RESEARCH ARTICLE

Lack of KRAS Gene Mutations in Chronic Myeloid Leukemia in Iran

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Abstract

Background: The single most common proto-oncogene change in human neoplasms is a point mutation in RAS genes. A wide range of variation in frequency of KRAS mutations has been seen in hematologic malignancies. Despite this, RAS roles in leukemogenesis remain unclear. The frequency of KRAS mutations in CML has been reported to be between zero an 10%. Many attempts have been done to develop an anti-RAS drug as a therapeutic target. . Materials and Methods: This cross sectional study was performed in Mashhad University of Medical Sciences, Mashhad, Iran from 2010-2012. In 78 CML patients (diagnosed according to WHO 2008 criteria) in chronic or accelerated phases, KRAS mutations in codons 12 and 13 were analyzed using a modified PCR-restriction fragment length polymorphism (RFLP) method. Results: We did not detect any KRAS mutations in this study. Conclusions: KRAS mutations are overall rare in early phase CML and might be secondary events happening late in leukemogenesis cooperating with initial genetic lesions.

Keywords: Leukemia - myelogenous - chronic - BCR-ABL positive - KRAS protein - mutation

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Introduction

A neoplasm is created by the clonal proliferation of a precursor cell with genetic damage. Four groups of normal regulatory genes including proto-oncogenes, tumor suppressor genes, genes regulating apoptosis and genes involved in DNA repair are the basic targets of this damage. Oncogenes are formed by genetic alteration such as mutations in proto-oncogenes and Proteins encoded by them, oncoproteins, can promote cell proliferation in the absence of growth signals. The RAS proto-oncogenes are consist of three genes including HRAS, KRAS and NRAS. RAS is a family member of small G proteins binded guanosine triphosphat (GTP) and guanosine diphosphate (GDP) (Stricker et al., 2010). In the inactive form, it binds GDP, but cell Stimulation by growth factors cause, inactive (GDP-bound) form is activated to a GTP-bound state. Activated RAS stimulates RAF and mitogen-activated protein (MAP) kinase cascade to transmit growth signals to the nucleus (Chan et al., 2004; Stricker et al., 2010). RAS has a basic role in signal transmission of growth factor receptors within cytoplasm and the activated form stimulates downstream regulators of proliferation.

The proper working of the RAS protein depends on two reactions: *i*) exchanging of GDP by GTP which activates RAS protein; *ii*) Hydrolysis of GTP which changes the GTP-bound, active form, to the GDP-bound, inactive form. These reactions are regulated by other proteins. The family of guanine nucleotide–releasing proteins catalyses exchanging of GDP by GTP and Conversely, GTPase-activating proteins (GAPs) accelerate the GTPase activity that is an intrinsic function in normal RAS proteins. Therefore, GAPs function inhibits uncontrolled RAS activity. RAS mutations can affect either the GTP-binding pocket or enzymatic area necessary for GTP hydrolysis resulting markedly reduction the GTPase activity of the RAS protein. The mutated variant of RAS leads to continuous stimulation of cells resulting independent proliferation of cells such as hematopoietic progenitor cells (Chan et al., 2004; Liang et al., 2006; Stricker et al., 2010).

The single most common proto-oncogenes disorder in human neoplasms is Point mutation of RAS genes. About 15-20% of tumors contain RAS oncoproteins. The RAS mutation frequencies vary in different tumors. For example, 90% of pancreatic adenocarcinomas and 30% of myeloid leukemia contain the RAS mutation (Stricker et al., 2010). The RAS protein is activated in many hematopoietic growth factor signaling and in hematologic neoplasms and a wide range of variation in frequency of RAS mutations have been observed in hematologic neoplasms (Ahuja et al., 1990; Braun et al., 2004); however RAS role in leukemogenesis is not completely clear (Baum and Ren, 2008). The RAS mutations are among the most common mutations in acute myeloid leukemia (AML) seen in about 25-44% of AML

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patients and among RAS mutations, KRAS mutations are observed in 10-15% of these patients (Chan et al., 2004). Frequencies of KRAS mutations in CML have been reported between zero to10% (LeMaistre et al., 1989; Ahuja et al., 1990; Serra et al., 1993). Because RAS is frequently mutated in varieties of neoplasms, many attempts have been done to develop an anti-RAS drug as a therapeutic target (Baum and Ren, 2008; McCubrey et al., 2008; Mansi et al., 2011; Zhu et al., 2012). The Ras/Raf/MAP/extracellular signal-regulated kinase (ERK) pathway often contributes in sensitivity and resistance to leukemia chemotherapy and abnormal expression of this pathway may cause drug resistance during leukemia therapy. For example failing or losing response to imatinib drug in CML patients may be due to RAS mutation (Pavlu et al., 2007). Therefore, detection and controlling the expression of this pathway could improve chemotrapy treatment in leukemia (Steelman et al., 2011; Stoppa et al., 2012). So, in this study we assessed the frequency of KRAS mutation (codon 12, 13) in CML.

Materials and Methods

Samples

This cross sectional study was financially supported by a research grant from Mashhad University of Medical Sciences and performed in molecular pathology and cytogenetic laboratory of Ghaem hospital (a major teaching hospital located in Mashhad, Northeast Iran) from 2010-2012. After approving by the local ethical committee, obtaining informed consent and a short medical history from patients, 10 mililiter blood was taken in EDTA-K2 tubes. For all patients complete blood counts (CBC) were performed and peripheral blood smears were prepared and differentiated cell counts were determined. After that, DNA and RNA were extracted according to standard methods and nested reverse transcriptase polymerase chain reaction (RT-PCR) analysis for BCR-ABL fusion gene was carried out by ABI Veriti PCR Machine (Applied Biosystems, USA). Patients with chronic or accelerated phase of CML were included in the study. According to WHO 2008 criteria for diagnosis of chronic myeloid leukemia (CML), BCR-ABL positive patients with leukocytosis and increased granulocytes and their precursors with or without thrombocytosis were diagnosed as CML (Bain et al., 2010). Patients who had WHO 2008 criteria for polycythemia vera, primary myelofibrosis, essential thrombocythemia, atypical CML (BCR-ABL negative) and other myeloprolifrative neoplasms were excluded from the study. Out of 88 patients, 10 patients were excluded from the study and finally KRAS mutations are assessed in 78 patients.

Detection of KRAS mutations

For the detection of KRAS point mutations, DNA samples were analyzed by using PCR-restriction fragment length polymorphism (RFLP) for both codon 12 and 13 according to a method presented by Nagasaka et al. (2004). Sensitivity of PCR-RFLP test was achieved by employing a two-stage procedure. The primers used (K12&13F and Kwt-R) For the first stage of PCR created restriction sites for a MvaI and a BglI for codon 12 and the wild allele of codon 13, respectively (Table 1). First stage of PCR was performed as 95°C for 11 minutes and then by 30 cycles of amplification as follow: denaturation at 95°C for 30s, annealing at 55°C for 30s, extension at 72°C for 30s and the final extension at 72°C for 5 minutes. After that, Aliquots (5μL) of the first product were digested with 10 units of MvaI (Fermentas, Lithuania) and 8 units of BglI (Fermentas, Lithuania) at 37°C for 3 hours, respectively.

For the second stage of PCR, aliquots (1μL) of MvaI and BglI digests were used for mutation detection in codons 12 and 13, respectively. For PCR of codon 12, K12&13F and 12mt-R primers and for codon 13, the K12&13F and 13mt-R primers were utilized (Table 1). PCR conditions in the second stage PCR was like the first one. Then, products of second-stage PCR for the KRAS codon 12 and 13 were digested at 37°C for more than 6

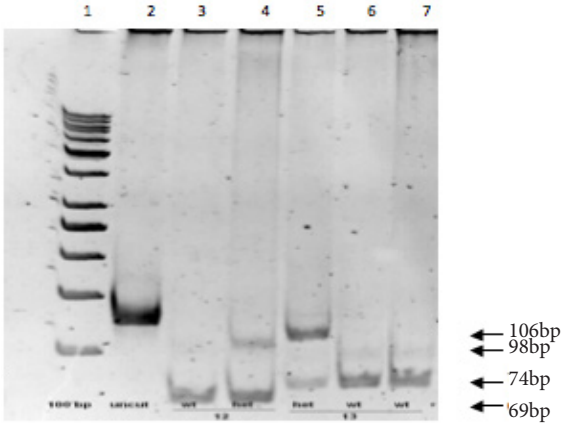

Figure 1. Photograph of Et- Br-stained 12.5% Polyacrylamide Gel Electrophoresis Demonstrating PCR-RFLP Analysis for Codon 12&13 of KRAS Mutations. RFLP analysis for KARS codon 12 led to generation of 69 bp , 29 bp and 22 bp fragmants after MVaI digestion in wild type (Wt) of codon 12 (Lane 3). [Bands of 29bp and 22bp are not seen] however, presence of 98bp and 69 bp bands as a positive control revealed heterozygous mutation states of coden 12 (Lane 4). Generation of 74bp fragment by Bg1I in the case of a wild type codon 13 is shown in lane 6 and 7 (bands of 32bp and 14bp are not seen) and 106bp and 74bp fragments bands in the case of heterozygous mutation status of codon 13 as a positive control is shown in lane 5

Table 1. Primer Sequences for PCR-RFLP Analysis for KRAS Gene Mutation in Codone of 12 and 13 in Chronic Myeloid Leukemia

Primer Name	Primer Sequence (5'-3')	Position*
K12&13F	ACTGAATATAAACTTGTGGTAGTTGGCCCT	18157260-18157289
Kwt-R	AACAAGATTTACCTCTATTGTTGGATCA	18157170-18157197
12mt-R	AACAAGATTTACCTCTATTCCTGGATCA	18157170-18157197
13mt-R	AACAAGATTTGCCTCTATGGCTGGATCA	18157170-18157197

*The nucleotide numbering for KRAS refers to NT_009714.16

hours with MvaI and BglI for codon 12 and codon 13, respectively. Amplification products were characterized after polyacrylamide gel electrophoresis. This analysis method for KRAS codon 12 mutation after MvaI digestion creates 69-bp, 29-bp, and 22-bp fragment if we do not have mutation and 98-bp and 22-bp fragments if we have. Similar analysis for codon 13 mutation creates 74-bp, 32-bp, and 14-bp fragments after BglI digestion if we do not have mutation and 106-bp and 14-bp fragments if we have.

Results

We studied 78 CML patients including 42 (54%) males and 36 (46%) females with an age range of 12-80 years and a mean (±SD) of 47.2 (±1.7) years. We didn't observe any KRAS mutations in codon of 12 and 13 in this study (Figure 1).

Discussion

The literature review shows a wide range of variation in the frequencies of RAS mutations in hematologic neoplasms. For example, the frequencies of RAS mutations vary in AML from zero to 50% (Ahuja et al., 1990; Baum and Ren, 2008; Preston et al., 2010; Sano et al 2012), MDS zero to 40% and CML zero to 33% (Ahuja et al., 1990; Baum and Ren, 2008). Although the reasons for this variability are not certain, it is likely that they reflect the variable sensitivities and specificities of the different techniques used. Alternatively, they may reflect heterogeneity in the patient populations studied or using a statistically insignificant number of patients in the study (Ahuja et al., 1990). Although we studied the higher number of patients compared to most other studies, however, we did not observe any KRAS mutation. This is consistent with other results. Ahuja et al. evaluated the pattern of RAS mutations in 10 patients with chronic phase of CML and 30 patients in blastic crisis by PCR and direct sequencing of exons 1 and 2 of RAS genes. They did not demonstrate any NRAS or KRAS mutation in chronic phase of CML, and NRAS mutation was observed only in two patients in blastic crisis (Ahuja et al., 1990). Tyner et al. (2009) sequenced all coding exons in KRAS, NRAS and HRAS in 329 AML patients, 32 chronic myelomonocytic leukemia (CMML) patients, and 96 healthy people and characterized four "noncanonical" point mutations in seven patients. They, therefore, concluded that RAS mutations, outside those seen at codons 12, 13, and 61, occur in myeloid leukemia and may play a greater role in leukemogenesis suggesting that screening for RAS mutations in neoplasms should include analysis of the all RAS coding area (Tyner et al., 2009).

Another study by LeMaistre et al. also revealed that RAS mutations are infrequent in CML occurring in late stage of CML, myeloid blast crisis (LeMaistre et al., 1989). Some other studies also confirm that KRAS mutation is very infrequent in CML and occurs in the late stage of the disease contributing in transformation to the blast crisis in some patients (Needleman et al., 1989; Watzinger et al., 1994; Serra et al., 1998). Some studies, as an in vitro, have also suggested that RAS activation is important for the lymphopoeisis and erythropoeisiss but not myelopoeisis (Baum and Ren, 2008).

KRAS mutations are seen in diverse myeloid neoplasms including AML with increased blast and bone marrow suppression, MPD that is associated with proliferation of one or more lineages with capability to differentiation and maturation and myelodyspslstic syndroms (MDS) that are characterized by cytopenia and ineffective hematopoiesis. The existence of RAS mutations in these various myeloid neoplasms shows that RAS mutations are not initiation event in leukemogenesis and probability they are secondary events cooperating with initial genetic damages (Braun et al., 2004). MDS and MPD commonly evolve to AML probably due to the acquisition of collaborating mutations. NRAS or KRAS mutations happen in about 20% of AML patients, and deregulation of RAS signaling by mutations in the FLT3 and c-Kit receptor tyrosine kinases genes are observed in an additional 25-40% of patients. Therefore, hyperactive RAS play a role in myeloid leukemogenesis (Braun and Shannon, 2008).

In conclusion, KRAS mutations are rare events in early stage of CML and they are probably secondary events occur late in leukemogenesis cooperating with initial genetic damages.

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