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Sequence Variation in Promoter Region of *Irf-4* Gene and the Risk of CML in the Kashmiri Population

Research Article

Gulzar Bhat[1], Ashaqullah Bhat[1], Wani Aadil[1], Ali Muzamil[1], Samoon Jeelani[2], Akbar Masood[1], Bashir Ahmad Ganai[1]*

[1]Department of Biochemistry, University of Kashmir, Srinagar, India

[2]Department of Clinical Hematology, SKIMS, Srinagar, India

***Corresponding author:** Bashir Ahmad Ganai, Department of Biochemistry, University of Kashmir, Srinagar, India; E-mail: bbcganai@gmail.com

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Abstract

A number of theories have been postulated to describe the etiology of Chronic Myeloid Leukemia (CML), such as genetic alterations and alterations in cytokine production. A combination of inflammatory cytokines has an important role in cancer development. The aim of this hospital based case-control study was to screen for variations located in promoter region of *irf-4* gene sequence in CML cases and healthy controls of Kashmiri population. We included sixty confirmed CML cases and an equal number of age, district and gender matched controls in this study. Sequencing of both the CML case and control samples for any potential variation in the nucleotide sequence in the promoter region of *irf-4* gene was done commercially. Sequence alignment with the wild type sequence revealed the substitution of T with G nucleotide at -991 position in the promoter region of *irf-4* gene. The allelic frequency for normal and variant allele in CML cases was 46.66% and 53.33%respectively. In controls, the allelic frequency observed for normal and variant allele was 86.66% and 13.33% respectively. Thus a statistically significant association was observed between the CML and the presence of variant allele (TG) (OR= 7.43, 95%CI: (3.9-14.1); p = 0.0001). Our study also revealed that CML is more prevalent in males as compared to females (ratio is 1.12:1, and the fruit consumption was very low in CML cases (72.33%). Furthermore 74.00% of the CML cases were found to be tobacco smokers. This is the first report on the sequence variation in promoter region of *irf-4* gene and the risk to CML in the Kashmiri population. However, the understanding gained through this study needs to be further substantiated by conducting a similar study on statistically significant sample size.

Keywords: CML; Cytokine; Philadelphia chromosome, IRF-4

Introduction

Chronic myelogenous leukemia (CML) is a clonal hematopoietic disorder caused by an acquired genetic defect in a pluripotent stem cell. Incidence increases with age, being exceptionally rare in infancy at 0.7/million/year at ages 1 to 14 years and rising to 1.2/million/year in adolescents [1]. It is slightly more common in males than in females. In general male predominance has been estimated to be 1.3-1.4 to 1. The hallmark genetic aberration of CML is a reciprocal chromosomal translocation t(9;22) leadingto expression of a BCR-ABL fusion gene, an aberrant activatedtyrosine kinase [2]. However there is mounting evidence that the deregulation of other genes, such as the transcription factor interferon regulatory factor 4 (IRF-4), MGMT, DAPK, p14ARF, p15INK4b, p16INK4a, BRCA1, CDH13 and APAF-1 is also implicated in the pathogenesis of CML [3-5]. 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. This hospital based case-control study was devised which was aimed to evaluate the promoter

region of the *irf-4* gene for any nucleotide variation in the CML patients and healthy controls of the Kashmir valley.

Interferon regulatory factors (IRFs) comprise a family of transcription factors that function within the JAK-STAT pathway to regulate interferon (IFN) and IFN-inducible gene expression in response to viral infection. IRFs play an important role in pathogen defense, autoimmunity, lymphocyte development, cell growth and susceptibility to transformation.IRF-4 is a lymphoid and myeloid restricted member of IRF transcriptional family [6,7]. The product of the *MUM1/IRF4* gene (also called PIP, LSIRF, ICSAT) [8-11] is a member of the interferon regulatory factor (IRF) family of transcription factors, known to play an important role in the regulation of gene expression in response to signaling by interferons and by other cytokines [12]. Besides Bcr-Abl translocation which is known to be causative genetic aberrationinchronic myeloid leukemia (CML), there is mounting evidence that the deregulation of interferon regulatory factor 4 (IRF-4), is also implicated in the pathogenesis of CML [13]. IRF-4 polymorphisms are associated with susceptibility to chronic lymphoid leukemia (CLL) and non-Hodgkin lymphoma (NHL) [14]. Mutations in the distal and proximal sites of this GC-rich sequence resulted in 62 and 81% reductions in the *irf-4* promoter activity, respectively [15].

Till date no study has been done to understand the etiology of CML in Kashmir. Therefore the aim of this hospital based case-control study was to screen for variations located in promoter region of *irf-4* gene sequence in CML cases and healthy controls in a sample of Kashmiri population.

Material and Methods

Patient characteristics

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 was 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. The patient characteristics are summarized in Table 1. A total of 60 CML cases (males 33 (55 %) and females 27(45 %) with a mean age of 44.7 ± 13.61) were recruited and during the same period 60 volunteer controls (males 42 (70%) and females 18 (30%) with the mean age of 31.83 ± 7.52) were also recruited. Three ml of venous blood was collected in a sterile EDTA coated vials and was stored at -20 ˚C. All patients and healthy individuals gave their written informed consent to participate in the study. Genomic DNA was isolated from the blood samples by using Phenol-Chloroform method (Sambrook 2001) and the isolated DNA was stored at -20 ˚C for future use. The study was approved by the Ethics Committee of the Sher-i-Kashmir Institute of Medical Science (SKIMS), India.

PCR amplification

After agarose gel electrophoresis it was found that concentration and purity of genomic DNA is desirable, the desired fragment of DNA i.e., *irf-4* promoter, was amplified by polymerase chain reaction (PCR). The technique was standardized for available environmental conditions. After standardizing all the parameters of PCR like varying annealing temperature, dNTP, primer and template concentration, the promoter regionof irf-4 gene was amplified.PCR was performed in total volume of 50µl. 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. The primer pair used for amplification of promoter region of *irf-4* was: forward primer: 5´-TTG AGA TGG AGT CTT GCT CTG T -3´; reverse primer: 5´- ATC ACT TCC AGA CTT CAG TTC ACC T -3´which on amplification produced a 314-bp product. Initial denaturation at 95 °C for 7 minutes was followed by 35 cycles of 50 second at 95 °C, 1 minute at 67 °C, 1 minute at 72 °C and final extension for10 minutes at 72 °C.

Table 1: Showing various demographic and clinical characteristics of CML cases.

Demographic Features		CML Cases n(60)
Age (Years)	30-40 40-50 50-60	20 13 11
Gender	Male Female	33 27
District	Srinagar Budgam Baramullah Others	18 8 6 30
Fruit Consumption	Low Moderate	36 24
Smoking	Ever Never	38 22
Economic status (INR)	≤ 6000 > 6000	37 23
BCR-ABL fusion gene	Present Absent	52 8
Hemoglobin(g/dl) **TLC X 10^3/ µl** **Platelet X 10^3/ µl**		10.7±2.518 19.31±40.71 194.3±139.3

Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by 2% agarose gel electrophoresis (Figure 1). The gel was visualized on UV-illuminator and photographed on the Gel Doc System.

Purification and DNA Sequencing

The purification and sequencing was commercially done using the services of Macrogen Inc. Soel, Korea. For purification and sequencing we send 50µl of unpurified PCR product samples along with 50µl of 20µM Forward & Reverse primers were sentto Macrogen, Korea. Alignment of all the sequences pertained to DNA samples of various cases and controls was done with respect to control sequence.

Statistical Analysis

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

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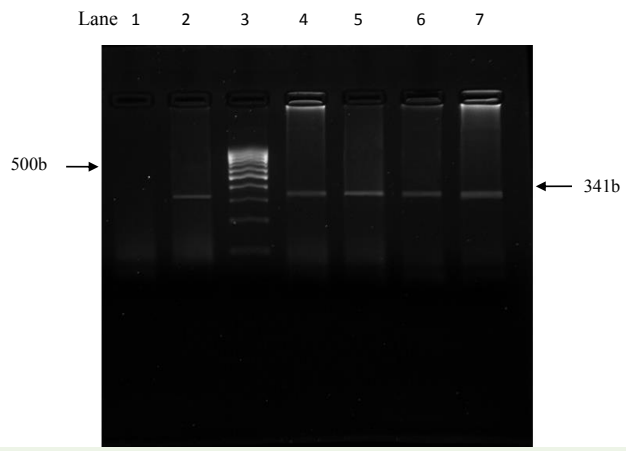

Figure 1: Representative photograph of the *irf-4*gene promoter region amplified by Polymerase chain reaction. Lane no. 1 represents negative control, Lane no. 3 represents 100bp ladder, Lane no. 2, 4,5,6,7 represents 341 bp fragment of *irf-4* promoter region amplified by PCR.

Results

Sequencing of all the samples for any potential variation in the nucleotide sequence of the promoter region of *irf-4* gene was done commercially using the services of Macrogen Inc. Seol, Korea. 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-4* promoter for any potential variations for affected and unaffected individuals are respectfully shown by chromatograms in Figure 2 and 3. These chromatograms indicate the presence of single TG polymorphism at -991 position in promoter region of *irf-4* gene. Since this T G polymorphism was seen in the promoter region of the gene, which might have an important role in the expression level of *irf-4*.

In present study we analyzed 60 confirmed CML cases and an equal number of nonmalignant controls belonging to Kashmir division for any potential SNP in the promoter region of *irf-4* gene. In CML cases, the allelic frequency for normal allele T was found to be 46.66% (56/120). The allelic frequency observed for T G type was found to be 53.33% (64/120). An equal number of non malignant age and gender matched controls were screened for the any potential SNP in the promoter region of *irf-4*gene. In controls, the allelic frequency observed for normal allele T was 86.66% (104/120). The allelic frequency in controls observed for TG type was found to be 13.33% (16/120).

Since the frequency observed for the variant allele (TG) was higher in CML cases (53.33%) than in normal controls (13.33%) and was found to be statistically significant (OR= 7.43, 95%CI:(3.9-14.1); p = 0.0001).The frequency of *irf-4* promoter alleles in CML patients and controls is summarized in Table 2.

Discussion

Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. The definite mechanism leading to the development of this disease is not known yet. The known genetic abnormality associated with the CML is the condition known as Philadelphia chromosome which occurs as a result of reciprocal translocation between chromosome 9 and 22 leading to juxtaposition of BCR-ABL gene [16]. 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. Only causative factor known to be associated with CML is exposure to radioactivity. Individual genotypic differences and also the level of expression of the carcinogen-metabolizing enzymes are crucial in determining the susceptibility of developing the cancer [17]. Interferon-regulatory factor-4 (*irf-4*) is an IRF family transcription factor important for hematopoietic development and immune processes. Irf-4 is expressed in lymphoid cells, dendritic cells, and macrophages, where it is associated with regulation of important cellular processes, including cell differentiation, apoptosis, DNA repair, and cytokine production [18]. irf-4 expression is reported to be down-regulated in patients with CML but restored in response to treatment with interferon-α (IFN-α), and higher *irf-4* expression is associated with a good response to IFN-α treatment [19,20]. By direct sequencing of the promoter region of *irf-4* gene, two base pair changes were found at the position -1081 (TC substitution), and at the position -1068 (AC substitution), which were later found not responsible for absent irf-4-expression. 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 the promoter region of the *irf-4* gene for any nucleotide variation in the CML patients and healthy controls of the Kashmir valley.

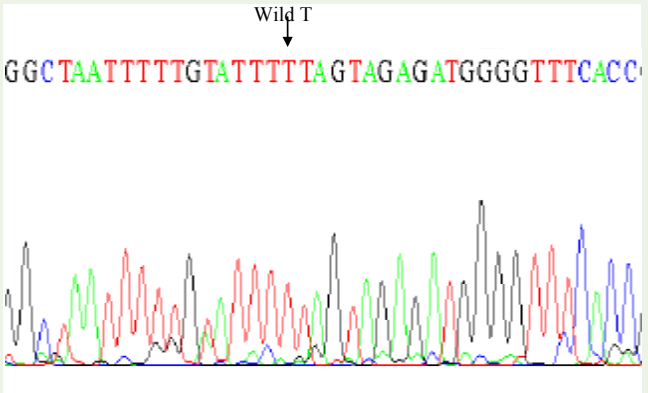

Figure 2: Representative chromatogram of direct sequencing for irf-4 unaffected individuals. The irf-4 of unaffected individuals contains a T nucleotide base at 991 position of promoter region.

Table 2: Illustrating frequency of *irf-4* promoter alleles in CML patients and controls.

Polymorphism		Cases n^s (%) 120	Control n^s(%) 120	OR (95% CI)	*p* Value
irf-4	T	56(46.66)	104 (86.66)	1	-
	G	64 (53.33)	16 (13.33)	7.43 (3.9-14.1)	0.0001

n^s= Number of Alleles
p<0.05 (Data statistically significant)

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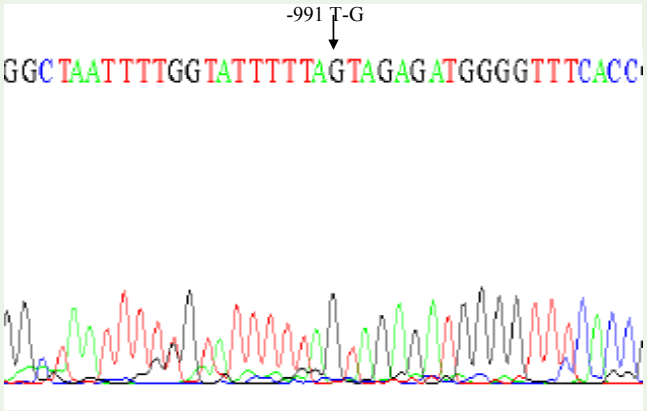

Figure 3: Representative chromatogram of direct sequenceing for irf-4 affected induviduals. The irf-4 affected individuals contain a T G substitution at -991position pf promoter region.

The present study consists of sixty confirmed CML cases and also equal number of age, gender and district matched controls. Thirty three percent (20/60) 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., [21]. The highest number of the CML patients in our study were from central part of Kashmir valley with district Srinagar accounting for 30% of the cases. The wide difference in the incidence rate of the CML across the different districts of the Kashmir valley suggests the role of some environmental exposure of the local population. The influence of such factors on the incidence rate if any needs to be studied by conducting well designed scientific studies. In addition we assessed all the CML patients for the presence of Philadelphia chromosome (BCR-ABL fusion gene) and observed that 52 (87%) of CML cases were positive. Our results are in agreement with other studies which have reported high prevalence of Philadelphia chromosome in CML patients [22,23]. In the present study we analyzed 60 confirmed CML cases and also equal number of gender, age and district matched nonmalignant controls belonging to Kashmir division for any mutation (SNPs) in the promoter region of *irf-4* gene by employing direct sequencing method. We found a single T to G substitution in the *irf-4* promoter region. In CML cases, the allelic frequency for normal allele (T) was found to be 46.66% (56/120) and the allelic frequency observed for TG type was found to be 53.33% (64/120). An equal number of non malignant age and gender matched controls were screened for the same mutation (SNPs) in the promoter region of *irf-4*gene. The allelic frequency observed in controls for normal allele (T) was 86.66% (104/120). The frequencies analyzed for TG allele was 13.33% (16/120). Since the frequency observed for T G allele was higher in CML cases (53.33%) than in normal controls (13.33%) and was found to be statistically significant (OR= 7.43, 95%CI:(3.02-18.29); p = 0.0001). It is reported that 62 and 81% reductions in the irf-4 promoter activity occurred due to mutations in the distal and proximal sites of the GC-rich sequence resulted in respectively [15]. *Irf-4* deficiency facilitates BCR/ABL-mediated transformation of B-lymphoid progenitors in vitro and accelerates progression of BCR/ABL-induced B-ALL in mice, and that forced expression of irf-4 effectively suppresses lymphoid leukemogenesis by BCR/ABL [18]. Since in our study T G polymorphism was found in the promoter region of the gene, which might have an important role in the expression level of Interferon regulatory factor 4 gene and hence on the risk to CML in Kashmiri population. This information collected by this study lays a foundation for further analysis and understanding the role of various other confounding factors which might be playing a role in the development of CML.

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