RESEARCH ARTICLE

Novel *IRF-1* Mutations in a Small Cohort of Leukaemia Patients From Saudi Arabia

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Abstract

Involvement of the Interferon Regulatory Factor 1 (IRF-1) gene in regulation of cell differentiation and proliferation made it a potential target in cancer research. IRF-1 acts as a tumor suppressor gene, and is inactivated in chronic (CML) and non-chronic myelogenous leukemia (non-CML). In the light of numerous reports on genetic changes in the noncoding region of the IRF-1 gene, this study aimed to explore possible genomic changes in coding and non-coding regions of IRF-1 in a random sample of leukemic Saudi patients, in order to obtain insights into potential impact of genetic changes on clinicopathological characteristics. Patients were classified into two major leukemia subtypes: CML (8 cases; 36.4%) and non-CML (14 cases; 63.6%). Sequencing results revealed two novel mutations in the coding area of the IRF-1 gene likely to influence the IRF-1/DNA binding affinity. In addition, three mutational sites in the non-coding region between exon 5&6 (8985(T>G), 8,990(T>G) and 8995(A>G) were identified. In conclusion, a larger representative study might help provide better understanding of the possible contribution of the identified genetic changes in IRF-1 to disease prognosis and outcomes in leukemic patients.

Keywords: IRF-1- AML- MDS- CML- MPD- Burkitt`s lymphoma

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Introduction

Leukemia is defined as a heterogeneous group of neoplastic proliferative disorders that are characterized by the overproduction of immature and mature leukocytes in the bone marrow and/or peripheral blood. Leukemia are classified into four major subgroups according to predominant cell morphology and degree of severity; acute myelocytic leukemia (AML) chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL) (Kumar, 2011). AML has been reported to be the most common type in adults while ALL is the most common type in children (Jin MW, 2016). A study conducted in the USA has predicted the prevalence of CML to increase from approximately 112,000 in 2020 up to 167,000 in 2040 (Huang et al., 2012). Up to date the exact cause for leukemia is unknown, however, multiple factors have been linked to the disease which include hereditary factors, congenital condition, and germline mutations (Khaled et al., 2016), therefore, scientists have been interested in investigating various approaches to identify some of the factors that may cause or affect the prognosis of leukemia.

Chromosomal aberrations is one of the common causes of leukemia specifically in chromosome 5, where monosomy 5 or deletion within long arm of chromosome 5 has been reported in many cases of leukemia and pre-leukemic myelodysplastic syndrome (MDS) (Willman et al., 1993). Interestingly, Interferon Regulatory Factor 1 (IRF-1) was mapped to the smallest commonly deleted region of chromosome 5 (5q31.1) of 7.72kb containing 10 exons and 9 introns (Cha et al., 1992). Initially, IRF-1 was known for its role as a DNA binding protein in the expression of interferon-β gene as a result of viral induced response (Miyamoto et al., 1988). More recently, IRF-1 has been shown to play a critical role in cell proliferation and cell differentiation by acting as a tumor suppressor gene (Harada et al., 1993; Tanaka et al., 1994; Passioura et al., 2005). Since the discovery of IRF-1 as a tumor suppressor, many studies have linked its role to various types of cancer such as, stomach carcinoma, esophageal carcinoma, gastric adenocarcinoma, breast cancer, skin melanoma, uterine endometrial carcinoma, cervical cancer, hepatocellular carcinoma, pancreatic cancer, and surprisingly leukaemia and pre-leukaemia myelodysplasia (Preisler et al., 2001).

IRF-1 inactivation in human cancer and thus attenuation of its transcriptional activity has been reported via several

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mechanisms. Alteration of IRF-1 gene locus at DNA level including loss of heterozygosity, and rearrangement has been reported in hematopoietic and solid tumors (Willman et al., 1993; Tamura et al., 1996; Sugimura et al., 1997; Peralta et al., 1998). IRF-1 attenuation at RNA level has been observed through "accelerated exon skipping" resulting in a splice variant of IRF-1 which misses one or more coding exons to alteri its transcriptional activity and thus its anti-oncogenic property; where exons 7, 8, and 9 been reported to be missed in IRF-1 transcribed RNA in cervical cancer (Lee et al., 2006). This is in contrast to another type of splice variant which misses exon 2 and 3 as reported in AML (Harada et al., 1994). However, the mechanism underlying "accelerated exon skipping" is not fully characterized.

Here, we aim to investigate the genomic status of IRF-1 gene by sequencing IRF-1 locus among leukemic patients so as to identify novel mutations in the coding region and non-coding region, and to investigate the exon-intron boundaries which might affect splicing mechanism and thus IRF-1 inactivation.

Materials and Methods

Study population and sampling

Patients referred to the haematology clinic at King Fahd Hospital, University of Dammam and suspected to have leukaemia or pre-leukemic disorders were investigated to confirm the diagnosis using bone marrow aspiration (performed using standard protocol by a medical haematologist) and routine blood tests (complete blood picture). Twenty-two patients were recruited to participate in the study based on the preliminary hematopathology report. Informed consent was obtained from all individual participants included in the study and the study was ethically approved by the IRB committee of the University of Dammam (IRB-2014-04-059) in accordance with the 1964 Helsinki Declaration and its later amendments. The initial diagnosis was further confirmed by karyotyping and Fluorescence In Situ Hybridization (Fish).

Karyotyping

The buffy coat from peripheral blood and bone marrow of leukemic patients were collected and cultured in MarrowMax medium according to the standard protocol followed in the cytogenetics laboratory at KFHU. The mitotic index of the cells was increased by applying a pokeweed and /or phytohemagglutinin as lymphoproliferative compounds. The cell cultures were treated with colcemid to arrest the chromosomes at metaphase. Karyotyping analysis was then performed on GTG-banded metaphase chromosomes using a synchronizing kit from Euroclone that generated 400–550 band resolutions. A minimum of 20 metaphases per patient were fully analyzed for numerical and structural aberrations using MetaSystem software from Ziess.

Fluorescence In Situ Hybridization (Fish)

Nineteen cases were evaluated by the appropriate FISH panel, as recommended by the clinical pathologist. FISH was not performed when karyotyping and routine blood tests confirmed diagnosis (case # 7, 16 and 18) in Table 1). The protocol was carried out according to manufacturer's instructions with slight modifications (Vysis, Germany). Briefly, cultured cells were treated with 0.2N HCl/2% pepsin for 20 min at 37°C prior to hybridization. Then 10µl of probe was placed on the target drop area, denatured at 73 °C and hybridized to the targeted cells at 37 °C for 16 h. Post hybridization wash was obtained using 0.4xSSC/0.3%NP-40 wash solution at 73±1°C followed by a washing step with 2XSSC/0.1%NP-40 at room temperature. The slides were dried and counterstained with DAPI. Signals were visualized under a Zeiss Axioskop microscope (Zeiss, Germany) using a FITC/Rhodamine dual band filter. A total of 20 metaphase or 200 nuclei were analyzed in each sample if available.

DNA isolation

DNA was isolated from white blood cells (WBCs) using QIAamp DNA Blood mini kit (Qiagen, Germany) with the elution volume of 50µl for each sample as per the manufacturer's instructions. DNA quantification was performed using the Qubit fluorometer (Life Technologies, U.S.A) and purity was checked with the Nanodrop spectrophotometer (Thermo Scientific, U.S.A).

PCR amplification

To sequence IRF-1 locus, 9 sets of primers were designed to cover all the 9 coding exons of IRF-1 and exon-intron boundaries to yield amplicon size ranging from 228 to 526bp. PCR was performed in 25.0µl volumes containing 1.25 units of GoTaq polymerase (Promega, U.S.A), 0.2mM of each dNTP, 1.5mM MgCl2, 1X Taq buffer, 20 pM of each primer and 50ng of DNA. The thermo-cycling conditions were 1 cycle of 5 min at 95◦C; 40 cycles of 30 sec at 95◦C, 30 sec at 54◦C, and 30 sec at 72◦C; and 1 cycle of 5 min at 72◦C. PCR product was analysed on 2% agarose gel which showed the expected amplicon for all products.

Capillary sequencing

The amplified product from the agarose gel was excised and purified using QIAquick Gel extraction kit (Qiagen, Germany) as per the manufacturer's instructions. BigDye terminator v3.1 kit was used for cycle sequencing (Life Technologies, U.S.A). The sequencing reaction was prepared on ice by mixing 1X Terminator ready reaction mix, 3.2pM of sequencing primer and 5–10ng of purified PCR product. PCR product was purified using BigDyeX Terminator purification kit (Life Technologies, U.S.A). Then 10µl of end product was loaded on 96 well plates and subjected to sequence detection using ABI 3500 Genetic Analyzer (Life Technologies, USA). All samples were sequenced in both directions. The sequences were analysed using ABI sequence analysis software. Sequence alignment was performed by the NCBI Align application with reference sequence of IRF-1 (NG 011450.1, Gene ID: 3659).

Statistical analysis

Statistical analysis was performed using SPSS statistical package (version 19). Differences were

considered significant if P<0.05. The relationships between mutation status and patient characteristics, clinical variables were analysed by Fisher's exact test.

Results

Karyotyping and FISH analysis

Some chromosomal abnormalities were spotted initially with karyotyping, which were later confirmed using FISH technique. Amongst these chromosomal aberrations, the Philadelphia chromosome t(9;22) was identified in 4 cases, in addition to deletions del(5)-(q13q33) in one case, and an insertion (5p) in another case (Table 1). The FISH results of the CML patients shows positive signals for the BCR/ABL probe or the Philadelphia chromosome (yellow fusion signal). The BCR/ABL probe is used to detect the translocation of the ABL gene (chromosome 9) to juxtapose the BCR gene (chromosome 22), when a green probe targeting BCR locus and a red probe targeting the ABL locus fused a yellow fusion signal is produced and confirms the presence of the Philadelphia chromosome t(9;22) as shown in Figure1.

IRF-1 gene mutations and genomic changes

The IRF-1 gene was sequenced and two-point mutations were identified in the coding region in exon 4 and 7 (Table 2). These two-point mutations resulted in the change of amino acid lysine to glutamic acid Lys75Glu (exon 4) or vice versa Glu222Lys (exon 7) as shown in Figure 2. The non-coding region of the IRF-1 gene was also sequenced to investigate if any identified genotypic changes were correlated with leukemia. There was no significant association between most genetic changes that were identified in the non-coding areas and leukemia (Table 4). However, results indicate that three genetic

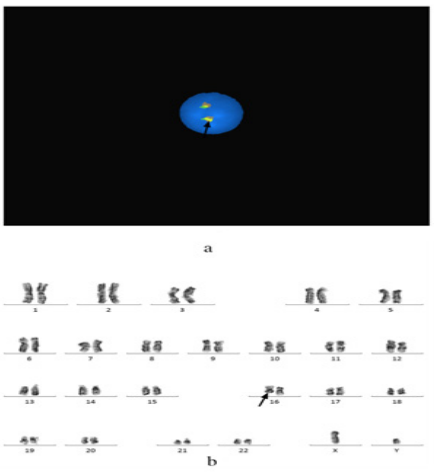

Figure 1. A Representative Figure of Karyotyping and FISH Analysis. BCR/ABL probe was used to detect the Philadelphia chromosome t(9;22) by FISH technique, a yellow fusion signal confirms translocation of the ABL gene to juxtapose the BCR gene (a). The karyotype (b) shows a deletion in chromosome number 16(del (16) (q23)).

Table 1. Patients Characteristics and Confirmed Diagnosis Using Karyotyping and FISH Technique. FISH was not performed when karyotyping and routine blood tests confirmed diagnosis as in cases 7, 16 and 18.

#	Age	Sex	Sample	Karyotype	FISH	Diagnosis
1	94	M	BM	46,XY/45,X,-Y	CEP X/Y-42% -XY and 52% X,-Y	MDS
2	95	M	BM	45,X,-Y	Monosomy of 13q14 area (7%)	MDS
3	40	M	BM	46,XY	Positive deletion of EGR1	MDS
4	61	M	BM	46,XY,inv(1),+3,t(7;9)	D20S108- (6%)-PML/RARA- (11%)	AML
5	67	M	BM	46,XY, t(9;22)	BCR/ABL (87%).-D13S319 deletion	CML
6	20	M	BM	46,XY,add(10p)/46,XY	EGR1 (5q31)/D5S23/D5S71, D20S108, 7S486/CEP7 (-/-)	MDS
7	72	M	BM	46,XY/45,X,-Y	---------	MDS
8	74	F	BM	46,XX,del(5)(q13q33),del(7)(p15)/46,XX	EGR1 (46%)	MDS
9	32	F	BM	46,XX,ins(5p)/46,XX	ETV6-(75%)	AML
10	42	F	BM	46,XX,der(13),t(1;13)(q12;q32),t(8;14) (q24.1;q32),46,XX	IGH (58%), MYC (48%)	Burkitt`s lymphoma
11	66	M	BM	46,XY,t(X;21;1)(q25;q33;q32)	EGR1, ETV6, D20S108, D7S486/CEP7 (-/-)	MDS/AML
12	44	F	BM	46,XX,t(9;22)	BCR/ABL (99%)	CML
13	44	F	BM	46,XX,t(9;22)	BCR/ABL (99%)	CML
14	66	M	BM	46,XY,t(X;21;1)(q25;q33;q32)	EGR1, 7Q31, D20S108 ETV6 (-/-)	MDS
15	25	F	BM	46XX,46,XX,t(9q;22q)	BCR/ABL (21%), PML/RARA (17%)	CML
16	40	F	BM	46,XX,t(3;12)(q25;p12)	----------	MDS
17	25	M	BM	46,XY,del(16)(q23)	BCR/ABL (34%)	CML
18	59	M	BM	46,XY,t(9;22)(q34;q11.2)/45,X,-Y,t(9;22) (q34;q11.2)	----------	CML
19	35	F	BM	46,XY,t(9;22)(q34;q11.2)/46,XX	BCR/ABL (90%)	CML
20	85	M	BM	46,XY/46,XY,+1,der(1;13)(q11;q11.1)	IGH, D13S319, TP53/CEP17 (-/-)	MPD
21	61	F	BM	46,XX/45,X, -X	MYB, ATM,MDM2,D13S319/LAMP, IGH, TP53/CEP17 (-/-)	MDS
22	38	M	BM	46,XY,t(16;22)(q24;q11.2)	BCR/ABL (75%)	CML

Table 2. Summary of Genotypic Changes in the Coding Area of IRF-1 Gene

Sample #	Amino acid change	Mutation status	Genotype change	Locus	group
8	Lys75Glu	Novel	AAG>GAG	Exon 4	Non-CML
9	Glu222Lys	Novel	GAA>AAA	Exon7	Non-CML
11	Glu222Lys	Novel	GAA>AAA	Exon7	Non-CML
12	Glu222Lys	Novel	GAA>AAA	Exon7	CML
13	Glu222Lys	Novel	GAA>AAA	Exon7	CML
19	Glu222Lys	Novel	GAA>AAA	Exon7	CML
20	Glu222Lys	Novel	GAA>AAA	Exon7	CML
21	Glu222Lys	Novel	GAA>AAA	Exon7	Non-CML
22	Glu222Lys	Novel	GAA>AAA	Exon7	Non-CML

Table 3. Correlation Analysis between Mutations in the Coding Area of IRF-1 and Hematologic Malignancies in a Random Sample of Leukemic Patients (N=22)

Variables	Total		P-value
	CML	N-CML	
Exon 2			NA
M	0	0	
NM	8	14	
Exon 3			NA
M	0	0	
NM	8	14	
Exon 4			1
AAG>GAG			
Lys75Glu Hetero			
M	0	1	
NM	8	13	
Exon 5			NA
M	0	0	
NM	8	14	
Exon 7			0.386
GAA>AAA			
Glu222Lys Hetero			
M	4	4	
NM	4	10	
Exon 8			NA
M	0	0	
NM	8	14	

*P < 0.05: statistical significance. M, mutation; NM, No mutation; CML, Chronic myelogenous leukemia; N-CML, Non chronic myelogenous leukemia (contain: MDS, AML, MPD, Burkitt`s lymphoma); MDS, Myelodysplastic syndromes; AML, Acute myelocytic leukemia; MPD, Myeloid proliferation disorders

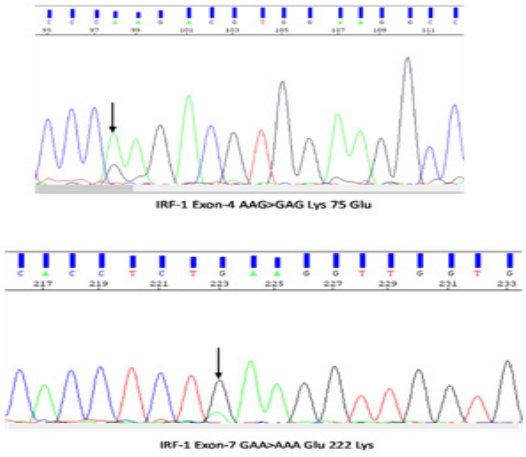

Figure 2. A Representative Electropherogram of IRF-1 Gene Sequencing (Coding Region). An electropherogram of IRF-1 gene sequencing showing genotypic changes in exon 4 and 7

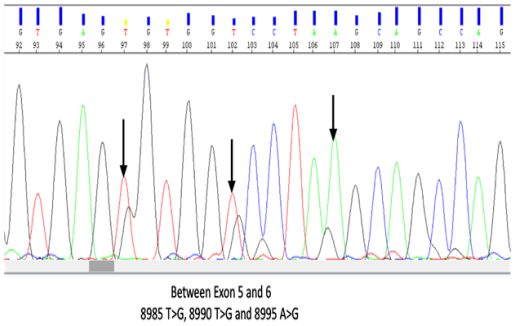

Figure 3. A Representative Electropherogram of IRF-1 Gene Sequencing (Non-Coding Region). An electropherogram of IRF-1 sequencing showing genotypic changes in non-coding region between exon 5 and 6.

variants were spotted between exon 5 and 6 (8985T>G, 8990T>G and 8995A>G), all of which were significantly correlated with leukemia (p=0.008) as represented in Figure 3.

IRF-1 gene mutations and genomic changes and their correlation with leukaemia

The correlation of the two-point mutations in the coding region of IRF-1 gene and hematologic malignancies was investigated. However, there was no correlation between the mutations and hematologic malignancy cases in this study (Table 3). On the other hand, the three mutantation sites between exon 5 and 6 were found to be significantly associated with the hematologic malignancies (Table 4). The potential relationship between those mutantation sites and the routine blood investigations were further investigated. Correlation analysis confirmed lack of any significant association between the presence of either mutantation sites and all blood indices (Table 5).

Table 4. Correlation Analysis between Genetic Changes in IRF-1 Noncoding Area and Hematologic Malignancies in a Random Sample of Leukemic Patients (N=22)

Variables	Total		P-value
	CML	N-CML	
Between Exon 5&6			0.008*
8985T>G			
Hetero Intronic			
M	6	2	
NM	2	12	
Between Exon 5&6			0.008*
8990T>G			
Intronic			
M	6	2	
NM	2	12	
Between Exon5&6			0.008*
8995A>G			
Intronic			
M	6	2	
NM	2	12	
Between Exon 6&7			0.704
9333A>G			
Intronic			
M	3	5	
NM	5	9	
Between Exon 6&7			0.704
9394T>C			
Intronic			
M	5	10	
NM	3	4	
Between Exon 7&8			1
9573G>A			
Intronic			
M	4	7	
NM	4	7	
Between Exon 7&8			0.704
9600A>C			
Intronic			
M	5	10	
NM	3	4	
Between Exon7&8			0.854
9678G>A			
Intronic			
M	5	9	
NM	3	5	
Between Exon 9&10			0.273
11486A>G			
Intronic			
M	0	3	
NM	8	11	

Table 4. Continued

Variables	Total		P-value
	CML	N-CML	
Between Exon 9&10			0.573
11489G>A			
Intronic			
M	6	10	
NM	2	4	
Between Exon 9&10			0.273
11492A>G			
Intronic			
M	0	3	
NM	8	11	
Between Exon 9&10			0.485
11496A>G			
Intronic			
M	6	11	
NM	2	3	
Between Exon 9&10			0.5
11666C>T			
Intronic			
M	5	10	
NM	3	4	
Between Exon 9&10			0.627
11668A>G			
Intronic			
M	5	9	
NM	3	5	

Discussion

The accumulation of genetic aberration in tumor suppressor genes and/or oncogenes is believed to initiate malignancy in normal cells, where the exact abnormality in these genes determines the morphology, histology, and severity of the malignancy. In leukaemia, several chromosomal aberrations have been identified. For example, the Philadelphia chromosome t (9;22) has been strongly associated with poor prognosis in leukaemia (Nowell, 2007). However, the specific role of these loci in oncogenesis and progression of leukaemia remains unclear. Previous studies have associated genetic aberrations in chromosome 5 with pre-leukemic and leukemic cases, which is the same region where IRF-1 was mapped (Willman et al., 1993).This preliminary study was an attempt to recognize the potential contribution of IRF-1 gene loss to leukemogenesis in a random sample of Saudi patients. The data presented in (Table 1) revealed that CML was the most common type of leukaemia in the study samples and accounting for 36.4% of cases.

To investigate the possible changes in the coding area of the IRF-1 gene and its role in leukemogenesis; capillary sequencing of all coding exons and flanking non-coding intronic regions of the IRF-1 gene was performed on twenty-two blood samples from the study population.

Table 5. Relationships between the Three Mutant Sites Correlated with the Disease Status and Routine Blood Test (N=22)

variables	Between Exon 5&6 8985 T>G (8990T>G, 8995 A>G) Hetero Intronic		P-value
	NM	M	
WBC			0.351
Normal	5	9	
Abnormal	1	7	
RBC			0.121
Normal	0	14	
Abnormal	2	6	
Hgb			0.117
Normal	1	13	
Abnormal	3	5	
HCT			0.121
Normal	0	14	
Abnormal	2	6	
MCV			0.309
Normal	12	2	
Abnormal	5	3	
MCH			0.662
Normal	5	9	
Abnormal	4	4	
MCHC			0.527
Normal	13	1	
Abnormal	6	2	
RDW			0.309
Normal	2	12	
Abnormal	3	5	
PLT			0.183
Normal	5	9	
Abnormal	6	2	

*P<0.05, statistical significance; WBC, White blood cell; RBC, Red blood cell; Hgb, Hemoglobin; CT, Red blood cell specific volume; MCV, Erythrocyte mean corpuscular volume; MCH, Mean red cell hemoglobin; MCHC, Red cell hemoglobin concentration; RDW, Red blood cell distribution width; PLT, platelet

Sequencing data of the current study helped to identify two novel mutations in exon 4 Lys75Glu and 7 and Glu222Lys of the IRF-1 gene (Table 2). Statistical analysis showed that these mutations are not significantly correlated to a practical group of leukemia (Table 3).

It is well established that cis-acting proteins shuttled into the nucleus to control gene expression by interacting with DNA that requires amino acids in the interacting protein domain to being mainly positively charged to facilitate the interaction with the negatively charged DNA. The most conserved exons in IRF-1 are present in exon 2, 3, and 4; those exons code for the DNA binding domain of IRF-1, which makes a pressing need to attain proper amino acid sequence and charge (Cha et al., 1992). In this study, one novel mutation, such as Lys75Glu, results in replacing lysine; a positively charged amino acid, to glutamine, a negatively charged amino acid, and this could influence the IRF-1/DNA binding affinity. Of note, the identified mutation in exon 7 was opposite to that identified in exon 4 (Glu222Lys), and has been reported in multiple samples (Table 2 and Figure 2). Exon 7 lies within IRF-1 transactivation domain where computer based analysis predicated that this region is loop-helix-loop-sheet, and any disturbance of this core region could interfere with the ability of wild-type IRF-1 to effectively induce apoptosis and inhibit cellular growth, possibly, through the formation of inactive homodimers with wild type IRF-1 or by competing for target promoters (Kim et al., 2003). Thus, exon 7 is coding for a critical domain that maintains the IRF-1 tumor suppressing properties, however, no significant correlation was found between the identified mutations in exons 4 and 7 and the disease status. In the same context, other studies have reported mis-sense mutation in exon 2 of IRF-1 in gastric adenocarcinoma Met8Leu, which was proposed to cause impaired transactivation capabilities of IRF-1 (Nozawa et al., 1998). Furthermore, A4396G single nucleotide polymorphism has been identified and found to be frequently expressed in human breast cancer cell lines (Bouker et al., 2007). Polymorphisms in IRF-1 gene has been linked to non-cancer status such as atopy and asthma; two silent mutations, A4396G in exon 7 and G6355A in exon 9, have been reported in samples from atopic asthmatics; though, no significant correlation has been found between IRF-1 and asthma development (Noguchi et al., 2000).

In addition, this study identified 14 genetic changes in the non-coding area of IRF-1 (Table 4). Of these, three genetic changes 8985T>G, 8990T>G, and 8995T>G are between exon 5 and 6, and were significantly correlated to the disease status (P = 0.008). These genetic changes were found in approximately 36.4% of the cases included in this study (8 out of the 22) and in approximately 71% of the identified CML cases in this study. However, no significant correlation was found between these genetic changes and routine blood tests (Table 5). Therefore, it is proposed that these genetic changes may contribute towards IRF-1 inactivation via accelerated exon skipping, as accelerated exon skipping and production of splice variant of IRF-1 was recently reported as an inactivation mechanism of IRF-1, which promotes tumorigenesis (Harada et al., 1994). In concordance with this, five splice variants of IRF-1 lacking a combination of exons 7, 8, and 9 have been reported in cervical cancer (Lee et al., 2006). Furthermore, three different exon regions (2, 3, and 4) are prone to deletion in leukemia (Harada et al., 1994; Khan et al., 2011). These variants display neither DNA binding nor tumor suppressing activity, and thus, their contribution to leukemogenesis appears to be resulting from accelerated exon skipping. The identified genetic changes in this study could contribute to the process of revealing the mechanism of accelerated exon skipping as they lay within exon-intron boundaries. In addition, they might have an impact on the pathophysiology of leukemic disorders.

In conclusion, the identification of these novel mutations is a step on the road to design larger scale

studies for wider investigation of the potential impact of these mutations on leukemic patients. Likewise, a larger and more representative study could help to explain the significant correlation between genetic changes that were identified between exon 5 and 6 of IRF-1 and the disease status.

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