Study of *IRF*- 8 gene in Kashmiri population and its relation with Chronic Myeloid Leukemia

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CERTIFICATE

Certified that the work in the dissertation entitled **"Study of *IRF*-8 gene in Kashmiri population and its relation with Chronic Myeloid Leukemia"** has been carried out by Mr. Muzamil Ali under the joint supervision of Prof. Mohammad Afzal Zargar and Prof. Bashir Ahmad Ganai (Department of Biochemistry, University of Kashmir) and the work is suitable for the award of M.Phil. Degree in Biochemistry.

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DECLARATION

I, Muzamil Ali, declare that the work embodied in this dissertation entitled **"Study of *IRF-8* gene in Kashmiri population and its relation with Chronic Myeloid Leukemia"** has been carried out by me in the Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

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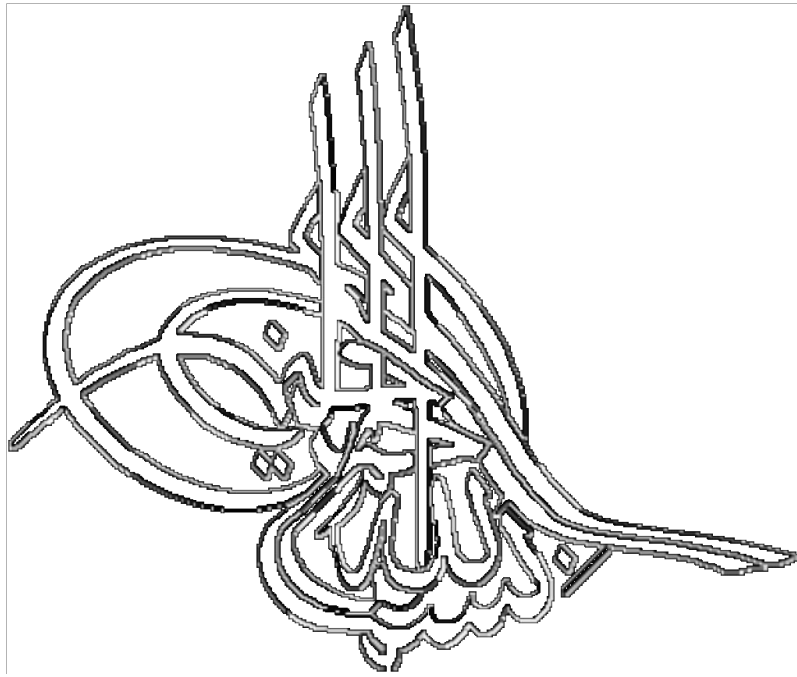
In the name of

Allah,

The most Compassionate,

The most merciful

Affectionately dedicated to my
beloved parents
and Abaan

ACKNOWLEDGEMENT

Abbreviations

µg	Microgram
µl	Microlitre
µM	Micromolar
ABL	Abelson murine leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
BCR	Breakpoint cluster region
bp	Base Pair
BPB	Bromophenol Blue
CGL	Chronic granulocytic leukemia
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
DDW	Double distilled water
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribosenucleotide –triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
Fig.	Figure
FISH	Flourescent in-sito hybridization
Gm	Grams

HHV-8	Human herpes virus-8
HSC	Hemopoietic stem cell
ICS	Interferon consensus sequence
ICSBP	Interferon consensus sequence binding protein
IFN	Interferon
IRF	Interferon regulatory factor
IRF-8	Interferon regulatory factor-8
ISRE	Interferon stimulated response element
Kb	Kilobase pair
KD	Kilo Dalton
LD	Linkage disequilibrium
M	Molar
METS	Mitogenic Ets transcriptional factor
mg	Miligram
min	Minutes
ml	Mililitre
mM	Mili molar
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NK	Natural killer

O.R	Odds ratio
ºC	Degree Celsius
PCR	Polymerase chain reaction
PCR	Polymerase Chain Reaction
PML	Promyelocytic leukemia
pmol	Picomole
rpm	Revolutions per minute
SNP	Single Nucleotide Polymorphsim
TAE	Trisacetic EDTA
Taq	Thermus Aquaticus DNA polymerase
TBE	Tris-Borate-EDTA
Tm	Melting Temperature
Tris	Tris(hydroxymethyl aminomethane)
UV	Ultra Violet

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ABSTRACT

Chronic myelogenous leukemia (CML) is a clonal hematopoietic disorder caused by an acquired genetic defect in a pluripotent stem cell. A number of theories have been postulated to describe the etiology of CML such as genetic alterations and alterations in cytokine production. A combination of inflammatory cytokines have an important role in cancer development. The aim of this study was to screen for mutations of IRF-8 gene in CML cases and healthy controls of the Kashmiri population. We included eighty confirmed CML cases and an equal number of age, district and gender matched controls in this study. HaeIII enzyme digestion cuts amplified product at 5'-GGCC-3' sequence and any mutation in it abrogates restriction digestion by this enzyme. Restriction results showed wild conditions with no mutation at any of the 6 positions where HaeIII cuts, which was confirmed by the sequencing results as well. Further sequencing results showed interesting single G→A substitution at position 92 of the amplified product. In CML cases, the allelic frequency for normal allele (G) was found to be 47.5% (76/160) and the allelic frequency observed for G→A type was found to be 52.5% (84/160). The allelic frequency observed in controls for normal allele (G) was 91.25% (146/160). The frequencies analyzed for G→A allele was 8.75% (14/160). Since the frequency observed for G→A allele was higher in CML cases (52.5%) than in normal controls (8.75%) and it was found to be statistically significant (OR= 11.52, 95%CI: (6.13-21.6); $p = 0.001$). Sequencing results further showed occasional deletion at the same position where transition was seen. Also we found that CML is prevalent more in males as compared to females (ratio is 1:1.12). Majority of the CML case were from district Srinagar of the Valley. Mean age of the cases and controls were found to be 44.7 years and 43.6 years respectively. We observed a higher representation of CML cases in the age group between 30 and 40. Furthermore, the presence of the Philadelphia chromosome (BCR-ABL fusion gene) was observed in 82.5% of CML cases in our study. This is the first report of the sequence variation in exon 7 region of IRF-8 gene and the risk to CML in the Kashmiri population. However, more insight need to be gained and further substantiated by conducting a similar study on statistically significant sample size.

Chronic myelogenous (or myeloid) leukemia (CML), also known as chronic granulocytic leukemia (CGL), is a clonal hematopoietic disorder caused by an acquired genetic defect in a pluripotent stem cell. A blood stem cell may become a myeloid stem cell or lymphoid stem cell. It is a form of clonal bone marrow stem cell disorder characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and thus accumulation of abnormal granulocytes (neutrophils, eosinophil's, and basophils) also called leukemic cells in the blood. The disease has the capacity to progress to a more-aggressive leukemia as a malignant clone loses the capacity for terminal differentiation. The leukemic cells can build up in the blood and bone marrow, so there is less room for healthy white blood cells, red blood cells and platelets. When this happens, infection, anemia or easy bleeding may occur **(Faderl et al., 1999)**. The number of blast cells in the blood and bone marrow and the severity of symptoms determine the phase of the disease. In the absence of intervention, CML typically begins in the *chronic* phase, and over the course of several years progresses to an *accelerated* phase and ultimately to a *blast crisis.* It is associated with a characteristic chromosomal translocation called the Philadelphia chromosome. In this translocation, parts of two chromosomes (the 9th and 22nd by conventional karyotypic numbering) switch places **(Faderl et al., 1999, Rowley et al., 1973)**. As a result, part of the BCR (breakpoint cluster region) gene from chromosome 22 is fused with the ABL gene on chromosome 9. This abnormal "fusion" gene generates a protein of p210 or sometimes p190. Because ABL carries a domain that can add phosphate groups to tyrosine residues (a tyrosine kinase), the BCR-ABL fusion gene product is also a tyrosine kinase **(Hehlmann et al., 2007)**. The BCR-ABL transcript is continuously active and does not require activation by other cellular messaging proteins. In turn, BCR-ABL activates a cascade of proteins that control the cell cycle, speeding up cell division. Moreover, the BCR-ABL protein inhibits DNA repair, causing genomic instability and making the cell more susceptible to developing further genetic abnormalities. This characteristic chromosomal abnormality can be detected by routine cyto-genetics, by fluorescent in-situ hybridization, or PCR for the BCR-ABL fusion gene **(Hehlmann et al., 2007)**.

The interferon regulatory factor (IRF) family, consisting of nine members in mammals, was identified in the late 1980's in the context of research into the type I interferon system **(Au et al., 1995)**. Indeed, many IRF members play central roles in the cellular differentiation of hematopoietic cells and in the regulation of gene expression in response to pathogen-derived danger signals. Moreover the role of several IRF family members in the regulation of cell cycle and apoptosis has important implications for understanding susceptibility to and progression of several cancers **(Tomohiko et al., 2007)**. IRFs also play an important role in pathogen defence, autoimmunity, lymphocyte development, cell growth and susceptibility to transformation. The IRF family includes IRF-1, IRF-2, ISGF3γ/p48, IRF-3, IRF-4 (Pip/LSIRF/ICSAT), IRF-5, IRF-6, IRF-7 and IRF-8/ICSBP. All IRF proteins share homology in their amino-terminal DNA binding domains and a divergent C-terminal region that serves as the regulatory domain. IRF family members regulate transcription through interactions with proteins that share similar DNA binding motifs, such as IFN-stimulated response elements (ISRE), IFN consensus sequences (ICS) and IFN regulatory elements (IRF-E) **(Honda et al., 2006)**. Interferon regulatory factor-8, also known as IRF-8 and ICSBP, encoded by IRF-8 gene, is a protein which in humans is a transcription factor of the interferon regulatory factor (IRF) family **(Huang et al., 2007)**. IRF-8/ICSCP is expressed predominately in hematopoietic cells and is further increased upon treatment with interferon **(Driggers et al., 1990, Weisz et al., 1992)**. IRF-8 can function as a transcription repressor of ICS-containing promoters **(Weisz et al., 1992)**. Expression of IRF-8 can lead to the down-regulation of the anti-apoptotic protein Bcl-2 **(Burchert et al., 2004)**. Originally described as being induced by IFN-γ, IRF-8 expression is also elevated by IRF-α as well as IL-12 in NK and T cells **(Lehtonen et al., 2003)**. IRF-8 is a critical regulator of myelopoiesis, which when deleted in mice results in a syndrome highly similar to human chronic myelogenous leukemia **(Seung-Hee et al., 2010)**. In human patients with CML and acute myeloid leukemia, IRF-8 expression is dramatically decreased **(Schmidt et al., 1998)**. These studies thus revealed that IRF-8 plays a pivotal role in regulation of leukemogenesis and functions as a tumor suppressor of certain myeloid malignancies. The molecular events involved in the control of leukemogenesis by IRF-8 are not fully understood. However, it has been shown that deficiency of IRF-8 in

hemopoietic cells leads to decreased spontaneous apoptosis and enhanced resistance to extrinsic apoptosis induction **(Burchert et al., 2004, Holtschke et al., 1996, Gabriele et. al., 1999)**, which suggests that acquisition of apoptosis resistance in myeloid cells might represent at least one of the molecular mechanisms involved in the pathogenesis of CML. Therefore, IRF-8 might function as a tumor suppressor through regulation of apoptotic cell death. Although no direct link between IRF-8 and inflammation-associated cancer promotion has been observed, IRF-8 has been shown to be involved in IFN-γ induced inducible NO synthase expression **(Xiong et al., 2003)**. Several other reports and studies of over two decades has now confirmed that transcription factors are commonly disrupted in CML either by their fusion as a result of chromosomal translocations or by point mutations.

CML accounts for approximately 15% of all leukemia's, with 4000 to 5000 new cases diagnosed in the United States annually. The incidence of CML is 1.6 to 2.0 cases per 100,000 persons per year, and the incidence is similar in all countries worldwide. No authentic study about the incidence of leukemia has been carried out so far, among the ethnic Kashmiri population. But as far as frequency of leukemia is concerned, it has been reported towards higher side. Main objective of the proposed work is to determine the mutations of IRF-8 gene in chronic myeloid leukemia (CML) and to generate information about role of IRF-8 gene in blood cancer susceptibility in Kashmir.

In the present study after the amplification of exon 7 region of IRF-8 gene, restriction digestion was followed by HaeIII enzyme, which cuts the amplified product at 5'-GGCC-3' sequence and any mutation in it abrogates restriction by the enzyme. For further confirmation, sequencing of both the CML case and control samples for any potential variation in the nucleotide sequence in the amplified product was done commercially. If we know the nature and position of mutation we will be able to benefit in the near future from different therapies that target specific mutations.

The term leukemia (Greek *leukos* - white, and *haima* - blood) coined by Rudolf Virchow in 1856, a renowned German pathologist, is a type of cancer of the blood or bone marrow characterized by an abnormal increase of white blood cells. Leukemia is a part of the broader group of diseases called hematological neoplasms.

An HSC can enter one of the two pathways –the lymphoid pathway or myeloid pathway to form common lymphoid progenitor cell or a common myeloid progenitor cell, respectively. If it forms the lymphoid progenitor, it can become either B progenitor or T progenitor. If the HSC becomes a myeloid progenitor, it can develop into erythrocyte progenitor, granulocyte-monocyte progenitor, eosinophils progenitor, basophil progenitor and megakaryocytic (Figure 1). The type of cell that has become cancerous, as well as the number of these cells, help to determine the type of leukemia. Clinically and pathologically, leukemia is subdivided into a variety of large groups. The four main types of leukemia are-

- **Acute lymphoblastic leukemia** (ALL) is the most common type of leukemia in young children.
- **Chronic lymphocytic leukemia** (CLL) most often affects adults over the age of 55.
- **Acute myelogenous leukemia** (AML) occurs more commonly in adults than in children, and more commonly in men than women.
- **Chronic myelogenous leukemia** (CML) occurs mainly in adults.

Chronic myelogenous leukemia (CML; also called *chronic myeloid leukemia* or *chronic granulocytic leukemia*) is a clonal hematopoietic disorder caused by an acquired genetic defect in a pluripotent stem cell. The disease has the capacity to progress to a more-aggressive leukemia as a malignant clone loses the capacity for terminal differentiation.

In CML, too many blood stem cells develop into a type of white blood cell called granulocytes. These granulocytes are abnormal and do not become healthy white blood cells. They may also be called leukemic cells. The leukemic cells can build up in the blood and bone marrow so there is less room for healthy white blood cells, red blood cells, and platelets. When this happens, infection, anemia, or easy bleeding may occur. CML is often divided into three phases based on clinical characteristics and

laboratory findings. In the absence of intervention, CML typically begins in the *chronic* phase, and over the course of several years progresses to an *accelerated* phase and ultimately to a *blast crisis.* These phases differ in number of blast cells ($\leq$ 10%, 10% to 20% and $\geq$ 20% respectively) in the blood and bone marrow and the severity of symptoms presented.

Most cases (85%) are diagnosed in the chronic phase; about 50% of cases are diagnosed by routine tests. Common findings at presentation are fatigue, weight loss, abdominal fullness, bleeding, purpura, splenomegaly, leukocytosis, anemia, and thrombocytosis, but about 40 percent of patients are asymptomatic, and in these patients, the diagnosis is based solely on an abnormal blood count. Three to five years after onset, CML progresses to the accelerated and blast phases (**Kantarjian et al., 1988**). The definition of the accelerated phase is vague. The blast phase is defined by the presence of 30 percent or more leukemic cells in peripheral blood or marrow or the presence of extramedullary infiltrates of blast cells (**Sokal et al., 1988**). In one third of cases, the blasts have a lymphoid morphology and express lymphoid markers such as terminal deoxynucleotidyl transferase or CD10 (common acute lymphoblastic leukemia antigen). The remaining two thirds of cases have a phenotype similar to that of acute myeloblastic leukemia and form a heterogeneous group (**Griffin et al., 1983**).

The disorderly expansion of myeloid progenitor cells appears to result from alterations in their proliferative capacity and a shift in the balance between self-renewal and differentiation toward differentiation, increasing the number of progenitor cells and reducing the pool of stem cells. Stem cells become part of the proliferating compartment, causing the neoplastic cell population to expand exponentially in later maturational compartments, where they may also be less responsive to growth-regulatory signals from either cytokines or the bone marrow microenvironment (**Strife et al., 1988, Clarkson et al., 1993**).
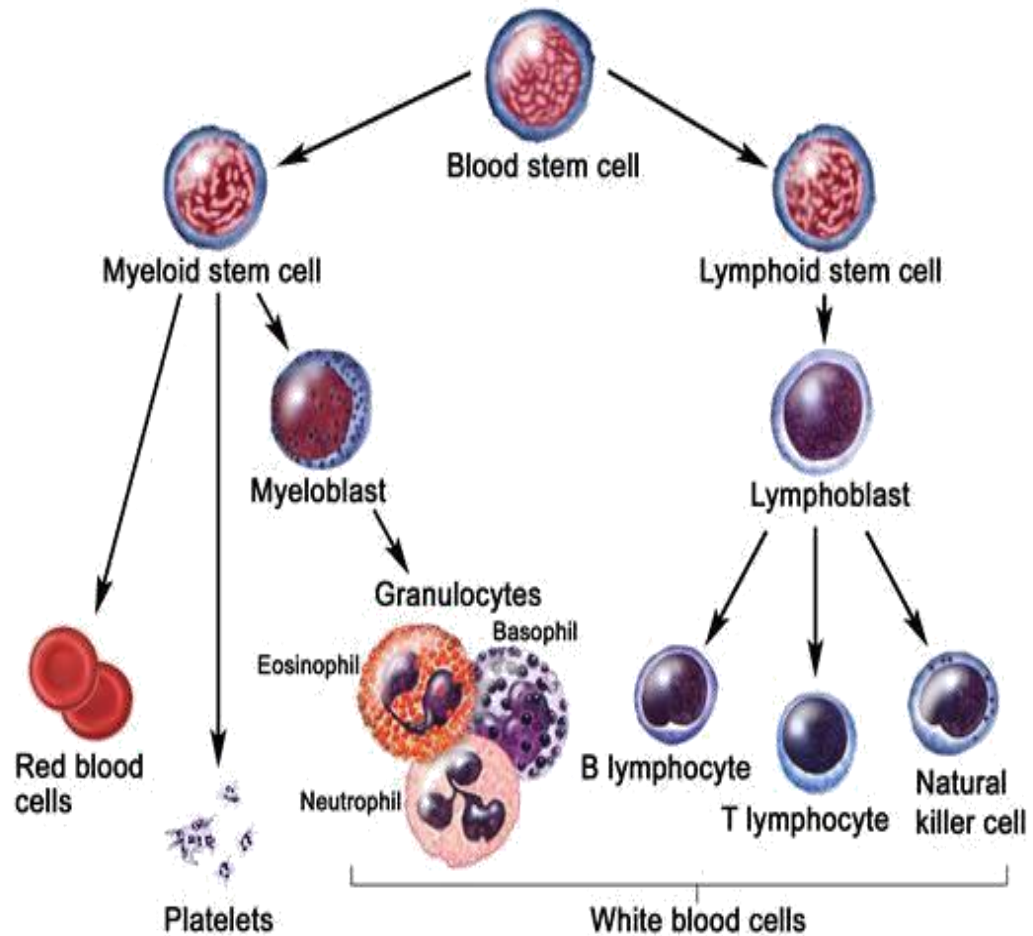
Figure 1: A blood stem cell goes through several steps to become a red blood cell, Platelet and white blood cell. (**Source**: www.health-reply.com)

2.1. The Biology of Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is part of a group of six myeloproliferative disorders in humans—that includes polycythemia Vera, chronic idiopathic myelofibrosis, essential thrombocythemia, chronic neutrophilic leukemia, and chronic eosinophilic leukemia—which are chronic diseases that can progress to acute leukemia **(Cortes et al., 2004, Beutler et al., 2001)**. CML is generally characterized by the cytogenetically detectable 9:22 translocation known as the Philadelphia (Ph) chromosome **(Faderl et al., 1999, Rowley et al., 1973)**. However, 5–8% of CML are Ph negative **(Kurzrock et al., 1988, Specchia et al., 1995)**. The Ph translocation adds a 3' segment of the ABL gene from chromosome 9q34 to the 5' part of the BCR gene on chromosome 22q11, creating a hybrid BCR–ABL gene that is transcribed into a chimeric BCR–ABL messenger RNA (mRNA) (Figure 2).

The ABL gene encodes a non-receptor tyrosine kinase with a molecular mass of 145kd (p145^{ABL}). It has 11 exons and spans over 230 kilobases (kb). The breakpoint in the ABL gene occurs usually 5' (toward the centromere) of exon 2 of ABL. The breakpoint locations within BCR fall either 5' between exons b2 and b3 or 3' (toward the telomere) between exons b3 and b4. A BCR–ABL fusion gene with a b2a2 or b3a2 junction is created and transcribed into an 8.5-kb mRNA. The fusion mRNA is translated into a chimeric protein of 210 kd called p210$^{BCR–ABL}$ **(Kurzrock et al., 1988)**. In most cases, CML cells have either b2a2 or b3a2 transcripts, but in 5 percent of cases, alternative splicing events allow the expression of both fusion products **(Melo et al., 1996)**.

Splicing out exons e1' and e2' forms a BCR–ABL transcript that is translated into a smaller BCR–ABL fusion protein of 190kd, termed p190 $^{BCR–ABL}$. A third breakpoint location in the BCR gene has been identified 3' from the M-BCR region between exons e19 and e20 (the μ-BCR); it translates into a fusion transcript with an e19a2 junction. The translation product is a protein of 230kd termed p230 $^{BCR–ABL}$ **(Pane et al., 1996)**. BCR–ABL transcripts cause factor-independent and leukemogenic cell growth in hematopoietic cell lines and can generate in mice a syndrome that closely resembles human CML **(Daley et al., 1990)**.
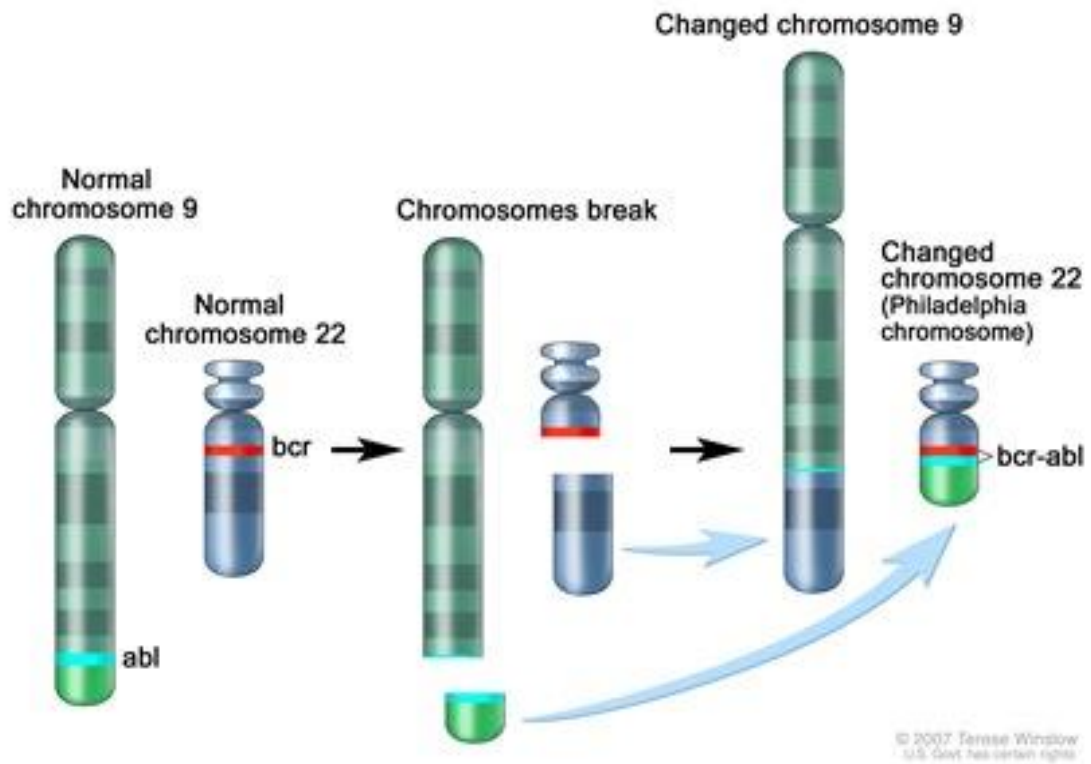
Figure 2: The translocation of t(9;22)(q34;q11) in CML. (**source: wikipedia**)

2.2. Pathways of BCR–ABL Signaling

ABL proteins are non-receptor tyrosine kinases that have important roles in signal transduction and the regulation of cell growth (**Wang et al., 1993**). The N-terminal segment of ABL includes two SRC homology domains (SH2 and SH3), which regulate the tyrosine kinase function of ABL, the catalytic domain, and a myristoylation sequence that connects ABL to proteins of the plasma membrane. Defects in the functional integrity of SH2 decrease phosphotyrosine binding and reduce the transforming capacities of ABL (**Gale et al., 1993**). SH3 has a negative regulatory effect on the tyrosine kinase function and deletion of SH3 facilitates transformation of ABL. The C-terminal part of ABL contains a DNA-binding domain, nuclear localization signals, and a binding site for actin (**Chung et al., 1996**). Various structural alterations of ABL and BCR facilitate the leukemogenic transformation of BCR–ABL.

The N-terminal coiled-coil motif of BCR increases its tyrosine kinase activity and enables binding of F-actin by ABL (**McWhirter et al., 1993**). The serine–threonine kinase domain of BCR activates signaling pathways mediated by ABL tyrosine kinase and $p210^{BCR–ABL}$ (**Reuter et al., 1994**). N-terminal fusion of BCR to ABL adds a large amino acid sequence to the SH2 segment of ABL (**Pendergast et al., 1991**). BCR interferes with the adjacent SH3 kinase regulatory domain, which in turn causes ABL to become constitutively active as a tyrosine phosphokinase. Both $p210^{BCR–ABL}$ and $p190^{BCR–ABL}$ have higher tyrosine phosphokinase activity than the normal ABL protein $p145^{ABL}$. The structure of $p210^{BCR–ABL}$ allows multiple protein–protein interactions and suggests the involvement of diverse intracellular signaling pathways. Several BCR domains serve to bind adapter proteins such as growth factor receptor–bound protein 2 (GRB2), CRK-oncogene–like protein (CRKL), casitas B-lineage lymphoma protein (CBL), and SRC homology 2–containing protein (SHC) (**Puil et al., 1994**). The SH2 domain of GRB2 binds to a conserved tyrosine residue (Y177) of BCR in $p210^{BCR–ABL}$. This links $p210^{BCR–ABL}$ to RAS, a guanosine triphosphate–binding protein involved in the regulation of cell proliferation and differentiation and at the core of the most prominent signaling pathway in the pathogenesis of CML (**Sawyers et al., 1995**). Signaling events downstream of RAS are not well characterized and may involve mitogen-activated protein kinases (MAPKs), such as the JUN kinase (JNK) pathway

(**Raitano et al., 1995**). Signaling cascades of p210$^{BCR–ABL}$ not involving RAS, such as c-Myc, have been identified**, (Afar et al., 1994)** but their role in the pathogenesis of CML is unclear.

2.3. The Cellular Biology of CML and Clinical Features

CML is a myeloproliferative disorder. Myeloid progenitor cells expand in various stages of maturation, are released prematurely into the peripheral blood, and home to extramedullary locations. Stem cells become part of the proliferating compartment, causing the neoplastic cell population to expand exponentially in later maturational compartments, where they may also be less responsive to growth-regulatory signals from either cytokines or the bone marrow microenvironment (**Strife et al., 1988, Clarkson et al., 1993**). Defective adherence of immature hematopoietic CML progenitors to marrow stromal elements may facilitate their release into the blood (**Gordon et al., 1984**). Normal hematopoietic progenitor cells adhere to the extracellular matrix or to immobilized growth-regulating cytokines. The attachment is mediated by cell-surface receptors on the progenitor cells, especially integrins. Integrins are cell-surface glycoproteins composed of two subunits, α and *β*. Whereas, the α- chain determines ligand specificity, the *β* chain initiates signal transduction pathways after binding to the ligand (**Verfaillie et al., 1997**). This signaling results in the recruitment of cytoskeletal adhesion proteins, the activation of adapter proteins, and the RAS– MAPK pathway (**Schlaepfer et al., 1994**). Defective cyto-adhesion of CML cells has been restored by pre-incubation of Ph-positive cells with antisense Oligonucleotides against p210 $^{BCR–ABL}$, tyrosine kinase inhibitors targeted against p210 $^{BCR–ABL}$, and treatment with interferon alfa (**Verfaillie et al., 1998**).

The suppression of pathways of programmed cell death, or apoptosis, has been implicated in the pathogenesis of CML. Hematopoietic progenitor cells that express p210$^{BCR–ABL}$ are able to escape dependency on growth factors and can withstand the noxious effects of cytotoxic drugs and irradiation (**Sirard et al., 1994 and McGahon et al., 1994**). The activation of anti-apoptotic mechanisms seems to depend on the phosphotyrosine kinase activity of p210$^{BCR–ABL}$ in addition to other structural domains of the fusion protein, including adapter protein-binding and phosphorylation sites (**Cortez et al., 1995**).

The expression of specific cytokine profiles may increase the expansion of CML progenitor cells. Serum from patients with CML can stimulate the proliferation of hematopoietic colony-forming cells **(Brown et al., 1986)**. Marrow cells from patients with CML who have advanced disease produce large amounts of interleukin-1*b*, and inhibition of interleukin-1*b* by either interleukin-1–receptor antagonists or soluble interleukin-1 receptors inhibits the proliferation of CML cells **(Estrov et al., 1991)**.

2.4. Diagnosis and Monitoring of CML

CML is often suspected on the basis on the complete blood count, which shows increased granulocytes of all types, typically including mature myeloid cells. Basophils and eosinophils are almost universally increased; this feature may help differentiate CML from a leukemoid reaction. A bone marrow biopsy is often performed as part of the evaluation for CML, but bone marrow morphology alone is insufficient to diagnose CML **(Tefferi et. al., 2006, Hehlmann et. al., 2007)**. Ultimately, CML is diagnosed by detecting the Philadelphia chromosome. This characteristic chromosomal abnormality can be detected by routine cytogenetics, by fluorescent in situ hybridization, or by PCR for the BCR-ABL fusion gene. However, the procedure is tedious and time consuming, and only 20 to 25 cells in metaphase are examined per sample.

Genomic polymerase-chain-reaction (PCR) and Southern blot analysis can determine the exact breakpoints of DNA fusion products. Reverse-transcriptase PCR (RT-PCR) and Northern blot analysis allow detection of BCR–ABL transcripts at the RNA level. The $p210^{BCR–ABL}$ protein can be demonstrated by using antibodies against the N-terminal region of BCR and the C-terminal region of ABL in immuneprecipitation or Western blot analysis **(Guo et al., 1991)**. Further Quantitative Southern-blot analysis is a powerful molecular tool for initial diagnosis and monitoring of CML patients during IFN-α therapy **(Skladny et al., 2010)**.

Monitoring patients who are receiving therapy is commonly done by PCR and fluorescence in situ hybridization for BCR–ABL. Quantitative RT-PCR is used for follow-up of patients after stem-cell transplantation **(Hochhaus et. al., 1998)**, but its use in that receiving interferon alfa therapy is more controversial. Fluorescence in situ hybridization allows analysis of both cells in metaphase and non-dividing cells in

Interphase, and the results are easily quantifiable. Peripheral-blood specimens can be analyzed by Interphase fluorescence in situ hybridization (averting the need for marrow aspiration). It is fast, allows analysis of more cells than is possible with conventional cytogenetic methods, and is reliable in assessing cytogenetic responses in CML (**Muhlmann et al., 1998**). However; it has a false positive rate of up to 10 percent and is not useful if fewer than 10 percent of cells contain a Ph chromosome. Hypermetaphase fluorescence in situ hybridization allows analysis of up to 500 cells in metaphase per sample in a time efficient manner and produces no false positive results, but it cannot be performed on peripheral blood samples (**Seong et al., 1995**). Another technique of fluorescence in situ hybridization that is applicable to blood samples uses double-color probes for the detection of Ph-chromosome–positive leukemias and has shown superior sensitivity and specificity (**Dewald et al., 1998, Buno et al., 1998**).

The detection of the BCR–ABL translocation is not only a diagnostic tool but also useful for assessing the response of patients to therapy with either stem-cell transplantation or interferon alfa and for evaluating the efficacy of treatment by monitoring residual disease. In most centers, PCR, because of its superior sensitivity, has become the diagnostic test of choice for monitoring residual leukemia. Cytogenetic relapse usually precedes hematologic relapse, and effective salvage therapy is available for patients whose relapse is detected early. Whereas 80 percent of patients in hematologic remission after stem-cell transplantation are negative for BCR–ABL transcripts by PCR, almost all patients treated with interferon alfa have these transcripts in their bone marrow (**Hochhaus et al., 1995**). If followed for a sufficiently long time, however, some patients treated with interferon alfa who have cytogenetically complete responses eventually have PCR tests that are negative for BCR–ABL (**Kurzrock et al., 1998**). The importance of PCR results for the predictability of relapse and survival is unclear, however, and dormant CML progenitor cells that are below the threshold of detection by PCR may still be present.

Monitoring the percentage of Philadelphia chromosome-positive cells is the best validated system for the assessment of the response to interferon-α and tyrosine kinase inhibitors, since the cytogenetic response is the best surrogate marker of survival (**O'Brien et al., 2003, Goldman, 2007**). For patients who achieve a complete

cytogenetic response to interferon-α, the 10-year survival is about 75% (**Baccarani et al., 2003**). For patients who achieve a complete cytogenetic response to imatinib, the 5-year survival rate is close to 100% (**Druker et al., 2006**) .The response is conventionally determined by chromosome banding analysis of marrow cell metaphases. A panel of experts appointed by the European LeukemiaNet recommended that at least two cultures should be performed, one for 24 hours and another for 48 hours (**Haferlach et al., 2007**).

2.5. Disease Transformation and Oncogene Cooperation

Disease transformation is often heralded by refractoriness to treatment, leukocytosis with increases in blood and marrow blasts, basophilia, increases or decreases in platelet counts unrelated to therapy, and clinical manifestations such as unexplained fever, splenomegaly, extramedullary disease, weight loss, and bone and joint pains. Cytogenetic and molecular changes occur in 50 to 80 percent of patients during the transition to the accelerated and blast phases. Minor cytogenetic changes include Monosomies of chromosomes 7, 17, and Y; Trisomies of chromosomes 17 and 21; and translocation t(3;21)(q26;q22) (**Mitelman et al., 1993**). Major changes include trisomy 8, isochromosome i(17q), trisomy 19, and an extra Ph chromosome (double Ph). Trisomy 8 is most common, and isochromosome i(17q) occurs almost exclusively in the myeloid type blast phase.

Molecular abnormalities may correspond to cytogenetic changes. These include abnormalities in p53 (on chromosome 17p13); RB1 (13q14); c-MYC (8q24); $p16^{INK4A}$ (9p21); RAS; and AML–EVI-1, a fusion protein resulting from translocation t(3;21) (q26;q22). Alterations of p53 (deletions, rearrangements, and mutations) occur in 20 to 30 percent of patients with CML in the blast phase (**Ahuja et al., 1989**) and are associated exclusively with myeloid transformation, (**Stuppia et al., 1997**) where as abnormalities of *RB1* are associated more with lymphoid transformation, although the association is weaker than it was between p53 and myeloid transformation. Mutations of p53 in the progression of CML are associated with an aberrant methylation status of CML cells (**Guinn et al., 1997**). The introduction of a methyl group causing transcriptional silencing of the calcitonin gene has been found in the transition of chronic-phase CML to blast-phase CML (**Malinen et al., 1991**). Altered methylation

was also described within the M-bcr of cells from patients with chronic-phase CML (**Litz et al., 1996**). Up to 50% of patients with lymphoid transformation have homozygous deletion of p16^{INK4A} (**Guo et al., 1991**). Alterations of RB1, amplifications of c-MYC*,* and mutations of *RAS* are less frequent.

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation and immune activation (**Vilcek et al., 1992**). The biomodulatory activities pertinent to this group of cytokines have been extensively exploited at the clinical level, and are used in therapy for many hematological malignancies and multiple sclerosis (**Gutterman et. al., 1994**). They act by binding their receptors, which leads to subsequent phosphorylation events and the association of activated transcription factors with different response elements in the promoter regions of IFN-regulated genes. Interferon alfa/beta (type I IFNs) and interferon gamma (type II IFN) mediate their action through distinct pathways and thus regulate various genes (**Pestka et al., 1987, Tanaka et al., 1992, Darnell et al., 1994, Schindler et al., 1995**). IFNs elicit their effects through the transcriptional activation of target genes that possess specific consensus DNA-binding recognition sites within their promoters. These genes are regulated through the JAK–STAT signaling pathway and through the interferon regulatory factors (IRFs), a growing family of transcription factors with a broad range of activities (**Nguyen et al., 1997**).

2.6. The Family of Interferon regulatory factors (IRFs)

A family of transcription factors, the interferon regulatory factors (IRF), was identified originally in the context of the regulation of the type I interferon (IFN)-α/β system. The original discovery of the first two members of the interferon (IFN) regulatory factor (IRF) family, IRF-1 and IRF-2, opened up new avenues of research in immunity and oncogenesis, which we may call 'the IRF world'. The transcription factor IRF-1 was identified originally as a regulator of the IFN system (**Miyamoto et al., 1988**). Following this at least nine structurally related members have been identified thus far, and currently constitute a family of IRF transcription factors which includes IRF-1, IRF-2, ISGF3γ/p48, IRF-3, IRF-4 (Pip/LSIRF/ICSAT), IRF-5, IRF-6, IRF-7 and IRF-8/ICSBP. These function to regulate interferon (IFN) and IFN-inducible gene expression in response to viral infection (**Taniguchi et al., 2001**). They play an

important role in pathogen defense, autoimmunity, lymphocyte development, cell growth and susceptibility to transformation. Loss of expression or function of IRF is observed in human cancers, whereas a certain IRF member is over expressed in hematological malignancy. Interestingly, human herpes virus (HHV)-8 encodes several proteins, termed vIRF, that are analogous to human IRF proteins and may be involved in the pathogenesis of Kaposi's sarcoma or other cancers **(Moore et al., 2003, Offermann et al., 2007)**. In the context of oncogenesis, we can therefore categorize several IRF family members into two types: antioncogenic IRF and oncogenic IRF. All IRF proteins share homology in their amino-terminal DNA binding domains which is characterized by having a winged-type helix-loop-helix motif with a signature tryptophan pentad. IRF family members regulate transcription through interactions with proteins that share similar DNA binding motifs, such as IFN-stimulated response elements (ISRE), IFN consensus sequences (ICS) and IFN regulatory elements (IRF-E) **(Honda et al., 2006)**. The secondary structures of the DNA-binding domains of IRF are similar to each other, suggesting that IRF members recognize similar, if not identical, DNA sequences. The C-terminal portion varies among these members and promotes versatile biological functions. In addition to their intrinsic transactivation potential, some IRF acquire a specific function by associating with another IRF member, other transcriptional factors, or cofactors. In addition, their transcriptional activities vary, resulting in activation, repression, or dual activity on their target genes. This is partly attributed to the partner proteins associated with IRF. These interactions are mediated by two types of association module of the C-terminal region: (1) IRF-associated domain-1 **(Sharf et al., 1997)**, which was initially found in IRF-8 and is conserved in all IRF (excluding IRF-1 and IRF-2); and (2) IAD2, which is shared only by IRF-1 and IRF-2. In most cases, these protein complexes enhance the ability of IRF to bind to target DNA sequences such as ISRE or IRF-E. For example, IRF-9 acts as a DNA-binding subunit that associates with STAT1 and STAT2 to form the ISGF3 heterotrimeric complex in response to type I IFN signalling **(Takaoka et al., 2006)**. IRF8 forms multiple protein complexes with both IRF-1 and IRF-2, resulting in increased binding activity to ISRE **(Sharf et al., 1997, Bovolenta et al., 1994)** IAD2 of IRF-1 and IRF-2 is an independent module for this interaction with IRF-8. The IRF-8

and IRF-1 complex generally functions as a suppressor of transcription. IRF-4 and IRF-8 interact with PU.1, a member of the ETS family, and this interaction allows them to bind to the immunoglobulin light-chain enhancer λB **(Brass et al., 1996, Eisenbeis et al., 1995)** for the subsequent activation of gene transcription. On the other hand, IRF-2, IRF-4, IRF-8, and IRF-7 suppress transcription from several ISRE promoters **(Harada et al., 1990, Nelson et al., 1993, Yamagata et al., 1996)**. However, these IRF also function as activators in other promoters **(Eisenbeis et al., 1995, Vaughan et al., 1995)**. IRF transcriptional activities are varied, resulting in either activation or repression. This variability enables classification of IRF proteins into three categories : activators (IRF-1, IRF-3, IRF-7, IRF-9), repressors (IRF-2, IRF-8) and those that are able to both, activate and repress gene transcription, depending on the target gene (IRF-2, IRF-4, IRF-5 and IRF-8) **(Shellacs et al., 2004)**.

2.7. The Interferon regulatory factor 8 (*IRF-8*)

Interferon regulatory factor-8, also known as IRF-8 and interferon consensus sequence binding protein (ICSBP), encoded by IRF-8 gene (located on long arm of chromosome 16) (Figure 3), is a protein which in humans is a transcription factor of the interferon regulatory factor (IRF) family **(Huang et al., 2007)**. Originally described as being induced by IFN-γ, IRF-8 expression is also elevated by IRF-α as well as IL-12 in NK and T cells **(Lehtonen et al., 2003)**. IRF-8 deficient mice have enhanced susceptibility to various pathogens and impaired production of interferons, as well as deregulated hematopoiesis that resembles chronic myelogenous leukemia **(Holtschke et al., 1996)**. It was reported that IRF-8 is expressed predominantly in hematopoietic cells, such as cells of myeloid and lymphoid lineages, and its gene expression is upregulated by IFN-γ. Because IFN-γ is a pivotal cytokine that is crucial for the clearance of not only virally infected cells but also cancerous cells, it can be presumed that IRF8 regulates tumor development. Of note, IRF-8 deficient mice exhibit marked expansion of granulocytes followed by a fatal blast crisis, which is quite similar to human CML **(Holtschke et al., 1996)**, a disease known to be caused by the constitutive kinase activity of the BCR-ABL (breakpoint cluster region-Abelson murine leukemia) oncoprotein. Particularly worth noting is that the IRF-8 expression level decreases markedly in CML and acute

myelogenous leukemia cells from patients **(Schmidt et al., 1998)** and that a return to normal levels was observed in patients in remission following treatment with IFN-γ. IRF-8 –/– myeloid progenitor cells have defects in both differentiation and growth. IRF-8 drives their differentiation toward macrophages whereas it inhibits granulocytic differentiation **(Scheller et al., 1999, Tamura et al., 2000)**. Moreover, IRF-8 inhibits myeloid cell growth and promotes apoptosis **(Tamura et al., 2000, Gabriele et al., 1999)**. Thus, the loss of IRF-8 results in the accumulation of granulocytes, and then presumably an additional genetic hit or hits in the progenitor cells causes clonal expansion of undifferentiated cells (i.e. blast crisis). Concerning the target genes of IRF-8, one report shows that some of these IRF-8 effects may be explained in part by an IRF8-mediated repression of bcl-2, a major antiapoptotic target of BCR/ABL, on a transcriptional and protein level **(Burchert et al., 2004)**. The results of another group indicate that some of the myeloleukemia suppressor activities of IRF-8 are mediated through the regulation of promyelocytic leukemia (PML), which is a tumor suppressor that serves as a scaffold protein for nuclear bodies **(Dror et al., 2007)**. In addition, IRF-8 has been shown to inhibit the growth of p210 BCR/ABL transformed myeloid progenitor cells. IRF8 suppresses c-Myc expression at least in part by direct activation of B-lymphocyte induced maturation protein-1 (Blimp-1) and mitogenic Ets transcriptional suppresor (METS), which may explain the mechanism of growth arrest induced by IRF-8 **(Tamura et al., 2000)**. The antagonistic role of IRF-8 against BCR/ABL is also supported by evidence that IRF-8 can ameliorate BCR/ABL-mediated murine myeloid leukemia *in vivo* **(Hao et al., 2000)**. These data indicate that the loss of IRF-8 expression may be a major event leading to the development of human CML, and that the restoration of IRF-8 expression can antagonize the oncogenic activity of BCR/ABL. In addition to the effect of IRF-8 in hematopoietic tumors described above, this factor has also been shown to manifest antitumor activity even in solid tumors. IRF-8 expression was found to be repressed by DNA methylation in human metastatic colon carcinoma cell lines and murine mammary carcinoma with lung metastasis *in vivo* **(Yang et al., 2007)**. It has been further shown that the overexpression of IRF-8 enhances apoptosis of cancer cells, whereas the disruption of IRF-8 function diminishes primary tumor cell sensitivity to apoptosis and can convert a poorly metastatic tumor to

a metastatic phenotype. Interferon regulatory factor 8 appears to exert its antileukemic activity not only by the direct control of cell growth, differentiation, and apoptosis but also by modulating antitumor immunity. Indeed, the coexpression of IRF-8 in BCR/ABL transformed BaF3 cells causes a CD8+ cytotoxic T-cell response to prevent the establishment of leukemia *in vivo* (**Deng et al., 2001**). Furthermore, human CML cells are sensitive to T cell-mediated immunity (**Lim et al., 1997**). Given the roles of IRF-8 in macrophages and DC, IRF-8 may also elicit antitumor immunity through its ability to support the differentiation and function of antigen-presenting cells.

IRF-8 and IRF-4 show a high degree of homology. They are expressed primarily in lymphocytes, macrophages, B cells and DC (**Eisenbeis et al., 1995, Politis et al., 1992**). These two proteins demonstrate only a weak DNA binding affinity, which can be increased by association with other transcription factors (**Marecki et al., 1999, Tailor et al., 2006**). Similar to IRF-8 and in contrast to its oncogenic activity in lymphoid cells, IRF-4 expression was shown to be down-regulated in patients with CML but restored in response to treatment with IFN-γ. Patients with higher IRF-4 expression had better responses to IFN-γ therapy (**Schmidt et al., 2000**). Despite many similarities in structure and function between IRF-4 and IRF-8, the described phenotype of IRF-4–deficient mice is of deficient B and T lymphocyte function (**Mittrucker et al., 1997**) and failure of development of certain dendritic cell subsets (**Tamura et al., 2005**), in contrast to the primarily myeloid phenotype seen in IRF-8-deficient animals. The deficiencies of IRF-4 and IRF-8 can cooperate in the development of both myeloid and lymphoid tumors. In B-cell development, IRF-4 and IRF-8 function redundantly at the pre-B cell development, IRF-4/8 double, but not single, deficiencies lead to blocking the transition from large, cycling pre-B cells to small, resting pre-B cells (**Lu et al., 2003**). IRF-4 and IRF-8 also have overlapping function in the myeloid system as the mice lacking both IRF-4 and IRF-8 develop, from a very early age, a much more aggressive CML-like disease than those lacking IRF-8 alone (**Tsujimura et al., 2002**).
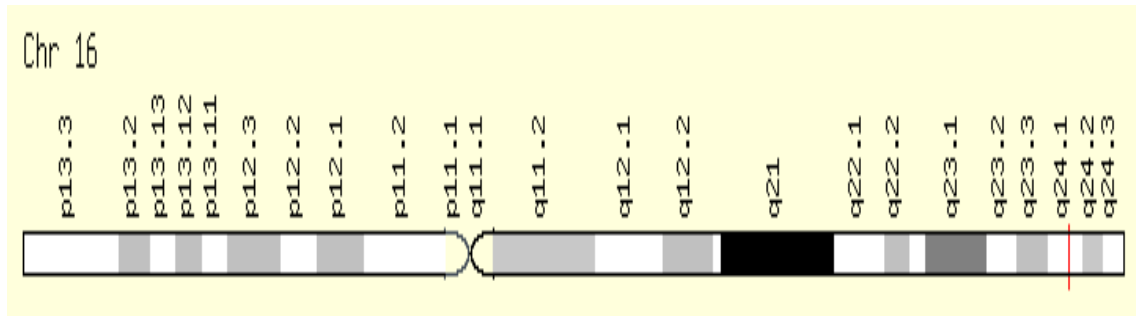
Figure 3: Schematic representation of *IRF-8* gene. (Source: **www.ncbi.net**)

Material and Methods

3.1. Study population

Patients with Chronic Myeloid Leukemia were evaluated at the department of Hematology of Sheri-Kashmir Institute of Medical Sciences (SKIMS), Soura. Clinical details were obtained by going through the medical records of the cases. The diagnoses of CML were based on the standard clinico-hematological criteria and the presence of Philadelphia chromosome (BCR-ABL fusion gene). Controls were taken from healthy individuals of Kashmir valley from Department of Hematology, SKIMS, Soura. Generally, Patients coming for treatment of minor ailments at the SKIMS Hospital, with no evidence of cancer were selected as controls.

3.1.1. Inclusion and Exclusion Criteria

These parameters are important part of any human study and are determined prior to commencement of the study. Following exclusion and inclusion criteria were adopted for the study.

3.1.1.1 Cases

No restrictions were made amongst the patients for cancer stage, gender or age.

Inclusion criteria

- Subjects should be from Kashmir population.
- CML proven cases by clinico-hematological and cytogenetic studies.
- Complete clinical history

Exclusion Criteria

- Patients below the age of 15 years were not included.
- Patients suffering from any chronic, debilitating disease and other blood disorders were not taken.

3.1.1.2 Controls

Controls were matched to the cases by gender and age.

Inclusion criteria

- Subjects not suffering from CML.
- Subjects should be from Kashmir population.
- Absence of any other blood disorders

3.2. Blood samples

Blood was collected from CML patients enrolled at Department of Hematology, SKIMS Soura. Clinical details were obtained by going through the medical records of the cases. About two ml (2 ml) peripheral blood of CML patient and normal control was collected in an EDTA coated sterilized plastic vials and stored at -20°C for further use. Proper consent was taken from all the subjects. Demographic characteristics of the cases and controls recruited for Study are shown in table 1:

Table 1: Demographic characteristics of the cases and controls recruited for study.

	Cases (80)	Controls (80)
Mean Age(years) **± SD**	44.78 ±13.61	31.83 ± 7.52
Males *n* (%) **Mean Age ± SD**	44 (55.0) 47.31 ± 14.43	56 (70.0) 31.33 ± 8.06
Females *n* (%) **Mean Age ± SD**	36 (45.0) 41.22 ± 11.14	24 (30.0) 33.00 ± 6.12

n= number of individuals
value represents ± SD

3.3. Chemicals used for the Study

Chemicals/Enzymes	Source
100bp Ladder	Sigma
Acetic acid	Qualigens
Agarose low EEO	Sisco Research Lab.Pvt.Ltd
Ammonium chloride	D.D. Fine Chem.Pvt.Ltd
Bromophenol blue	Himedia
Chloroform	Qualigens
dNTPs	Sigma
EDTA-disodium	CDH
Ethanol	Jiangsu Huaxi International Co.Ltd
Ethidium bromide	Himedia
HaeIII	Fermentas
Isoamyl alcohol	Qualigens
Isopropanol	Qualigens
Magnesium chloride	Sisco Research Lab
Oligonucleotides	Sigma
Phenol	Qualigens
Proteinase K	Sigma
SDS	Sigma
Sodium acetate	S.D. fine chem.pvt ltd
Sodium chloride	Sisco Research Lab.Pvt.Ltd
Sodium hydroxide	RANKIN
Sucrose	Sisco Research Lab
Taq DNA polymerase	GENETAQ^{TM}

Tris – base   Himedia

Tris-HCl   Himedia

3.4. Reagents

3.4.1. Reagents for DNA extraction

Chloroform isoamyl alcohol (CIA)

Chloroform   24.0 ml

Isoamyl alcohol   1.0 ml

Chloroform: isoamyl alcohol, in the ratio 24:1 was prepared by mixing 24 ml of Chloroform and 1 ml of isoamyl alcohol. The solution was stored at 4°C in dark bottle.

DNA storage buffer:

0.5 M EDTA   0.01 ml

1 M Tris   0.5 ml

Final volume was made 50 ml with sterile distilled water.

Lysis buffer

1 M Tris   2.0 ml

0.5M   EDTA   400 µl

10% SDS   30.0 ml

Final volume of the solution was made 100ml with sterile distilled water.

Lysis solution

1 M Ammonium chloride   15.5 ml

1M Potassium bicarbonate   1.0 ml

0.5 M EDTA   200 µl

Final volume was made 100ml with sterile distilled water.

Proteinase K

Proteinase K   10 mg

Proteinase k was dissolved in 1 ml of deionized water and stored at -20˚C in aliquots of 1 ml each.

SE Solution

5 M Sodium chloride   3 ml

0.5 M EDTA   8 ml

Final volume was made 200ml with sterile distilled water. pH was adjusted to 8.

SDS (10%)

SDS   10 g

SDS was dissolved in 100 ml sterile distilled water.

Sodium acetate (3M)

Sodium acetate   40.83 g

Sodium acetate was dissolved in 100ml sterile distilled water; pH of the solution was adjusted to 5.0 using acetic acid.

Saturated phenol

0.2% β- mercaptoethanol

8-hydroxy quinoline

0.1 M Tris chloride buffer   800 ml

0.5 M Tris chloride buffer   1000 ml

Phenol   1000 ml

The mixture obtained by adding equal volume of 0.5M Tris-Cl buffer and melted phenol was stirred for 15 min on magnetic stirrer. Two phases were allowed to separate. Upper aqueous phase was removed and equal volume of 0.1 M Tris-chloride buffer was added. The saturation with 0.1 M Tris-Cl buffer was repeated till phenol >7.8 pH was

obtained. A pinch of β-mercaptoethanol and 8-hydroxy quinoline was added. Phenol was stored at 4˚C in a dark bottle.

3.4.2. Reagents for Agarose gel electrophoresis

Agarose (1%)

Agarose	1.0 g
Buffer	100 ml
EtBr	5.0 µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. EtBr was then added to the solution.

Bromophenol blue

Bromophenol blue	0.4 g
Sucrose	20.0 g

Bromophenol blue was dissolved in 100ml of distilled water.

From the above stock solution 31.25ml was taken and sucrose was added. Final volume was made 50ml with distilled water.

Ethidium bromide
Ethidium bromide	10 mg

Ethidium Bromide was dissolved in 1ml of distilled water. The solution was stored in a dark bottle at 4˚C.

50x TAE (pH 8.0)

Tris base	242 g
0.5M EDTA	100 ml
Glacial acetic acid	57.1 ml

Final volume was made 1000ml with distilled water.

1x TAE (pH 8.0)

50 x TAE   20 ml

Final volume was made 1000ml with distilled water.

3.4.3. Reagents for PCR

Stock

Deoxyribose nucleotide triphosphate (dNTP) 10mM each dATP, dGTP, dCTP and dTTP

Taq polymerase (5U/ml)

10x Taq buffer Primers: 100mM in sterile demonized water (Sigma)

100bp DNA ladder (100µg/ml)

3.5. Genetic Analysis

For the analysis of mutation, DNA was extracted from whole blood and was followed by PCR amplification of desired fragment. These amplified products were digested by Hae III enzyme and further analyzed by nucleotide sequencing.

3.5.1. DNA extraction by phenol-chloroform method

Blood samples (2ml) were obtained from the patients in the EDTA coated plastic vials and genomic DNA was isolated by Phenol-chloroform method **(Sambrook and Rusell, 2001)**.

1. First cells were lysed with 4ml of freshly prepared Lysis buffer (155mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) with gentle mixing and kept at -20^oC for 15 minutes.

2. The tubes were then centrifuged at 8000 rpm for 10 minute and then supernatant was discarded.

3. The pellet was resuspended in 2 ml of SE, 100µg/ml Proteinase K and 100 ml of 10% SDS. The solution was incubated at 37^oC overnight.

4. On the next day, equal volumes of Tris-saturated-phenol (pH 8) was added to the sample in the tube and mixed thoroughly by inverting the tube for 15-20 minutes.

5. The tubes were then centrifuged at 6000 rpm for 10 minutes. The aqueous supernatant was transferred to fresh tubes and to it was added an equal volume of phenol-CIA (chloroform-isoamyl alcohol).

6. The tubes were shaken gently for 15 minutes and then centrifuged at 7000 rpm for 10 minutes.

7. The supernatant formed was transferred to fresh tube and to it is added an equal volume of CIA.

8. The tubes were again shaken gently for 15-20 minutes and then centrifuged at 8000 rpm for 10 minutes.

9. To the supernatant was added an equal volume of chilled ethanol and 0.1 ml of sodium acetate, the DNA precipitated immediately.

10. After retrieving the DNA, it was washed thrice with 70% ethanol. DNA was then dissolved in 5 ml of DNA storage buffer and stored at $4^{o}C$ for future use.

3.5.2. Qualitative and Quantitative Analysis Genomic DNA

Qualitative Analysis

The quality of the genomic DNA was examined by gel electrophoresis using 0.8% agarose gel. Initially, 0.8g of agarose and 100ml of 1x TAE buffer were taken in conical flask and heated on the heating mantle for 5-10 min. After that 2μl of ethidium bromide (10mg/ml) were added and gel solution was poured into the tray and allowed to set for 20 minutes. The gel was submerged in 1x TAE buffer. 2μl of each DNA sample was mixed with 1μl of 1x DNA loading dye (1x loading dye consists of 4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose in 1ml water) and was loaded in the gel. Electric current was applied at 20 volt until DNA enters in to the gel and was raised to 50 volt for rest of the run. Run was stopped when the dye had travelled nearly two third of the gel. Gel was visualized by a Gel doc system (AlphaimagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system (Figure 4).
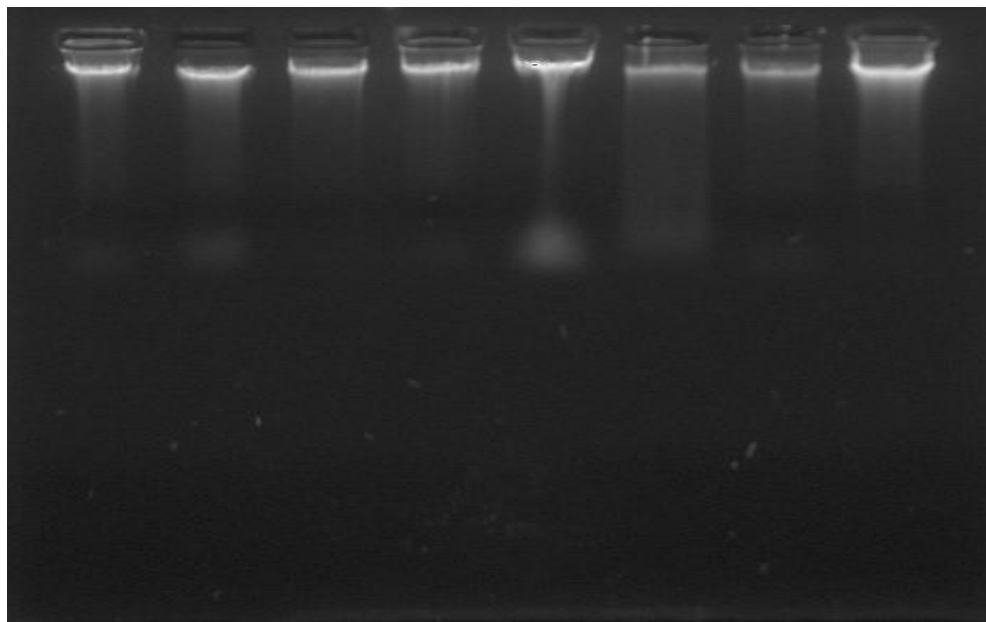
Figure 4: Representative photograph of the genomic DNA isolated from the human blood samples analyzed by 0.8% agarose gel electrophoresis

Quantitative Analysis

The quantity of the DNA was estimated by making appropriate dilutions to determine the optical density at 260nm and 280 nm by double beam spectrophotometer (Spectron 2206) and the concentration was determined using equation

$$DNA\ (\mu g/ml) = A_{260} \times 50 \times dilution\ factor$$

The ratio of $A_{260/280nm}$ was calculated and the DNA samples for which the ratio was 1.6-1.9 was considered for the future use. The DNA was stored at $4^{o}C$ for a short time but the vials were kept at $-20^{o}C$ for longer duration storage.

3.5.3. Genotyping of IRF-8 gene

After Agarose gel electrophoresis it was found that concentration and purity of genomic DNA is desirable, the desired fragment of DNA i.e., *IRF-8 (exon 7)* was amplified by polymerase chain reaction (PCR). After standardizing all the parameters of PCR like varying annealing temperature, dNTP, primer and template concentration, fragment of interest was amplified. PCR was performed in total volume of 25 μl for restriction digestion and 50μl for direct sequencing. The PCR reactions were composed of 100 ng of genomic DNA, 0.2mM dNTPs, 0.4 pmoles/μl of each primer and 0.2 U/μl of Taq polymerase in 1x PCR buffer (Table 2). PCR tubes containing reaction mixture ware mixed and placed in a 96 well automated thermal cycler (Applied) for amplification. After placing the tubes within thermal cycler, different temperatures were set as given in the table 3. The primer pair designed and used for amplification includes

Forward primer: 5´- GGCACCAAGCTGTATGGG -3´

Reverse primer: 5´- AGAACTGGCTGGTGTCGAAG -3´

Lyophilized primer stocks were diluted first to 100pg/μl concentration and then working was made 20pg/μl using miliQ water. Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by 2% agarose gel electrophoresis. The gel was visualized on UV-illuminator and photographed on the Gel Doc System. The final PCR product of 271 bp was obtained and was digested by restriction enzyme HaeIII (Fermntas) for 4 h at 37 ^{0}C. Digested products were separated on a 3% agarose gel. HaeIII cuts the fragment of interest at 6 positions (5, 18, 26, 52, 173, and 216), giving 121bp and 55bp as major fragments. For further confirmation of results sequencing was commercially done using the services of SciGenomics Kerala. 50 μl of unpurified PCR product samples were send along with 50μl of 20μM Forward & Reverse primers for purification and sequencing.

Table 2: Volume of different Reagents used for 25µl Reaction

Reagent	Volume used
10x PCR reaction buffer	2.5 µl
$MgCl_2$	2.2 µl
dNTP mix	0.6 µl
Forward Primer	0.3 µl
Reverse Primer	0.3 µl
Genomic DNA	3.0 µl
Taq DNA polymerase	0.3 µl
MilliQ water	15.8 µl
Total Volume	**25 µl**

Table 3: PCR cycling parameters

Steps	Temperature ^{o}C	Time	
Initial Denaturation	95	5 minutes	
Denaturation	95	50 seconds	
Annealing	62.1	50 seconds	35 Cycles
Extension	72	50 seconds	
Final extension	72	10 minutes	

3.5.4. Purification and DNA Sequencing

The purification and sequencing was commercially done using the services of SciGenomics kerela. For purification and sequencing we send 50 µl of unpurified PCR product samples along with 50 µl of 20 µM Forward & Reverse primers.

3.5.5. Statistical Analysis

The χ^2-test was used to examine the differences in the distribution of genotypes between cases and controls. OR with 95% CI were computed using unconditional logistic regression (GraphPad Prism 5) and adjusted for age and gender.

CML is a myeloproliferative disorder but definite mechanism leading to this carcinogenesis is yet to be understood completely. Only causative factor known to be associated with CML is exposure to radioactivity. Individual genotypic differences and also the level of expression of various signaling molecules are crucial in determining the susceptibility of developing the cancer. In this hospital based case-control study we evaluated the exon 7 region of IRF-8 gene for any nucleotide variation in the CML patients and healthy controls of the Kashmir valley.

On analyzing the data it comes out that out of 80 CML patients registered, 44 were males and 36 were females and thus the male to female ratio comes out to be 1:1.12 (Figure 5). Out of 80 cases, 30 (37.50%) were found in the age group of 30-40 years, 17(21.25%) of cases were found in the age group of 40-50 years and 14(17.5%) were in the age group of 50-60 years (Figure 6).

Of all the ten districts of the Kashmir division, highest number of CML cases turned out from the central district of Srinagar, with total no of 20 CML cases (25%) recruited for the study (Figure 7). We observed that the fruit consumption in most (65%) of the CML cases was very low. Also the use of tobacco in the form of cigarette smoking and traditional *Hukka* was seen in about 60% of cases (Table 4).

A good proportion of the CML cases (63.75%) that were recruited for this study had a monthly income of less than 6000 INR (Table 4).

Taking presence of Philadelphia chromosome (BCR-ABL fusion gene) into account it was found that 66 (82.5%) of cases were found positive, while as only 14(17.5%) of cases were negative (Figure 8).

Table 4: Showing various demographic characteristics of CML cases.

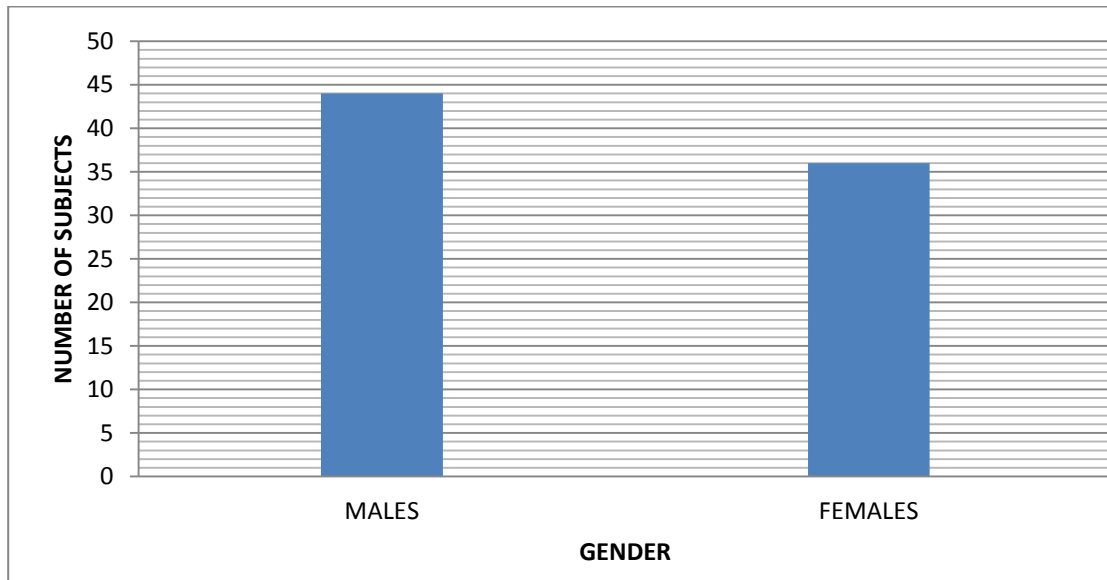| Demographic Features		CML Cases n(80)
Age (Years)	30-40	30
	40-50	17
	50-60	14
Gender	Male	44
	Female	26
District	Srinagar	20
	Budgam	10
	Baramulla	8
	Others	42
Fruit Consumption	Low	52
	Moderate	28
Smoking	Ever	48
	Never	32
Economic status (INR)	≤ 6000	51
	> 6000	29
BCR-ABL fusion gene	Present	66
	Absent	14

Figure 5: Gender distribution of CML patients included in the study.

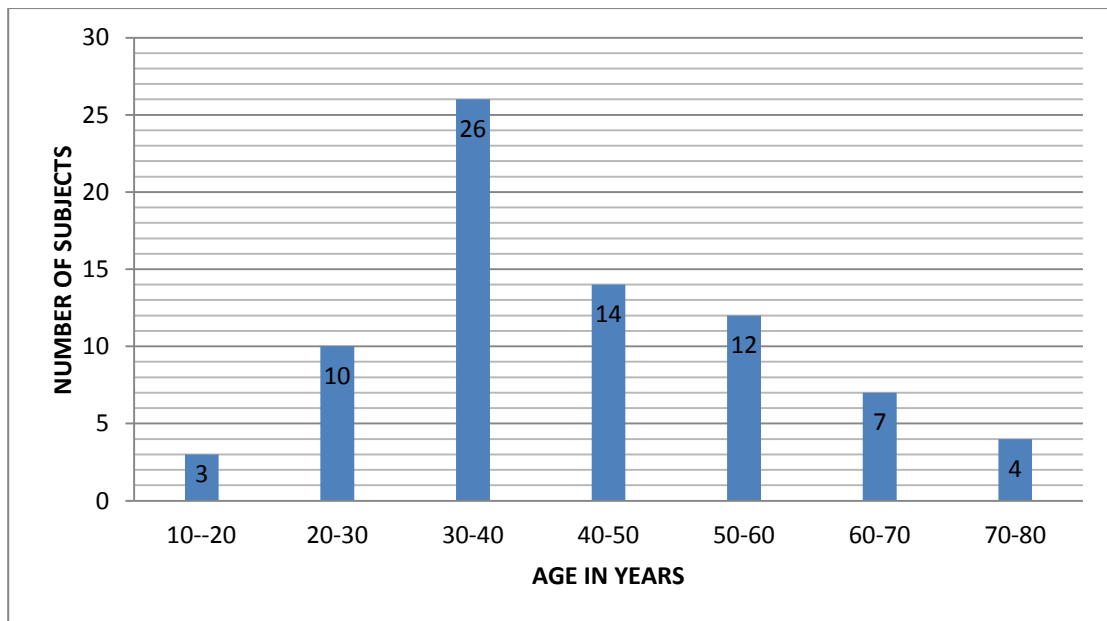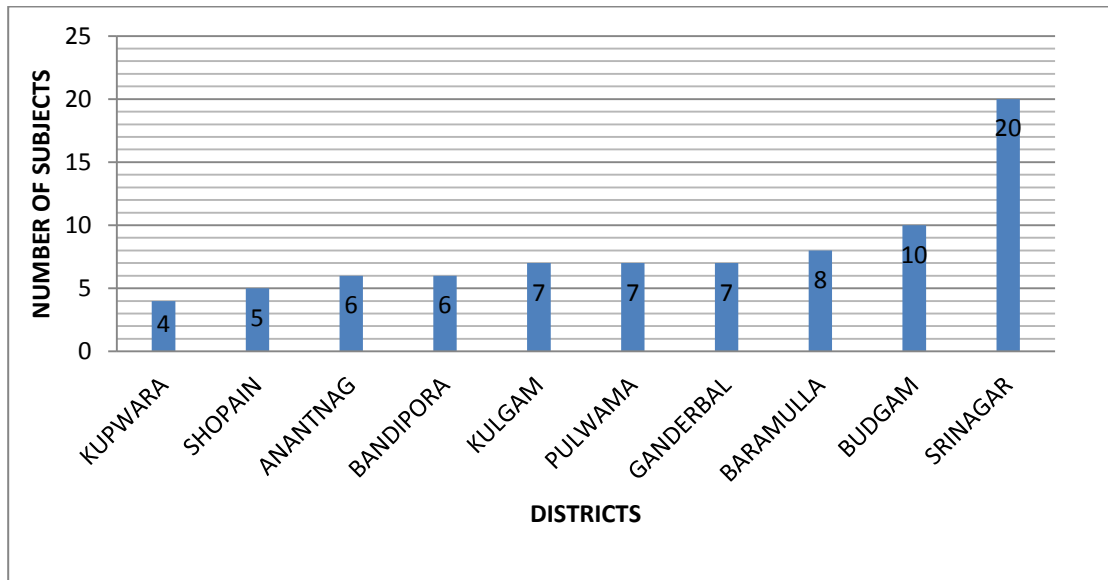**Figure 6:** Age distribution of CML patients included in the study.

Figure 7: District-wise distribution of CML patients included in the study.
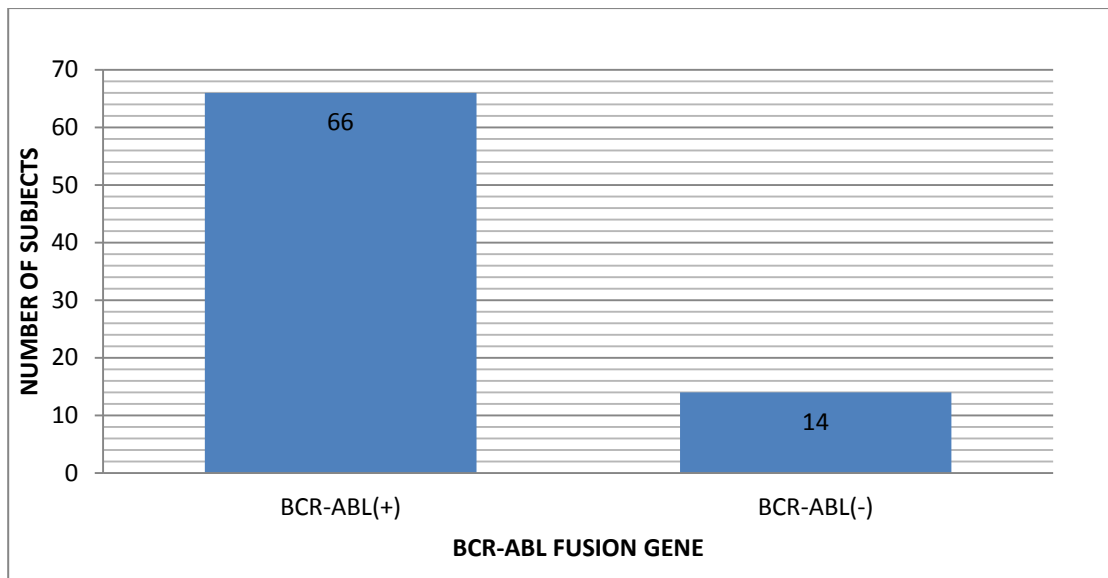

Figure 8: BCR-ABL status of CML patients included in the Study

4.1. Clinical features of CML patients of Study population

The patients with CML were diagnosed in the Department of Hematology of Sheri-Kashmir Institute of Medical Sciences (SKIMS), Soura. The diagnosis of CML was based on the standard clinico-hematological criteria and the presence of Philadelphia chromosome (BCR-ABL fusion gene). The number of patients with CML (n=80) comprised 44(55%) males and 36(45%) females with mean age of 44.7 years. The number healthy controls (n=80) consisted of 56(70%) males and 24(30%) females with mean age of 31.8 years.

Clinical and Biochemical characteristics of Chronic Myeloid Leukemia patients who were genotyped for exon 7 region of *IRF-8* gene are shown in Table 5.

Table 5: Clinical characteristics of Study Subjects

Variables	CML patients

Sex
      Male
      Female
	44
	36

Hemoglobin(g/dl)	10.7±2.518
TLC $\times 10^3/$ µl	19.31±40.71
Platelet $\times\ 10^3/$ µl	194.3±139.3
BCR-ABL (%age)	67.81±32.41

n= number of individuals
value represents ± SD

4.2. Genetic Analysis

4.2.1. PCR Amplification of *IRF-8* (exon 7)

A single primer set was designed and used to amplify the *IRF-8* (exon 7) which gave 271 bp size amplicon. The reaction conditions and PCR program which was used for amplification is described in methodology section. The results showed that all the genomic DNA samples were amplified successfully, producing specific amplicon of expected size. After amplification, 5µl of PCR products were visualized under UV exposure after the gel was stained with ethidium bromide as shown in Figure 9.
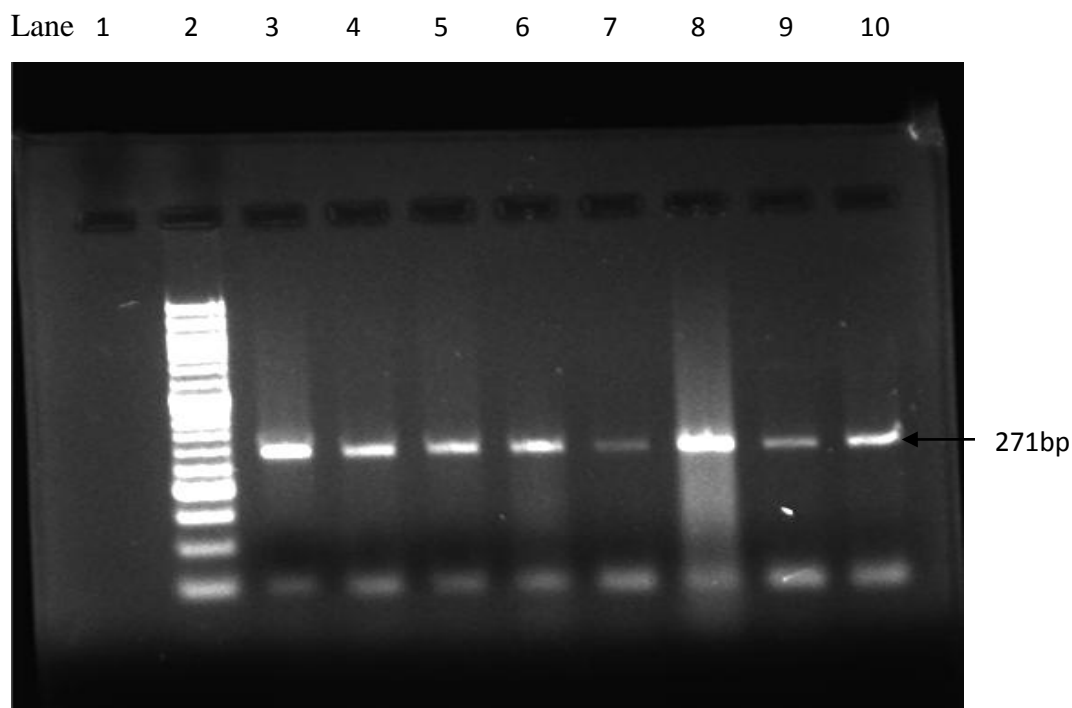

Figure 9: Representative photograph of the *IRF-8 (exon7)* amplified by Polymerase chain reaction. Lane no. 1 represents negative control Lane no. 2 represents 50 bp ladder, Lane no. 3-10 represents 271 bp fragment of exon 7 of *IRF-8* gene amplified by PCR.

4.2.2. Restriction digestion

The amplified product of exon 7 region of IRF-8 gene was subjected to restriction digestion by HaeIII enzyme. It cuts amplified product at 6 positions (5, 18, 26, 52, 173 and 216) giving digested products of 13bp, 8bp, 26bp, 121bp, 43bp and 55bp. The results showed only 121bp and 55bp restriction fragments on 3% agarose gel which was visualized under UV exposure after the gel was stained with ethidium bromide as shown in Figure 10.

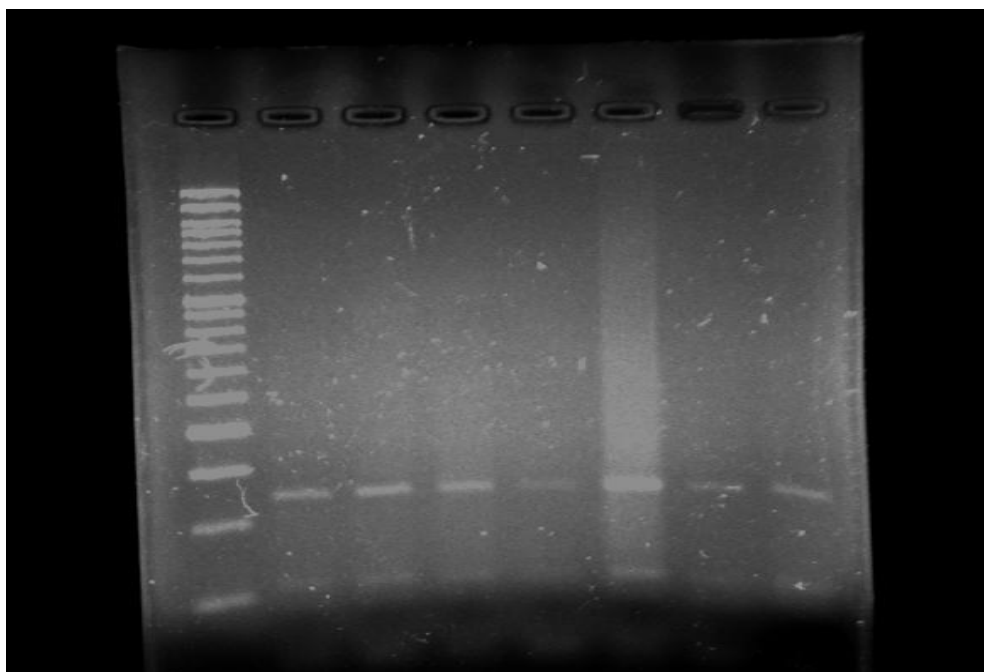Lane   1   2   3   4   5   6   7   8

Figure 10 : Representative photograph of the *IRF-8 (exon7)* restriction fragments. Lane no. 1 represents 50 bp ladder, Lane no. 2-8 represents 121 bp and 55 bp fragments each of exon 7 of *IRF-8* gene restriction by HaeIII .

4.3. Sequence Analysis

Sequencing of all the samples for any potential variation in the nucleotide sequence of the *IRF-8* gene (exon 7) was done commercially using the services of SciGenomics kerela. Alignment of all the sequences pertained to DNA samples of various cases and controls was done with respect to control sequence. Sequencing results for *IRF-8* amplicon for potential mutation for cases and controls are respectively shown by chromatograms in Figure 11 and 12. These chromatograms indicate the presence of single G→A transition position 92 in amplified exon 7 of my gene of interest which might have an important role in the expression level of *IRF-8 gene*. The representative chromatograms of wild type T allele and the variant allele with G → A change are given in the Figure 13 and 14 respectively.

 In CML cases, the allelic frequency for normal allele G was found to be 47.5% (76/160). The allelic frequency observed for G →A type was found to be 52.5% (84/160). An equal number of non malignant age and gender matched controls were screened for the any potential mutation for the same region. In controls, the allelic frequency observed for normal allele G was 91.25% (146/160). The allelic frequency in controls observed for G→A type was found to be 8.75% (14/160).

Since the frequency observed for the variant allele (G→A) was higher in CML cases (52.5%) than in normal controls (8.75%) and was found to be statistically significant (OR= 11.52, 95% CI: (6.13-21.6); p = 0.001). The frequency of *IRF-8* (exon 7) in CML patients and controls is summarized in Table 6.
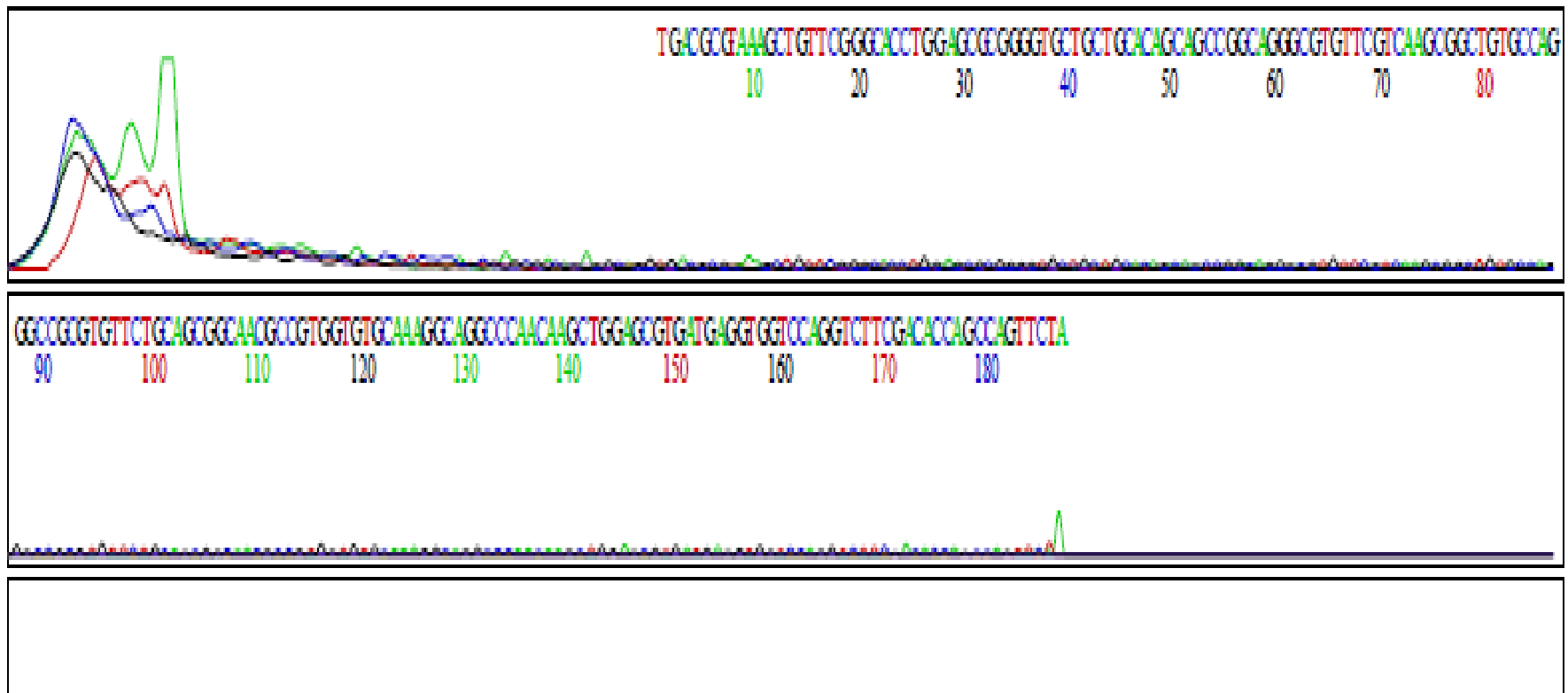
Figure 11: Representative Chromatogram of direct sequencing for *IRF-8 (exon 7)* in CML patients.
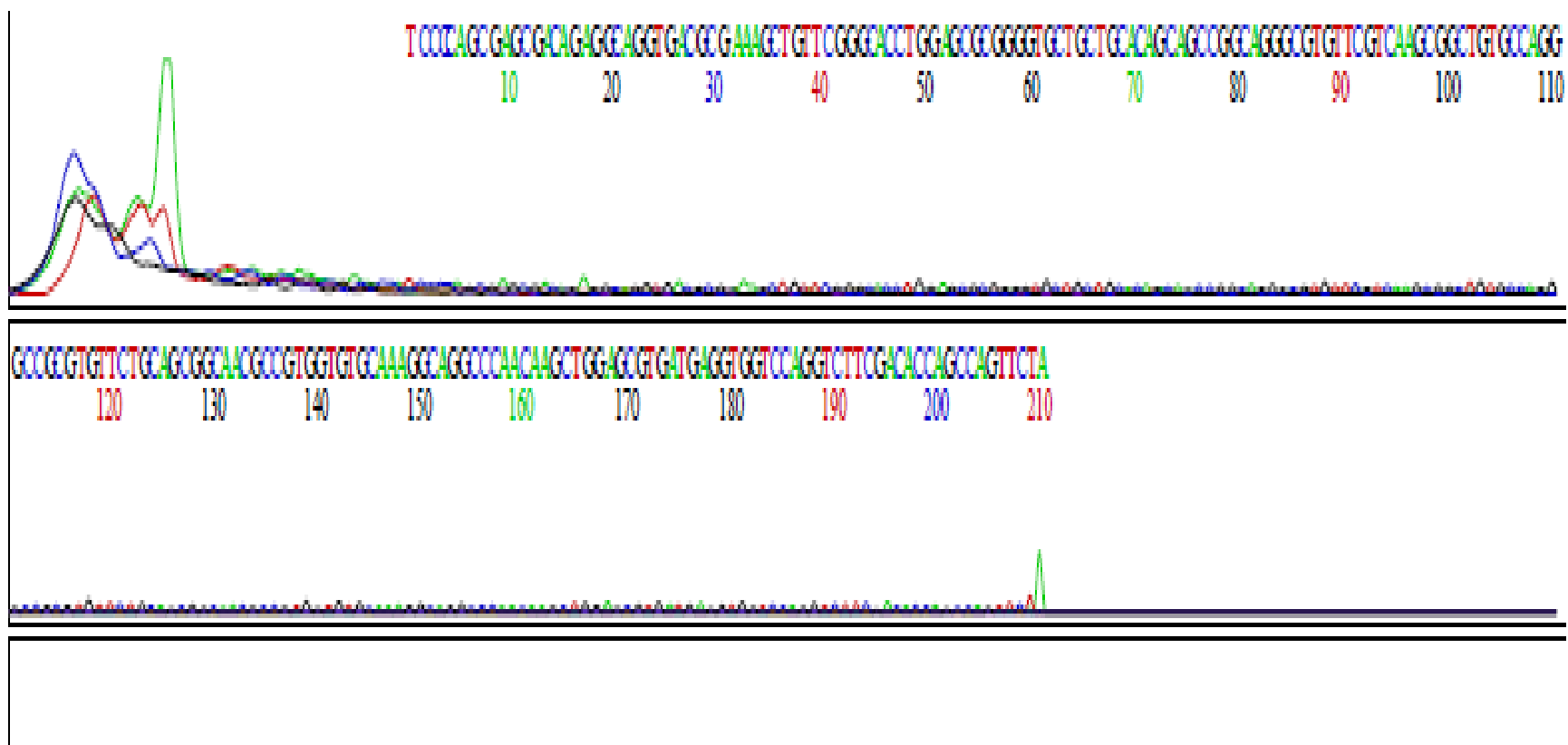
Figure 12: Representative Chromatogram of direct sequencing for *IRF-8 (exon 7)* in controls.
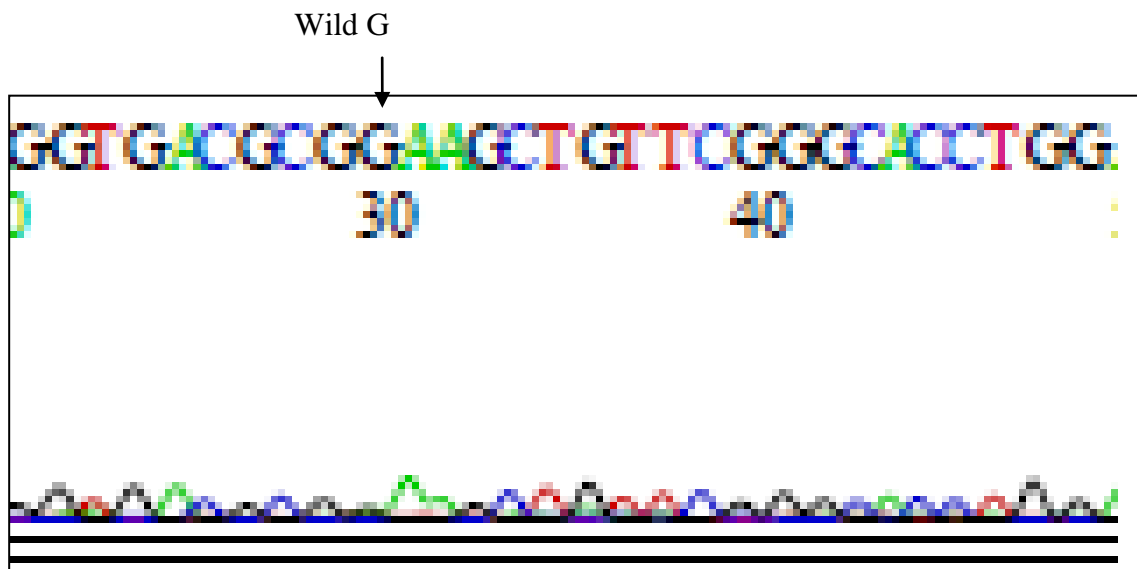
Figure 13: Representive chromatogram of Direct sequencing for *IRF-8* unaffected individuals.The *IRF-8* of unaffected individuals contains a G nucleotide base at position 92 in amplified exon 7.
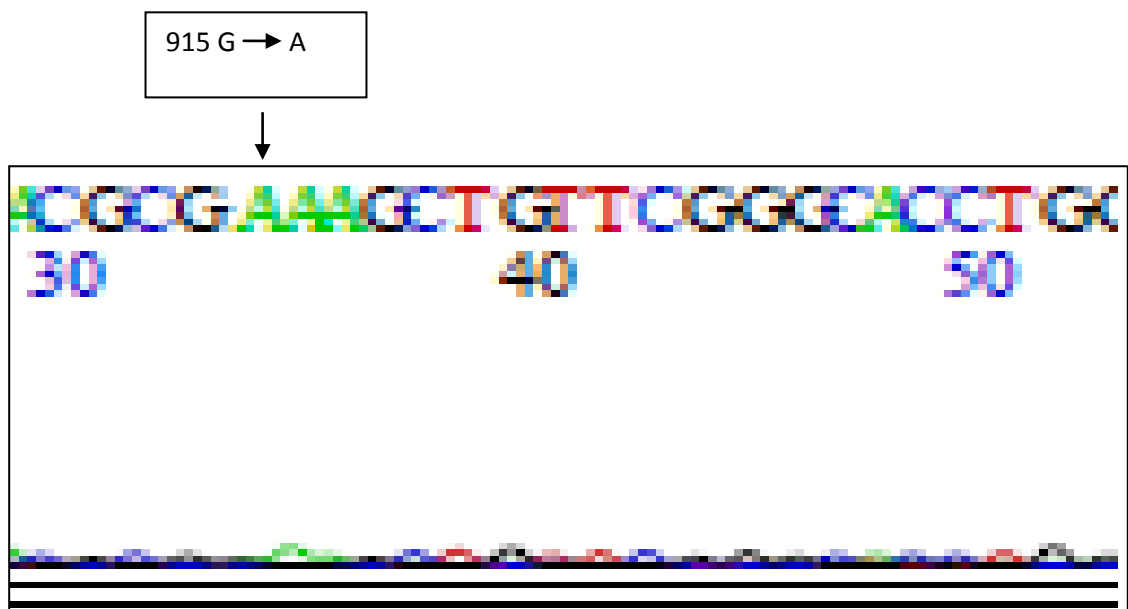
Figure 14: Representive chromatogram of Direct sequencing for *IRF-8* affected individuals . The *IRF-8* of affected individuals contains a G ➝ A sub- stitution at position 92 in amplfied exon 7.

Table 6: Illustrating frequency of *IRF-8* (exon 7) alleles in CML patients and controls.

Polymorphism		Cases n^s (%) 160	Control n^s (%) 120	OR (95% CI)	*p* value
irf-8	G	76(47.5)	146(91.25)	1	-
	A	84(52.5)	14(8.75)	11.52 (6.13-21.6)	0.001

n^s = Number of Alleles

$p<0.05$ (Data statistically significant)

Chronic myeloid leukemia (CML) is myeloproliferative disorder whose definite mechanism of development is yet unknown. It is associated with a characteristic Philadelphia chromosome leading to reciprocal translocation between chromosome 9 and 22 resulting in juxtaposition of BCR-ABL gene (**Faderl et al., 1999, Rowley et al., 1973**). Four major mechanisms have been implicated in the malignant transformation by BCR-ABL, namely altered adhesion to stroma cells and extracellular matrix (**Gordon et al., 1987**), constitutively active mitogenic signaling (**Puil et al., 1994**), reduced apoptosis (**Bedi et al., 1994**) and proteasome-mediated degradation of ABL inhibitory proteins (**Dai et al., 1998**). Only a limited number of studies have been so far conducted in which the role of other possible confounding factors like environment has been studied together with genetic analysis. IRF-8/ICSCP is expressed predominately in hematopoietic cells and is further increased upon treatment with interferon (**Driggers et al., 1990, Weisz et al., 1992**). Expression of IRF-8 can lead to the down-regulation of the anti-apoptotic protein Bcl-2 (**Burchert et al., 2004**). Originally described as being induced by IFN-γ, IRF-8 expression is also elevated by IRF-α as well as IL-12 in NK and T cells (**Lehtonen et al., 2003**). IRF-8 –/– myeloid progenitor cells have defects in both differentiation and growth. IRF-8 drives their differentiation toward macrophages whereas it inhibits granulocytic differentiation (**Scheller et al., 1999, Tamura et al., 2000**). Moreover, IRF-8 inhibits myeloid cell growth and promotes apoptosis (**Tamura et al., 2000, Gabriele et al., 1999**). These studies reveal that IRF-8 plays a pivotal role in regulation of leukemogenesis and functions as a tumor suppressor of certain myeloid malignancies. By direct sequencing of the exon 7 region of *IRF-8* gene, a single base pair G ➔ A transition was found at position 92 of the exon amlified. Only few studies have been conducted on CML in Kashmir and the cause of the high incidence rate is yet a mystery. Thus a hospital based case-control study was devised which was aimed to evaluate exon 7 region of the *IRF-8* gene for any nucleotide variation in the CML patients and healthy controls of the Kashmir valley.

The present study consists of eighty confirmed CML cases and also equal number of age, gender and district matched controls. Fifty five percent (44/80) of CML patients were men thus giving the male to female ratio to be 1:1.12. We did not find any significant difference in the mean age of the cases (44.7 years) and controls (43.6

years). We observed a higher representation of CML cases in the age group between 30 and 40. Similar pattern of age at diagnosis was also observed in studies conducted by Mendizabal et al., 2010.

The highest number of the CML patients in our study were from central part of Kashmir valley with district Srinagar accounting for 25% of the cases. The wide difference in the incidence rate of the CML across the different districts of the Kashmir valley may be suggestive of timely access to the only tertiary care centre owing to its easy availability and health consciousness, although, role of some environmental exposure of the local population may not be discarded.

We assessed all the CML patients for the presence of Philadelphia chromosome (BCR-ABL fusion gene) and observed that 66 (82.5%) of CML cases were positive. Our results are in agreement with other studies which have reported high prevalence of Philadelphia chromosome in CML patients **(Kurzrock et al., 2003, Goldman et al., 2003)**.

Restriction results showed wild conditions with no mutation at any of the 6 position where HaeIII cuts, which was confirmed by the sequencing results as well. Further sequencing results showed interesting single G⟶ A substitution at position 92 of the exon amplified. In CML cases, the allelic frequency for normal allele (G) was found to be 47.5% (76/160) and the allelic frequency observed for G⟶ A type was found to be 52.5% (84/160). The allelic frequency observed in controls for normal allele (G) was 91.25% (146/160). The frequencies analyzed for G ⟶A allele was 8.75% (14/160). Since the frequency observed for G⟶ A allele was higher in CML cases (52.5%) than in normal controls (8.75%) and it was found to be statistically significant (OR= 11.52, 95%CI: (6.13-21.6); p = 0.001). Sequencing results further showed occasional deletion at the same position where transition was seen. IRF-8 is a critical regulator of myelopoiesis, which when deleted in mice results in a syndrome highly similar to human chronic myelogenous leukemia **(Seung-Hee et al., 2010)**. In human patients with CML and acute myeloid leukemia, IRF-8 expression is dramatically decreased **(Schmidt et al., 1998)**. This study may be augmented by the fact that any aberrations in the gene of interest which might be having significant effect in expression pattern or folding phenomenon would ultimately result in CML.

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