex30 (D)	R1B	337		AML[24]
				*CBF*ex5/*MYH11*ex29 (E)	R1B	544		AML[24]
				*CBF*ex4/*MYH11*ex34 (F)	R1A	174		AML[24]
				*CBF*ex4/*MYH11*ex30 (G)	R1B	241		AML[4]
				*CBF*ex4/*MYH11*ex29 (H)	R1B	348		AML[4]
				*CBF*ex5/*MYH11*ex32 (I)#	R1A	591		AML[25]
				*CBF*ex5/*MYH11*ex33[nt88]+ins7#	R1A	402		AML[26]
t(X;11)(q13.1;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*AFX*ex1	R1C	344		P
	AFX(Xq13.1)	*FOXO4*	ENST00000374259	*MLL*ex9/*AFX*ex1	R1C	231(476)		P
				*MLL*ex10/*AFX*ex1	R1C	345(590)	Karpas-45[28,29]	ALL[28,29]
				*MLL*ex11/*AFX*ex1	R1C	492(737)		P
t(1;11)(q21;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*AF1q*ex2	R5E	400		AMMOL[30]
	AF1q(1q21)	*MLLT11*	ENST00000368921	*MLL*ex9/*AF1q*ex2#	R5E	287(532)		AML[99]
				*MLL*ex10/*AF1q*ex2	R5E	401(646)		P
				*MLL*ex11/*AF1q*ex2	R5E	548(793)		P
				*MLL*ex9[nt115]/*AF1q*ex2+ins27#	R5E	542		AML[31]
t(1;11)(p32;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*AF1p*ex2	R2A	301	TZ-1[33]#	ALL[32],AMOL[33]#
	AF1p(1p32)	*EPS15*	ENST00000371733	*MLL*ex9/*AF1p*ex2	R2A	188(433)		P
				*MLL*ex10/*AF1p*ex2	R2A	302(547)		P
				*MLL*ex11/*AF1p*ex2	R2A	449(694)		P
t(1;19)(q23;p13)	*E2A*(19p13)	*TCF3*	ENST00000262965	*E2A*ex16/*PBX1*ex3 (I)	R3A	376	PC,697[34,35],SUP-B27[34]#	ALL[35]
	PBX1(1q23)	*PBX1*	ENST00000420696	*E2A*ex16/*PBX1*ex3+ins27 (Ia)	R3A	403		ALL[35]
t(2;5)(p23;q35.1)	*NPM*(5q35.1)	*NPM1*	ENST00000296930	*NPM*ex4/*ALK*ex20	R8D	296	SUP-M2[36],L82[37]	ALCL[36],T/B-cell lymphomas[38]
	ALK(2p23)	*ALK*	ENST00000389048					
t(3;5)(q25.1;q35.1)	*NPM*(5q35.1)	*NPM1*	ENST00000296930	*NPM*ex6/*MLF1*ex2	R8F	276		MDS[39], AML[39]
	MLF1(3q25.1)	*MLF1*	ENST00000619577					
t(3;21)(q26.2;q22.3)	*AML1*(21q22.3)	*RUNX1*	ENST00000300305	*AML1*ex5/*MDS1*ex2	R4B	446	SKH1[40]	CML-BC[40], AML[41], MDS[42]
	MDS1(3q26.2)	*MECOM*	ENST00000494292	*AML1*ex6/*MDS1*ex2	R4B	638	PCO	CML-BC[40], AML[41], MDS[42]
t(4;11)(q21;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*AF4*ex4	R5A	317	KOCL45[43]#	ALL[43,44]
	AF4(4q21)	*AFF*	ENST00000307808	*MLL*ex9/*AF4*ex4	R5A	204(449)	RS4; 11[43]	ALL[43,44]
				*MLL*ex10/*AF4*ex4	R5A	318(563)		ALL[44,46]
				*MLL*ex11/*AF4*ex4#	R5A	465(710)		ALL[45]
				*MLL*ex8/*AF4*ex5	R5A	272	MV-4-11[44]	ALL[44,46]
				*MLL*ex9/*AF4*ex5	R5A	159(404)		ALL[43,44,46]
				*MLL*ex10/*AF4*ex5	R5A	273(518)		ALL[43,46]
				*MLL*ex11/*AF4*ex5§	R5A	420(665)		ALL
				*MLL*ex8/*AF4*ex6#	R5A	185		ALL[47]
				*MLL*ex9/*AF4*ex6	R5A	72(317)		ALL[46]
				*MLL*ex10/*AF4*ex6	R5A	186(431)	KOCL-69[43]#	ALL[43,44,46]
				*MLL*ex11/*AF4*ex6§	R5A	333(578)	PC	ALL
t(5;12)(q33;p13)	*TEL*(12p13)	*ETV6*	ENST00000396373	*TEL*ex4/*PDGFR*ex9#	R6D	657		CMML[48]
	PDGFR(5q33)	*PDGFRβ*	ENST00000261799	*TEL*ex4/*PDGFR*ex11	R6D	321	PCO	CMML[48,49], MDS[49]
t(5;17)(q35.1;q22)	*NPM*(5q35.1)	*NPM1*	ENST00000296930	*NPM*ex4/*RARA*ex2	R8E	165		APL[50]
	RARA(17q22)	*RARα*	ENST00000394081	*NPM*ex5+129bp/*RARA*ex2	R8E	294		APL[51]
t(6;9)(p22.3;q34.1)	*DEK*(6p22.3)	*DEK*	ENST00000397239	*DEK*ex9/*CAN*ex18	R7A	320	FKH-1[52]#	AML[52]
	CAN(9q34.1)	*NUP214*	ENST00000359428					
t(6;11)(q27;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex7/*AF6*ex2#	R1D	234	SHI-1[53#]	AML[53]
	AF6(6q27)	*AFDN*	ENST00000400822	*MLL*ex8/*AF6*ex2	R1D	308	PC,ML-2[3]SHI-1[53,54]#	AML[23], ALL[3]
				*MLL*ex9/*AF6*ex2	R1D	195(440)	CTS[55]#	AML[23]
				*MLL*ex10/*AF6*ex2	R1D	309(594)		P
				*MLL*ex11/*AF6*ex2§	R1D	456(741)		AML
t(8;21)(q22;q22.3)	*AML1*(21q22.3)	*RUNX1*	ENST00000300305	*AML1*ex5/*ETO*ex3	R4A	353	PC,Kasumi-1[56],SKNO-1[57]#	AML[56]
	ETO(8q22)	*RUNX1T1*	ENST00000523629					
t(9;9)(q34.11;q34.13)	*SET*(q34.11)	*SET*	ENST00000372692	*SET*ex7/*CAN*ex17#	R7B	552		T-ALL[58]
	CAN(q34.13)	*NUP214*	ENST00000359428	*SET*ex7/*CAN*ex18	R7B	393	MEGAL[58]#,LOUCY[58]#	AUL[59],AML[60]#,T-ALL[61]#
t(9;11)(p22;q23)	*MLL*(11q23)	*KMT2A*	ENST00000534358	*MLL*ex8/*AF9*ex6	R5C	321	THP-1[62]¶,UG3[63]#	AML[43]
	AF9(9p22)	*MLLT3*	ENST00000380338	*MLL*ex9/*AF9*ex6	R5C	208(453)	Mono-Mac-6[3],KOPB-26[43]#	AML[64]
				*MLL*ex10/*AF9*ex6	R5C	322(567)	Mono-Mac-6[3],KOPB-26[43]#	AML[64]
				*MLL*ex11/*AF9*ex6§	R5C	469(714)		AML
				*MLL*ex7/*AF9*ex7#	R5C	171(521)		AML[65]
				*MLL*ex8/*AF9*ex9	R5D	365		AML[66]
				*MLL*ex9/*AF9*ex9	R5D	252(497)		P
				*MLL*ex10/*AF9*ex9	R5D	366(611)		P
				*MLL*ex11/*AF9*ex9§	R5D	513(758)		AML
				*MLL*ex8/*AF9*ex10#	R5D	293		AML[67]

t(9;12)(q34.1;p13)	*TEL*(12p13) *ABL*(9q34.1)	*ETV6* *ABL1*	ENST00000396373 ENST00000318560	*TEL*ex4/*ABL*ex2 *TEL*ex5/*ABL*ex2#	R6C R6D	366 912	ALL-VG[68]#, PCO ALL-VG[68]#	ALL[69,70],CML[71]#,T-cell lmphoma[72]# ALL[70],AML[70],CML[71],T-cell lymphoma[72]
t(9;22)(q34;q11)	*BCR*(22q11) *ABL*(9q34)	*BCR* *ABL1*	ENST00000305877 ENST00000318560	*BCR*ex1/*ABL*ex2 (e1a2) *BCR*ex1/*ABL*ex3 (e1a3) *BCR*ex13/*ABL*ex2 (b2a2) *BCR*ex13/*ABL*ex3 (b2a3) *BCR*ex14/*ABL*ex2 (b3a2) *BCR*ex14/*ABL*ex3 (b3a3)	R6A R6A R6B R6B R6B R6B	320 146 397 223 472 298	NALM-29[73]# PC,BV-173[73]#,MOLM-1[73]# K562[73]¶ 	ALL[74] P CML[74] P CML[74] P
t(10;11)(p12;q23)	*MLL*(11q23) *AF10*(10p12)	*KMT2A* *MLLT10*	ENST00000534358 ENST00000307729	*MLL*ex7/*AF10*ex16 *MLL*ex8/*AF10*ex15 *MLL*ex9/*AF10*ex15 *MLL*ex10/*AF10*ex15 *MLL*ex11/*AF10*ex15 *MLL*ex7/*AF10*ex14 *MLL*ex8/*AF10*ex10 *MLL*ex9/*AF10*ex10 *MLL*ex10/*AF10*ex10 *MLL*ex11/*AF10*ex10 *MLL*ex8/*AF10*ex9 *MLL*ex9/*AF10*ex9 *MLL*ex10/*AF10*ex9 *MLL*ex11/*AF10*ex9 *MLL*ex8/*AF10*ex6 *MLL*ex9/*AF10*ex6 *MLL*ex10/*AF10*ex6 *MLL*ex11/*AF10*ex6	R2C R2C R2C R2C R2C R2C R2D R2D R2D R2D R2D R2D R2D R2D R2E R2E R2E R2E	202 388 275(520) 389(634) 536(781) 493 268 155(400) 269(514) 416(661) 364 251(496) 365(610) 512(757) 267 154(399) 268(513) 415(660)	PC	AML[75,76] AML[75,76] P P P AML[3] AML[75,76] AML[76] P P AML[76] AML[76] P P AML[76] P P P
t(11;17)(q23;q21)	*MLL*(11q23) *AF17*(17q21)	*KMT2A* *MLLT6*	ENST00000534358 ENST00000621332	*MLL*ex7/*AF17*ex11 *MLL*ex8/*AF17*ex7-del615# *MLL*ex8/*AF17*ex8-del615# *MLL*ex9[nt74]/*AF17*ex11[nt94]#	R2B R2B R2B R2B	281 839 671 335		AML[77] AML[78] AML[78] AML[78]
t(11;17)(q23.1;q21)	*PLZF*(11q23.1) *RARA*(17q21)	*ZBTB16* *RARα*	ENST00000335953 ENST00000394081	*PLZF*ex3/*RARA*ex2 *PLZF*ex4/*RARA*ex2	R8A R8A	315 402		APL[79] APL[79]
t(11;19)(q23;p13.1)	*MLL*(11q23) *ELL*(19p13.1)	*KMT2A* *ELL*	ENST00000534358 ENST00000262809	*MLL*ex8/*ELL*ex2 *MLL*ex9/*ELL*ex2 *MLL*ex10/*ELL*ex2 *MLL*ex11/*ELL*ex2 *MLL*ex8/*ELL*ex2+ins120 *MLL*ex9/*ELL*ex2+ins120 *MLL*ex10/*ELL*ex2+ins120 *MLL*ex11/*ELL*ex2+ins120 *MLL*ex9/*ELL*ex3#	R1E R1E R1E R1E R1E R1E R1E R1E R1E	330 217(462) 301(576) 448(723) 450 337(582) 451(696) 598(845) 169	PC	P AML[80],ALL§ P P P AML[80] AML[80] P AML[81]
t(11;19)(q23;p13.3)	*MLL*(11q23) *ENL*(19p13.3)	*KMT2A* *MLLT1*	ENST00000534358 ENST00000252674	*MLL*ex8/*ENL*ex2 *MLL*ex9/*ENL*ex2 *MLL*ex10/*ENL*ex2 *MLL*ex11/*ENL*ex2	R5B R5B R5B R5B	186 73(318) 187(432) 334(579)	PCO KOCL-44[43],KOCL-51[43]# KOCL-44[43],KOCL-51[43]# 	ALL[46] ALL[46] ALL[46] P
t(12;21)(p13;q22.3)	*TEL*(12p13) *AML1*(21q22.3)	*ETV6* *RUNX1*	ENST00000396373 ENST00000300305	*TEL*ex5/*AML1*ex3 *TEL*ex5/*AML1*ex2 *TEL*ex5+ins33/*AML1*ex2#	R3C R3C R3C	298 337 370	 REH[82]¶ 	ALL[83] ALL[83] ALL[27]
t(15;17)(q22;q21)	*PML*(15q22) *RARA*(17q21)	*PML* *RARα*	ENST00000268058 ENST00000394081	*PML*ex7+ins110/*RARA*ex2[nt46]# *PML*ex7/*ADAMTS17*ex15/*RARA*ex2# *PML*ex6/*RARA*ex2 (BCR1) *PML*ex6+ins29/*RARA*ex2 (BCR2) *PML*ex6+ins25/*RARA*ex2 (BCR2) *PML*ex6[nt205]/*RARA*ex2 (BCR2) *PML*ex6[nt113]/*RARA*ex2 (BCR2)# *PML*ex6[nt101]/*RARA*ex2 (BCR2) *PML*ex5/*RARA*ex2# *PML*ex4/*RARA*ex2 *PML*ex4/ins58/*RARA*ex2# *PML*ex4+ins9/*RARA*ex1[nt413]# *PML*ex3-del42-ex4/*RARA*ex2# *PML*ex3/*RARA*ex2 (BCR3) *PML*ex3-del54/*RARA*ex2	R8B R8B R8B R8B R8B R8B R8B R8B R8B R8C R8C R8C R8C R8C R8C	544 601 427 456 392 373 280 269 168 464 522 549 422 393 339	 NB-4[86,91]¶ NB-4[86]¶ 	APL[84] APL[85] APL[86,5] APL[5] APL[5] APL[5] APL[87] APL[5] APL[88] APL[86] APL[89] APL[90] APL[87] APL[86] APL[5]
t(16;21)(p11.2;q22.3)	*TLS*(16p11.2) *ERG*(21q22.3)	*FUS* *ERG*	ENST00000254108 ENST00000417133	*TLS*ex8/*ERG*ex10# *TLS*ex7/*ERG*ex9# *TLS*ex6+44bp+ex7/*ERG*ex12 (a) *TLS*ex7/*ERG*ex12 (b) *TLS*ex6/*ERG*ex12 (c) *TLS*ex6/*ERG*ex10 (d) *TLS*ex6/*ERG*ex9 (e)	R4C R4C R4C R4C R4C R4C R4C	412 448 318 274 239 344 413	 UTP-L12[94] UTP-L12[94],JIH-4[95]# UTP-L12[94] PC 	AML[92] ALL[93] AML[94] AML[94] AML[94],ALL[94] ALL[3] ALL[3]
t(17;19)(q22;p13.3)	*E2A*(19p13.3) *HLF*(17q22)	*TCF3* *HLF*	ENST00000262965 ENST00000226067	*E2A*ex16/*HLF*ex4+ins137# *E2A*ex16/*HLF*ex4+ins90# *E2A*ex16/ins59/*HLF*ex4 (I) *E2A*ex16/*HLF*ex4 (I) *E2A*ex15/*HLF*ex4 (II) *E2A*ex14/*HLF*ex4#	R3B R3B R3B R3B R3B R3B	468 421 390 331 207 48	YCUB-2[96]# YCUB-2[96]# HAL-01[97] YCUB-2[96]# 	ALL[96] ALL[96] ALL[97] ALL[97] ALL[97] ALL[98]

Abbreviations: HGNC, HUGO Gene Nomenclature Committee; No, number; act, activation; del, deletion; dup, duplication; inv, inversion; t, translocation; p, short chromosome arm; q, long chromosome arm; ex, exon; ins, insertion; nt, nucleotid; bp, base pair; BCR1/2/3, breakpoint cluster region 1/2/3; PC, positive control obtained from patient material; PCO, positive control obtained from patients who are not member of this study; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CML, chronic myeloid

leukemia; BC, blast crisis; AUL, acute undifferentiated leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; AMOL, acute monocytic leukemia; AMMOL, acute myelomonocytic leukemia; P, possible fusion variant in terms of theoretical.
Symbols: *Letters and numbers in brackets and parentheses respectively indicates alternative breakpoints and name of fusion variants. ‡R1 to R8 indicate multiplex reaction number, and A to F indicate split-out reaction with individual primer pairs. ¥Number in the parentheses suggested the size of PCR product when co-amplified from the *MLL* exon 7 primer, just for *MLL*ex7/*AF9*ex7 is related to the co-amplification from the *AF9* exon 11 primer. ″Related references shown on upper right corner. †This variant of *HOX11* transcript has been predicted in NCBI Reference Sequence as transcript variant X1 and not described on ENSEMBL. #Novel fusion variant, cell line and observation in other study. ¶Available cell lines employed as positive control. §Novel fusion variant and disease observation in this study.

Table 2: Primers used in the GAP-PCR and NM-PCR

Group	Gene	ENSEMBL Transcript ID	Forward Primer (5'>3')	Reverse Primer (5'>3')	PCR Mix No.	Product Length	Tm (^{0}C)
A							
	GAPDH	ENST00000229239.9	CATGGCCTCCAAGGAGTAAG	GGTTGAGCACAGGGTACTTTA	-	219bp	57
B							
	AF4	ENST00000307808	-	GTTTTTGGTTTTGGGTTACAGAACT	R5A	-	58
	SET	ENST00000372692	CACCGAAATCAAATGGAAATCTG	-	R7B	-	56

Group A: primer pair used for GAP-PCR protocol. Forward primer annealed to the exon9 and reverse primer placed on 3' UTR of GAPDH transcript.

Group B: optimized primers for NM-PCR. Reverse primer of *AF4* is for second step of NM-PCR primer set. Forward primer of *SET* is related to first step of NM-PCR primer set. Other primers were used as provided sequence in the past [3].

Table 3

Table 3: Preanalytic features and MRT-PCR results of 53 subjects with hematological malignancies

Group	Case No.*	Status	Sex	Age (year)	Diagnosis	IBC (%)	Abnormality	Related Gene or Fusion Variant	Additional details
A									
	1*	present	M	40	T-ALL	11	act(10q24) act(3q26)	Activation of *HOX11* Activation of *EVI1*	New case
	7*	present	F	32	Pre-B-ALL	18	t(1;19)(q23;p13) act(3q26)	*E2A*ex16/*PBX1*ex3 (I) Activation of *EVI1*	New case
	11*	present	F	12	ALL	21	act(10q24) act(3q26)	Activation of *HOX11* Activation of *EVI1*	New case
	20	present	M	26	Pre B-ALL	4	act(3q26)	Activation of *EVI1*	New case
	39*	present	M	6	T-ALL	17	t(16;21)(p11.2;q22.3) act(10q24) act(3q26)	*TLS*ex6/*ERG*ex10 (d) Activation of *HOX11* Activation of *EVI1*	New case
	41*	present	M	7	ALL	24	t(11;19)(q23;p13.1) t(4;11)(q21;q23) t(4;11)(q21;q23) t(4;11)(q21;q23) act(3q26)	*MLL*ex9/*ELL*ex2 *MLL*ex10/*AF4*ex6 *MLL*ex11/*AF4*ex6 *MLL*ex11/*AF4*ex5 Activation of *EVI1*	New case
	43	present	M	4	Pre B-ALL	-	t(4;11)(q21;q23)	*MLL*ex11/*AF4*ex6	New case
	47*	present	M	5	Pre B-ALL	13	t(6;11)(q27;q23) t(12;21)(p13;q22.3) act(3q26)	*MLL*ex8/*AF6*ex2 *TEL*ex5/*AML1*ex2 Activation of *EVI1*	New case
	48*	present	F	2	Pre B-ALL	12	t(12;21)(p13;q22.3) t(4;11)(q21;q23) act(3q26)	*TEL*ex5/*AML1*ex3 *MLL*ex11/*AF4*ex4 Activation of *EVI1*	New case
	6	remission	M	4	Pre B-ALL	-	Negative		The initial diagnosis was performed by Immunophenotyping. During sampling, patient was received chemotherapy.
	12	present	M	23	T-ALL	-	Negative		New case
	14	present	F	15	ALL	-	Negative		New case
	44	present	F	4	Pre B-ALL	-	Negative		New case
	45	present	F	8	Pre B-ALL	-	Negative		New case
	51	remission	M	5	Pre B-ALL	-	ND		The initial diagnosis was performed by Immunophenotyping. During sampling, patient was received chemotherapy.
B									
	3*	present	F	16	AML	31	t(6;11)(q27;q23) t(11;19)(q23;p13.1) t(9;11)(p22;q23) t(9;11)(p22;q23) act(3q26) t(15;17)(q22;q21)	*MLL*ex11/*AF6*ex2 *MLL*ex9/*ELL*ex2 *MLL*ex11/*AF9*ex9 *MLL*ex11/*AF9*ex6 Activation of *EVI1* *PML*ex3/*RARA*ex2	New case
	5*	present	M	56	AML-M1	6	dup(11q23) act(3q26)	*MLL*ex9/*MLL*ex2 Activation of *EVI1*	New case
	8	present	M	43	AML with Pancytopenia	7	act(3q26)	Activation of *EVI1*	New case
	9	present	M	70	AML-M5	10	act(3q26)	Activation of *EVI1*	New case
	10	remission	M	30	AML-M3	7	act(3q26)	Activation of *EVI1*	The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Partial remission was achieved after 6 months.
	18*	present	M	24	AML-M3	35	act(3q26) t(15;17)(q22;q21)	Activation of *EVI1* *PML*ex3/*RARA*ex2	New case
	19	present	M	41	AML	6	act(3q26)	Activation of *EVI1*	New case
	21	present	M	19	AML-M2 with Pancytopenia	-	t(8;21)(q22;q22)	*AML1*ex5/*ETO*ex3	New case
	22*	present	M	45	AML-M3	18	act(3q26) t(15;17)(q22;q21)	Activation of *EVI1* *PML*ex3/*RARA*ex2	New case
	24	present	M	29	AML-M4Eo	-	inv(16)(p13.11;q22.1) inv(16)(p13.11;q22.1)	*CBF*ex5/*MYH11*ex34 (A) *CBF*ex5/*MYH11*ex33+ins7	New case
	25*	present	M	18	AML-M3 with Pancytopenia	-	t(10;11)(p12;q23) t(15;17)(q22;q21)	*MLL*ex7/*AF10*ex16 *PML*ex6/*RARA*ex2 (BCR1)	New case
	26*	present	M	35	AML-M3	36	t(9;11)(p22;q23) act(3q26) t(15;17)(q22;q21)	*MLL*ex8/*AF9*ex10 Activation of *EVI1* *PML*ex6+ins25/*RARA*ex2	New case
	27	present	M	28	AML-M4Eo	-	inv(16)(p13.11;q22.1)	*CBF*ex5/*MYH11*ex34 (A)	New case
	28*	remission	M	32	AML-M3	7	act(3q26) t(15;17)(q22;q21)	Activation of *EVI1* *PML*ex6[nt205]/*RARA*ex2	The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Partial remission was achieved after 8 months.
	29	remission	M	29	AML-M3	15	act(3q26)	Activation of *EVI1*	The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment

									was started by ATRA. Partial Remission was achieved after 6 months.
	30*	present	F	32	AML-M3	-	t(8;21)(q22;q22) t(15;17)(q22;q21)	*AML1*ex5/*ETO*ex3 *PML*ex6/*RARA*ex2	New case
	31	relapse	M	35	AML-M2	-	t(8;21)(q22;q22)	*AML1*ex5/*ETO*ex3	The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Relapsed after 4 months with t(8;21)(q22;q22) and diagnosed as AML-M2.
	35	present	M	15	AML-M3	-	t(15;17)(q22;q21)	*PML*ex3/*RARA*ex2	New case
	4	present	M	29	AML	-	Negative		New case
	13	present	M	44	AML	-	Negative		New case
	15	remission	M	45	AML	-	Negative		The initial diagnosis was performed by Immunophenotyping. During sampling, patient was received chemotherapy.
	17	relapse	F	21	AML-M4	-	Negative		The initial diagnosis was performed by Immunophenotyping. Partial remission was achieved after 6 months. Then, Relapsed after 2 months.
	32	remission	M	68	AML-M3	-	Negative		The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Complete remission was achieved after 7 months.
	36	present	M	79	AML	-	Negative		New case
	38	remission	M	35	AML-M3	-	Negative		The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Complete remission was achieved after 5 months.
	46	remission	M	37	AML-M3	-	Negative		The initial analysis showed AML-M3 with t(15;17)(q22;q21) and treatment was started by ATRA. Complete remission was achieved after 5 months.
C									
	2*	present	F	43	CML	11	t(9;22)(q34;q11) act(3q26)	*BCR*ex13/*ABL*ex2 Activation of *EVI1*	New case
	16*	relapse	F	41	CML	29	t(9;22)(q34;q11) act(3q26)	*BCR*ex13/*ABL*ex2 Activation of *EVI1*	The initial analysis showed CML with t(9;22)(q34;q11). Treatment was started by Imatinib. Relapsed after 7 months. Without effective point mutation in ABL1 kinase domain.
	23	present	M	53	CML-BC	-	t(9;22)(q34;q11)	*BCR*ex13/*ABL*ex2	New case
	33	relapse	M	34	CML	-	t(9;22)(q34;q11)	*BCR*ex14/*ABL*ex2	The initial analysis showed CML with t(9;22)(q34;q11). Treatment was started by Imatinib. Relapsed after 6 months. A point mutation related to T315I amino acid change was detected in ABL1 kinase domain.
	40*	present	F	36	CML	10	t(9;22)(q34;q11) act(3q26)	*BCR*ex13/*ABL*ex2 Activation of *EVI1*	New case
	42*	present	F	26	Pro-CML	6	t(9;22)(q34;q11) act(3q26)	*BCR*ex13/*ABL*ex2 Activation of *EVI1*	New case
	49	present	F	49	CML-BC	-	t(9;22)(q34;q11)	*BCR*ex13/*ABL*ex2	New case
	52	present	M	92	CML	16	act(3q26)	Activation of *EVI1*	New case
	53*	present	F	62	CML	17	t(9;22)(q34;q11) act(3q26)	*BCR*ex13/*ABL*ex2 Activation of *EVI1*	New case
	37	remission	M	25	CML	-	Negative		The initial analysis showed CML with t(9;22)(q34;q11). Treatment was started by Imatinib. Complete remission was achieved after 4 months.
	50	remission	M	28	CML	-	Negative		The initial analysis showed CML with t(9;22)(q34;q11). Treatment was started by Imatinib. Complete remission was achieved after 6 months.
D						-			
	54*	present	M	47	HES	28	dup(11q23) act(3q26)	*MLL*ex10/*MLL*ex2 Activation of *EVI1*	New case
	34	present	M	16	LL	-	Negative		New case

Group A, patients diagnosed as ALL subtypes. Group B, patients diagnosed as AML subtypes. Group C, patients diagnosed as CML subtypes. Group D, patients with other hematological malignancies.

Abbreviations: IBC, immature blast cell; M, male; F, female. Pre B-ALL, precursor B-cell acute lymphoblastic leukemia; Pro-CML, progenitor cell-chronic myeloid leukemia; HES, hypereosinophilic syndrome; LL, Lymphoblastic Lymphoma; ND, not done; ATRA, all trans-retinoic acid. Other abbreviations are similar to Table 1.

Symbols: *Subjects with MA situation.

Table 4: The CBC test results in subjects with novel fusion variant.

Case No.	IBC (%)	Hem (g/dL)	Platelet /µL	WBC /µL	Neut (%)	Mono (%)	Eos (%)	Lym (%)
3*	31	6.9	24×10^3	16.21×10^3	88.2	8.6	-	2.1
11*	21	11.1	26×10^3	3.94×10^3	23.1	2.3	1.3	72.8
41*	24	11.6	66×10^3	3.77×10^3	11.2	11.1	0.3	76.9

Abbreviation: Hem, hemoglobin; WBC, white blood cell; Neut, neutrophil cell; Mono, monocyte cell; Eos, eosinophilia cell; Lym, Lymphocyte cell. Other abbreviations and Symbols are similar to Table 1 and Table 3.

Fig1

Click here to download Figure Fig1.tif

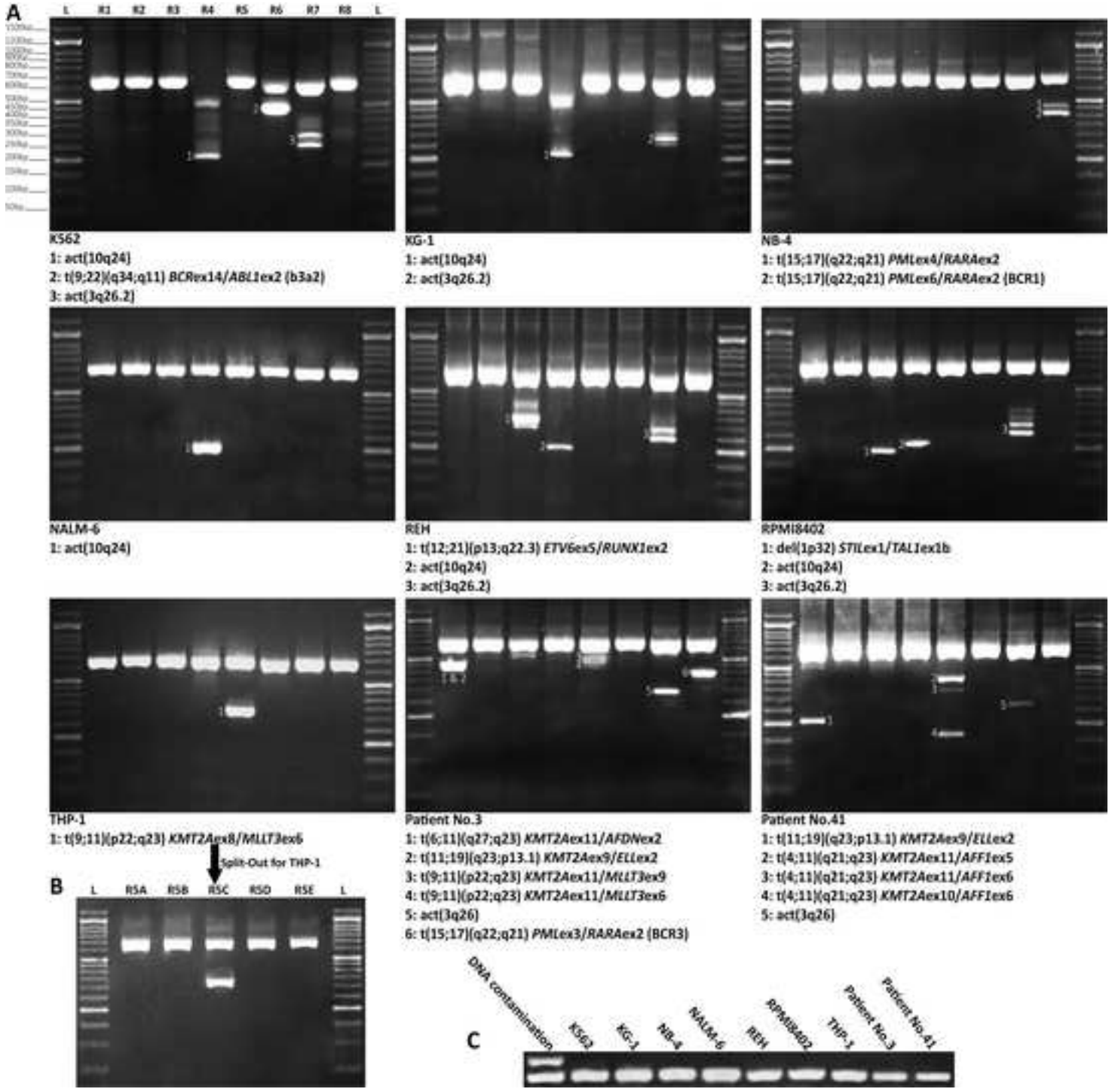

Fig2

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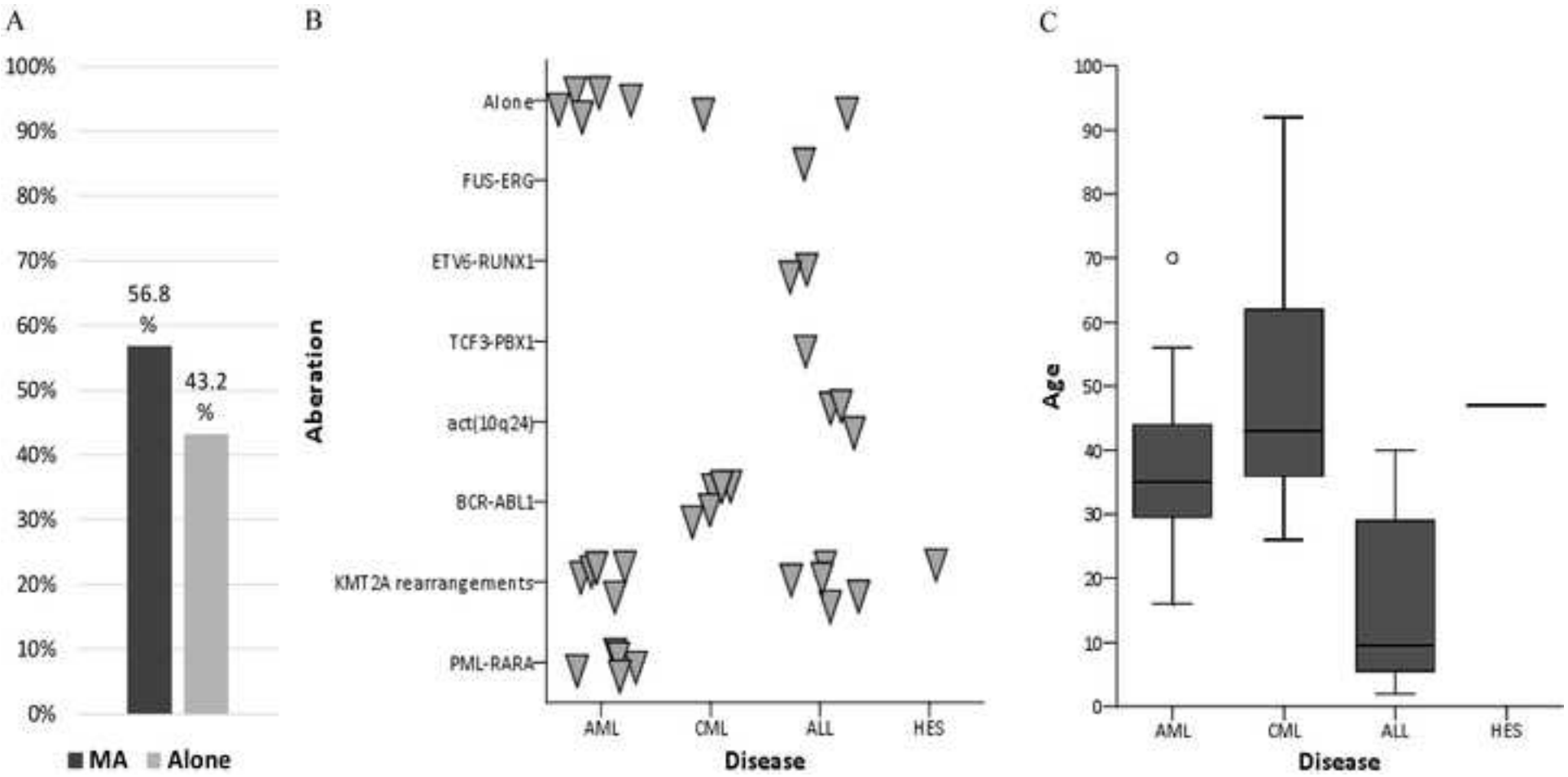
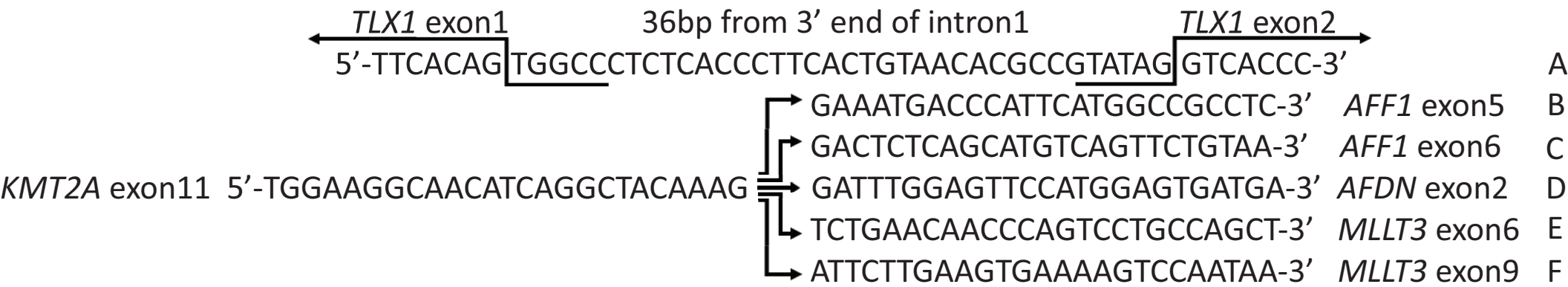


Fig3


TLX1 exon1
36bp from 3’ end of intron1
TLX1 exon2
5’-TTCACAG TGGCCCTCTCACCCTTCACTGTAACACGCCGTATAG GTCACCC-3’
A
GAAATGACCCATTCATGGCCGCCTC-3’ *AFF1* exon5 B
GACTCTCAGCATGTCAGTTCTGTAA-3’ *AFF1* exon6 C
KMT2A exon11 5’-TGGAAGGCAACATCAGGCTACAAAG
GATTTGGAGTTCCATGGAGTGATGA-3’ *AFDN* exon2 D
TCTGAACAACCCAGTCCTGCCAGCT-3’ *MLLT3* exon6 E
ATTCTTGAAGTGAAAAGTCCAATAA-3’ *MLLT3* exon9 F