

RESEARCH COMMUNICATION

Relation between IRF-1 gene and Acute Myelocytic Leukemia in Kashmiri population

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Abstract

The IRF-1 protein, a mammalian transcriptional factor encoded by a gene located in 5q23-q31, has antioncogenic properties. Involved in regulation of differentiation and proliferation, IRF-1 acts as a tumor suppressor gene and is inactivated by deletion of its one or more exons (exon skipping) in many hematological malignancies, including acute myelocytic leukemia (AML) and myelodysplastic syndromes (MDS). DNA samples, extracted from peripheral blood, taken from 50 Kashmiri AML subjects, were analysed using the polymerase chain reaction and compared with examples of age and gender matched healthy controls from the same population. Three different exon regions (2, 3 and 4) of the IRF-1 gene that were previously shown to be prone to deletion were selected for amplification and analysis. Deletion was observed in 31(62%) out of 50 AML patients ($p=0.016$). Exon 3 was most frequently deleted (58%), followed by exon 2 (28%), while exon 4 was least affected (12%), providing insights into critical roles in leukemogenesis. The number of deleted exons was variable, but single exon deletions were more frequent (30%). Of interest, IRF-1 gene deletions were not observed in 19 (38%) patients. In our study, the frequency of deletions of these three exons was slightly higher than in an Indian population (52%), but lower than in Sweden in Europe (95%). This study also explored the prevalence and clinical profile of IRF-1 deletions in AML patients. Adults had a significantly higher incidence than children ($p=0.0168$) and IRF-1 deletions were associated with low Hb ($p<0.0001$), high TLC ($p=0.0033$) and a low platelet count ($p=0.0076$).

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Introduction

Acute myeloid leukemia (AML), a cancer of the myeloid series of cells, characterized by the proliferation and accumulation of abnormal immature cells (blasts) in the bone marrow and blood, is the most common form of leukemia in adults. It accounts for approximately 80% of acute leukemia in adults and 20% in children (Weinstein, 1999). In 2000, approximately 256,000 children and adults, worldwide, developed leukemia, and 209,000 died from it. This represents about 3% of almost seven million deaths due to cancer that year (World Health Organization report, 2000). The incidence of AML progressively increases with age, and in adults over the age of 65 years is approximately 30 times that in children (Bhatia and Neglia, 1995). Geographic variations in the incidence and subtype of AML have been reported. The highest incidence of AML in adults is in N. America, Western Europe and Oceania and the lowest in Asia and Latin America (Miller and Daonst, 2000).

AML is unfortunately difficult to treat, with only about 20-30% of people cured. The disease is most likely to affect those who are elderly; average age of onset is 63. As yet, a cure for older patients who get the disease remains elusive. Although of the order of 75-85% of patients

achieve complete remission after induction chemotherapy, long-term survival is still relatively low.

Interferon regulatory factor-1 (IRF-1) is a mammalian transcription factor, encoded by the IRF-1 gene in humans (Itoh et al., 1991). The IRF-1 gene (7.72 kb) was mapped to human chromosome 5 and contains 10 exons and 9 introns (Cha et al., 1992). By linkage analysis based on RFLP, the gene was localized to chromosome 5q23-q31 (Itoh et al., 1991). The deduced amino acid sequences were compared among different species; the most conserved exons were 2, 3, and 4, in which the putative DNA binding domain for the IRF-1 protein is located (Cha et al., 1992).

IRF-1 has been shown to be involved in the regulation of genes expressed during inflammation, immune response, hematopoiesis, cell proliferation and differentiation (Nguyen et al., 1997). Recent studies suggest that IRF-1 plays an important role in myeloid differentiation. An in vivo study using mice with a null mutation in the IRF-1 gene has provided evidence for the involvement of IRF-1 in the early phases of granulo-monocytic differentiation (Boulwood et al., 1993). IRF-1 is expressed in immature myeloid bone marrow cells (Liebermann and Hoffman, 2002) and its expression significantly increases during granulocytic differentiation of normal human and murine myeloid progenitors (Coccia et al., 2000; Stellacci et al.,

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2004). IRF-1 induction appears to represent a limiting step in the early events of myeloid differentiation (Testa et al., 2004).

The mechanism of human leukemogenesis may be partly associated with no or low-expression of IRF-1 gene (Xia et al., 1998). IRF-1 is functionally inactivated in many types of AMLs (Preisler et al., 2001). The IRF-1 gene is located on chromosome 5q23-q31, close to the region commonly deleted in myelodysplasia, AML, and acute promyelocytic leukemia (APL) (Willman et al., 1993; Green et al., 1999). These deletions often result in inactivation of one or both IRF-1 alleles (Green et al., 1999). "Exon-skipping" in IRF-1 gene transcription has been reported; it produces IRF-1 mRNA lacking exons 2 and 3, rendering the consequent protein dysfunctional (Guzman et al., 2001; Harada et al., 1994). Deletion 5q is one of the most frequent chromosomal abnormalities in myeloid leukemia, in Indian population (Dakshinamurthy et al., 2005).

Materials and Methods

Study population and blood sampling

Blood samples were collected from 50 AML patients from the Department of Clinical Hematology and Medical Oncology of Sheri-Kashmir Institute of Medical Sciences (SKIMS). Clinical details were obtained by going through the medical records of the cases. The first clue to the diagnosis of AML was made on the basis of abnormality in complete blood count (CBC). A bone marrow aspiration and biopsy is required to make a definitive diagnosis. Marrow or blood is examined via light microscopy as well as by flow cytometry to diagnose the presence of leukemia, to differentiate AML from other types of leukemia (e.g. acute lymphoblastic leukemia), and to classify the subtype of disease. According to the widely used WHO criteria, the diagnosis of AML is established by demonstrating involvement of more than 20% of the blood and/or bone marrow by leukemic myeloblasts. Diagnosis and classification is also established with the help of immunophenotyping.

Whole blood was collected in EDTA vials and was stored at -20°C for further analysis. Proper consent was taken from all the subjects. Patients coming for treatment for minor ailments at the SKIMS Hospital, with no evidence of cancer were selected as controls (n=50). Other inclusion criteria were that subjects be from the Kashmiri population, maximum clinical data and sufficient biological material be available. The recruited AML patients comprised 29 males and 21 females. Informed consent was obtained from all patients, included in the study, and also from the Institution.

Method

Modified salting out method was used for DNA extraction (Nasiri et al., 2005). The quantity and purity of genomic DNA was checked by spectroscopy. The purity was checked as absorbance ratio of (1:200) diluted DNA solution at 260nm and 280nm. The samples having absorbance ratio (A_{260nm} / A_{280nm}) between 1.7-1.9 are considered pure.

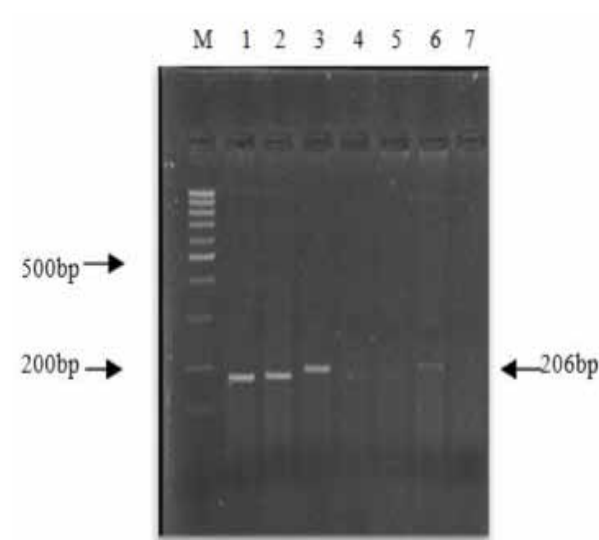


Figure 1. Agarose gel of PCR amplification results.

Lane no. 1, 2 and 3, represents a healthy control (age and gender matched), and lane no. 4, 5 and 6, represents an AML patient. AML patient contains deletion of the two exons (no band in lane no. 4 and 5). M represents the 100bp ladder. Lane no. 1, 2 and 3 contains an amplicon of 174bp, 177bp and 206bp, resp.

Genotyping of IRF-1 gene deletion

Once it was confirmed that the genomic DNA is present, and the concentration and purity is also desirable, the desired fragment of DNA i.e., the exon 2, 3 and 4 of the IRF-1 gene, was amplified by Polymerase Chain Reaction (PCR). The standard protocol for PCR was used; however the technique was standardized or optimized for available environmental conditions. PCR was performed in total volume of 50µl. The PCR conditions were selected after extensively standardizing all the PCR parameters. Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by agarose gel electrophoresis on 1.5% gel. After the electrophoresis, the gel was visualized on UV-illuminator and photographed on a Gel Doc (see Figure 1).

Statistical Analysis

Results were statistically analyzed and data was expressed as mean ± SE. Hematological Tests were compared between AML patients and normal subjects; and AML males and females, using the t-test and ANOVA (one way). ANOVA (one way) was also used for comparing deletion of three exons.

Results

Several clinical and biochemical parameters of 50 AML subjects (29 males and 21 females), were examined. The mean age of AML subjects was 27.1 years, very low as compared to the population in west (Bhatia and Neglia, 2005). The AML population was severely anemic (Mean Hb=7.9g/dL), also having leukocytosis (Mean TLC=32.7 x 10³/µL) and thrombocytopenia (Mean platelet count=41.9 x 10³/µL). Immunophenotyping reveals the presence of myeloid markers (CD13+, CD33+) and absence of lymphoid markers (CD3-, CD5-, CD7-, CD19-, CD22-). The three blood components varied significantly between AML subjects and controls. Also, the comparison

was drawn between AML male and female subjects, who only vary significantly between their Hb concentrations; the other two components i.e. TLC and platelet count, along with age, vary insignificantly.

Amplification of three Exon regions (2, 3 and 4) of the IRF-1 gene

Three DNA fragments, each encompassing an exon and few bases of its flanking introns from the IRF-1 gene, were enzymatically amplified. A primer set was designed and used to amplify exon 2, 3 and 4 from fifty DNA (genomic) samples of AML patients and fifty DNA (genomic) samples of controls (gender and age matched). The amplified products were visualized under UV exposure, using the ethidium bromide as visualizing agent. The results of PCR of two subjects, i.e. AML and control were compared. Three types of bands of length 206bp, 177bp and 174bp were amplified. Some PCR amplifications from AML subjects, shows absence of one or more bands when compared to control, which confirms the deletion of that particular exon/exons in that AML patient. Also gel densitometry technique was used for quantifying DNA.

Discussion

Leukemia, being a malignant disease, is primarily developed because of genetic abnormality in hematopoietic cells. Acute myelocytic leukemia is a malignancy of hematopoietic myeloid progenitor cells with different molecular genetic abnormalities, clinical characteristics, and variable outcomes with currently available treatments. In 50-60% of patients with AML, numerous recurrent chromosomal abnormalities have been described (Haferlach et al., 2004). These genetic alterations contribute to the leukemic transformation of hematopoietic stem cells or their committed progenitors by changing cellular functions (Gilliland, 1998; Ferrando and Look, 2000).

Cytogenetic analysis performed at diagnosis is considered to be the most valuable prognostic factor in AML. Interstitial deletion within chromosome 5q is one of the most frequent cytogenetic abnormalities in human acute leukemia and myelodysplasia. IRF-1 (5q31.1), a gene whose product manifests anti-oncogenic activity, was consistently found deleted at one or both alleles in acute leukemia (Willman et al., 1993).

The mechanism of human leukemogenesis may be partly associated with no or low-expression of IRF-1 gene (Preisler et al., 2001). Increased frequency of alternate IRF-1 transcripts, in which exon 2 (containing the AUG initiation codon) and 3, and sometimes even exon 4, have been reported to be deleted in bone marrow and peripheral mononuclear cells, from patients with myelodysplastic syndrome or myeloid leukemias, as a result of exon skipping (Harada et al., 1994; Green et al., 1998; Tzoanopoulos et al., 2002).

This accelerated exon skipping cause the inactivation of IRF-1, as it neither displays DNA binding nor tumor suppressive activity; thereby contribute to the development of human hematopoietic malignancies.

These alternate transcripts are missing in the normal translational start site, which is found in exon 2 (Harada et al., 1994). Patients expressing high levels of aberrantly spliced IRF-1 mRNAs were also found to express reduced levels of full-length IRF-1 transcript compared with healthy individuals, suggesting that exon skipping may be an important mechanism of tumor-suppressor gene inactivation in hematopoietic malignancies.

IRF-1 is a tumor suppressor gene (Tanaka et al., 1994; Yim et al., 1997) and its role in AML has already been verified in a number of other studies, in different populations. Accelerated exon skipping was seen in 28.88% of myeloid leukemia patients in a study by Green et al. All primary acute promyelocytic leukemia (APL) samples lacked IRF-1 protein and most exhibited accelerated exon skipping.

In our study, IRF-1 gene deletion frequency (for exon region 2, 3 and 4) was studied in 50 AML patients. Out of this, IRF-1 gene deletion was observed in 31(62%) AML cases ($p=0.016$). It is slightly higher than observed in other Indian population (52%) (Dakshinamurthy et al., 2005; Bram et al., 2003), but very low as compared to Swedish population, 95% (Bram et al., 2003).

In our cases, exon 2 was deleted in 14(28%) cases, exon 3 was deleted from 29(58%) cases and exon 4 was absent from only 6(12%) cases. The ratio of deletions of the three exon regions being-E2: E3: E4 = 2.33: 4.83: 1

From above it was clear that the effect of exon skipping was mostly on the exon 3, as is evident from the fact that 30% of AML cases studied had deletion of exon 3 alone. So, it can be concluded that deletion of exon 3 has more effect on the leukemogenesis of AML (Harada et al., 1994).

Out of 50 AML patients selected for study, only 4(8%) had deletion in all the three exons studied; 15(30%) had a single deletion of exon 3; 8(16%) had a deletion of 2 and 3 exon; 2(4%) had deletion of exon 3 and 4; and 2 patients (4%) had a deletion of exon 2 alone. Of interest, IRF-1 gene deletion was not observed in 19(38%) patients. Exon 3 deletion is involved in all the cases of deletion (except for 2 where other exons were involved), providing insight into its critical role in leukemogenesis.

The overall frequency of IRF-1 gene deletion, in our study, was found more in males than in females ($p=0.0184$), in consistence with the fact that more males are effected by AML than females. The dwelling of the patient, whether rural or urban had no significance with IRF-1 deletion ($p=0.16$). Most studies have found a higher incidence of AML in males although the male predominance is not as distinct as in ALL. In our series also, a slight male preponderance was present, with a male to female ratio of 1.38:1.

The incidence of AML progressively increases with age, and in adults (over the age of 65 years), the incidence is approximately 30 times the incidence of AML in children (Bhatia and Neglia, 1995). In our study, 17(34%) patient fall in the age group of 2-18yr; 20(40%) in the age group of 19- 36yr; and 13(26%) in the age group of 37-55yr. These figures indicate that AML cases in Kashmiri population fall in a younger age group, with mean age of 27.18year, very low in comparison to western populations,

where AML is considered as a disease of elderly. Age of male and female AML patients in our study, differ insignificantly from each other ($p=0.8$).

The commonest AML subtype in our series was AML-M2 at 64% which is quite higher than frequency of 27-29% reported in the literature. A higher frequency of AML-M2 in both pediatric and adult population was also recorded in a study conducted on Indian population at TATA Memorial Hospital (Advani et al., 1983; Ghosh et al., 2003; Jaffe et al., 2001). The incidence of AML-M1 is 14%, less than the frequency of 20% reported. The incidence of APML in our study was 12% and slightly higher than the reported frequency in the literature at 5-10% (Weinstein, 1999; Miller and Daoust, 2000; Jaffe, 2001). 6% of the cases were biphenotypic and 2(4%) cases were each of M0 and M7. There was no case of AML-M4 and M5 in our series.

The complete blood count of leukemia patients differ significantly from the controls. Mean Hb in case of AML patients was 7.898 (indicating anemia), significantly less from the mean of controls, 11.8 ($p<0.0001$). The mean TLC of AML patients was $32.71 \times 10^3/\mu\text{L}$ (leukocytosis), which as expected, was very high in comparison to the mean of controls, $6.632 \times 10^3/\mu\text{L}$ ($p=0.003$). Mean platelet count in AML patients was 96.19 (revealing thrombocytopenia) which, as in case of Hb, is significantly low in comparison to controls 148 ($p=0.007$). The comparison of these three components among AML men and women was insignificant (Greenberg et al., 1997).

Our study, to the best of our knowledge, is the first study on leukemia at molecular level in Kashmir. We analyze the effect of IRF-1 deletion on AML patients, among Kashmiri population. But need is to conduct such studies on a large population, as they provide insights into the biology and molecular basis of AML which would result in the improvement of the understanding of the disease as well as help to develop newer treatment strategies.

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