

RESEARCH ARTICLE

Frequency of K-RAS and N-RAS Gene Mutations in Colorectal Cancers in Southeastern Iran

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Abstract

Background: K-RAS and N-RAS gene mutations cause resistance to treatment in patients with colorectal cancer. Based on this, awareness of mutation of these genes is considered a clinically important step towards better diagnosis and appropriate treatment. **Materials and Methods:** Fifty paraffin-embedded blocks of colorectal cancer were obtained from Imam Reza Hospital of Birjand, Iran. Following DNA extraction, the samples were analyzed for common mutations of exons 2, 3 and 4 of KRAS and NRAS genes using real time PCR and pyrosequencing. **Results:** According to this study, the prevalence of mutations was respectively 28% (14 out of 50) and 2% (1 out of 50) in KRAS and NRAS genes. All the mutations were observed in patients >50 years old. **Conclusions:** Mutations were found in both KRAS and NRAS genes in colorectal cancers in Iranian patients. Determining the frequency of these mutations in each geographical region may be necessary to benefit from targeted cancer therapy.

Keywords: Colorectal cancer - mutation - KRAS - NRAS - Birjand

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Introduction

Colorectal cancer (CRC) that refers both to the colon and rectum cancers is known as the third common cancer as well as the second leading cause of mortality in the world (Abuli et al., 2011). This disease is the third cause of mortality among women after lung cancer and breast cancer. It is also known as the second cause of death next to prostate cancer in men. The risk of developing this disease is approximately 6% for any individual (Chen et al., 2014). Colorectal cancer is the third common cancer in women and the fifth type of cancer common among men (Golfam et al., 2013).

Genetic and environmental factors are regarded as the main contributors to the pathogenesis of this disease (Abuli et al., 2011; Chen et al., 2014). Furthermore, approximately 10% of all colon cancers are hereditary and result from specific genetic changes (Shen et al., 2013).

The early stages of this type of cancer start with colon polyps, but after a while these polyps lead to disorders in the growth of the cells and colon wall repair. The genetic mechanism to convert normal epithelium to proliferating epithelium in this pathway starts with mutation in Adenomatous Polyposis Coli (APC) gene. Moreover, as the proliferating epithelium is grown into adenoma, this mechanism is associated with changes in DNA

methylation. Mutations also occur in KRAS and NRAS genes in the adenoma stage, and finally transformation of adenoma to carcinoma results from mutation in P53. For their high prevalence in colorectal tumors, mutations in APC, KRAS, and P53 are known as molecular diagnostic markers for CRC. However, as small adenomas can often have mutations in APC and more than 90% of adenomas do not lead to malignancy, APCs are not suitable diagnostic markers for colorectal cancer. P53 mutations also occur at the advanced stages of malignancy; hence, they are not suitable for diagnosis at the early stages. However, KRAS mutation is diagnosable in the benign adenoma stage which is highly important in disease control and prevention (Edalat et al., 1385).

KRAS and NRAS are GTPase proteins which transmit extracellular signals to the nucleus (Trevisiol et al., 2006; Neumann et al., 2009; Tan and Du, 2012). KRAS acts as a membrane-bound intracellular molecular switch and transforms the external received signals to intracellular signals through cell membrane receptors (i.e. it relays signals from outside the cell to the nucleus of the cell). These signals are thought of as commands to the cell to grow, divide and, in some cases, to be mature. Therefore, disorder in this pathway is an indicator of the risk of developing cancer.

KRAS gene is located on the short (p) arm of

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chromosome 12 and NRAS is located on the chromosome 1 at position 13.1 (Alshahid et al., 2013; De Stefano and Carlomagno, 2014). Compared to NRAS mutations with a frequency of 3-5%, KRAS mutations with a 40% presence in colorectal cancers play a more significant role in the incidence of CRC (4). Around 90-95% of KRAS mutations are found in codons 12 and 13 (exon 2) and the mutation of other codons such as 61 (exon 3), 117 and 146 (exon 4) are less common (Douillard et al., 2013; Er et al., 2014). Mutation in NRAS gene also causes an apoptosis function which leads to the development of colorectal cancer (Wang et al., 2013). These mutations mostly occur in codons 12 and 13 (exon 2), 61 (exon 3), 117 and 146 (exon 4) (Douillard et al., 2013; Er et al., 2014). Moreover, there is a little chance for cancer cells with mutated forms of KRAS and NRAS genes to respond to treatment by anti-EGFR monoclonal antibodies, and cancer and metastasis may continue to progress in spite of medication. Examples of such antibodies are cetuximab and panitumumab. Therefore, there appears to be no favorable response to treatment in individuals eligible for NRAS and KRAS gene mutation due to drug resistance (Er et al., 2014; Pietrantonio et al., 2015). Based on this, the presence or absence of mutations of KRAS and NRAS genes in a patient must be primarily determined by the specialist prior to treatment to avoid excessive costs and time expenditure associated with inefficient medication. For this reason, the Food and Drug Administration (FDA) allocated a label for the given medications in 2009 which indicated that they are not useful for patients with mutations in NRAS and KRAS genes (Er et al., 2014).

In sum, awareness of the presence and absence of mutation in KRAS and NRAS genes in CRC patients can be an efficient guide for specialist physicians in adopting the best treatment alternatives during different stages of the disease, especially in the primary steps. Moreover, awareness of the frequency and typical forms of mutation in KRAS and NRAS genes in a group of patients, that can be a predictor of the effect (or lack of effect) of a medication, reduces the time required to determine the appropriate treatment protocol for a patient, and hence reduction of time waste during treatment leads to increased chance for recovery. Therefore, as regards the inaccessibility of information on the frequency and type of mutations in KRAS and NRAS genes in the population of Birjand – a fact which originates from the lack of a study on this issue in Birjand – the present research was designed to investigate the given mutations in CRC patients in this eastern city of Iran. In doing so, the study sought to take a positive step towards determining the most appropriate treatment for patients referring to Birjand-based healthcare clinics based on their genetic characteristics.

Materials and Methods

Sampling

This study investigated the FFPE sample tissues of CRC patients who referred to Imam Reza Hospital of Birjand during 2009-2014. The pathologist had already confirmed the presence of malignant cancer in the patients. The samples of the study included 50 tissue sections (2-4

mm). The definitive diagnosis of cancer was the main criterion for including the patients in the study. After coding, the samples were transferred to the laboratory of Research Center of Birjand University of Medical Sciences. Given the unavailability of the patients, it was considered impossible to take the written consent. The study protocol was approved by the Ethics Committee of the university in conformity with the “Guide for the Practice of Ethics in Research” (Available: <http://www.kacst.edu.sa/ar/depts/bioethics/Pages/home.aspx>.) (Ethics Code Number IR.BUMS.1394.89). Based on this, it was agreed that no written consent was necessary for this retrospective study, that patients’ information would remain absolutely confidential, and that the study would cause no harm to the patients.

Dna extraction and determining the genotype

DNA extraction from the samples was conducted using salting out procedure and proteinase K based on Sengüven’s method (Sengüven et al., 2014). During the extraction stages, first the tissues were deparaffinized

Table 1. Demographic Characteristics of Patients under Study

Factor	Frequency	Percent	P-value	
Sex	Man	35	70%	
	Woman	15	30%	P=0/36
Age	50≥	43	86%	
	50<	7	14%	P=0/24
	Colon	26	52%	
Site of Tumor	Cecum	5	10%	
	Sigmoid	3	6%	P=0/34
	Rectal	13	26%	
	Ileum	3	6%	
	Vessels	12	24%	
Metastasis	Nerves	1	2%	
	Muscle	5	10%	
	Vessels and nerves	3	6%	
	Serous	2	4%	P=0/52
	Other tissue was not observed	1	2%	
	Unknown	10	20%	
	T3	16	32%	
	T3N0	11	22%	
	T3N1	3	6%	
	B	2	4%	
	B2	1	2%	
A	2	4%	P=0/56	
Stage	C1	1	2%	
	T2	1	2%	
	T2N0	2	4%	
	Unknown	1	2%	
	Well differentiated	26	52%	
	moderately differentiated	15	30%	
	differently differentiated	18	36%	
Grade	differently differentiated	4	8%	P=0/81
	not seen			
	Unknown	13	26%	

Table 2. Frequency of the Observed Mutations in KRAS and NRAS Genes in CRCs in Birjand Patients

Gene	Mutation	Exon	Codon	Frequency (percent)		mean (age) \pm SD	P-value
KRAS	P.Gly12Asp (c.35G> A)	2	12	Man	3(8/6%)	69/3 \pm 10/5	P=0/38
				Woman	2(13/3%)	64/5 \pm 10/6	
	Gly13ASP (c.38G> A)	2	13	Man	2(5/7%)	68/5 \pm 20/5	
				Woman	2(13/3%)	55/5 \pm 7/7	
NRAS	P.Gly12Val (c.35G> T)	2	12	Man	5(14/3%)	70 \pm 8/3	P=0/3
				Woman	0	0	
	A146Thr(c.436G>A)	3	146	Man	0	0	
				Woman	1(6/7%)	46	

using absolute xylene and then varying concentrations of ethanol (50%, 80%, and 100%) were added to remove paraffin and to gradually hydrate the tissues. Next stages included adding Tissue Lysis Buffer (TLB), K proteinase, cold NaCl and isopropanol. Finally, after the complete extraction of DNA, the samples were transferred to freezer (-20°C). The concentration and quality of the extracted DNAs were assessed using Nanodrop Spectrophotometer BioTek, USA EPOCH which was provided by the Research Laboratory of Birjand University of Medical Sciences.

Exons 2, 3 and 4 of KRAS gene (including codons 12, 13, 59, 61, 146 and 117) were focused on to study mutations G12R(c.34G>A), C12S(c.34G>A), G12C(c.34G>T), C12D(c.35G>A), G12A(c.35G>C), G12V(c.35G>T), G13D(c.38G>A) in exon 2, mutations A59T(c.175G>A), A59G(c.175C>A), Q61E(c.181C>G), Q61K(c.181C>A), Q61L(c.182A>T), Q61R(c.182A>G), Q61H(c.183A>C), Q61H(c.183A>T) in exon 3, and mutations A146P(c.436G>C), A146T(c.436G>A), A146V(c.437C>T), K117N(c.351A>C), K117N(c.351A>T) in exon 4 of KRAS. The investigations were performed in Tehran Partolab Pathobiology and Molecular Diagnostics laboratory by Real Time PCR and Roche PCR 480. The pyrosequencing was conducted using PyroMark Q96 ID QIAGEN sw 2.5 system.

Similarly, mutations G12R(c.34G>A), C12S(c.34G>A), G12C(c.34G>T), C12D(c.35G>A), G12W(c.[34G>T,36G>T]), G12A(c.35G>C), G12D(c.35G>A), G13D(c.38G>A), G12S(c.34G>A), G13C(c.37G>T), G13E(c.37G>C), G13R(c.37G>C), G13V(c.38G>T) and G12N(c.34-35GG>AA) in the region of exon 2 and mutations A59T(c.175G>A), A59G(c.175C>A), Q61E(c.181C>G), Q61K(c.181C>A), Q61L(c.182A>T), Q61R(c.182A>G), Q61H(c.183A>C), Q61H(c.183A>T) in the region of exon 3, and mutations A146P(c.436G>C), A146T(c.436G>A), A146V(c.437C>T), K117N(c.351A>T) for exon 4 of NRAS gene were also examined in Partolab laboratory.

Data concerning the relationship between mutation in KRAS and NRAS genes and factors like age, sex, location of tumor, tissue under attack, stage of disease, and tumor grade were analyzed by SPSS (version 22) using Crosstabs-Chi square test. The significance level was set at P<0.05.

Results

This study was conducted on the cancer tissues of 50

patients with colorectal cancer who had referred to Imam Reza Hospital of Birjand. The mean age of the patients was 62.17 \pm 14.18 and 59.4 \pm 10.2 respectively for men and women. The other demographic characteristics of the patients are offered in Table 1.

Out of 50 samples that were sequenced, a total of 15 patients (i.e. 30% of the whole population) were detected with mutation in KRAS and NRAS genes. Of the observed mutations, 20% related to KRAS gene and 10% were associated with NRAS gene. As regards the KRAS gene, the observed mutations had only occurred in exon 2 (codons 12 and 13) and mutation in NRAS gene was only found in exon 3 (codon 146). In sum, the frequency of men with mutation in this exon was quite higher than women (20% men vs. 8% women). Based on the investigations conducted, there was no significant difference between factors as age, sex, tumor location, attacked tissue, stage of disease and tumor grade with the observed mutations (P>0.05) (Table 1). Table 2 shows the frequency of the observed mutations in KRAS and NRAS genes for the population under study in terms of gender. The results showed that 100% of the mutations occurred in patients above 50 years of age.

Discussion

CRC is a multi-factor disease that develops under the influence of genetic and environmental factors. Mutation in NRAS and KRAS genes are among the most important genetic contributors to the occurrence of the disease. Mutation in NRAS gene is also found in other types of cancer besides CRC and is linked to different stages of disease including tumor growth, development, spread, metastasis, as well as response to treatment (Lari et al., 1390; Abuli et al., 2011). Such a mutation causes the spread of cancer through antiapoptotic function. However, as NRAS mutations have been reported only in about 3-5% of colorectal cancers, they play a less important role in CRC compared with KRAS mutations (Arrington et al., 2012; Shen et al., 2013; Wang et al., 2013).

In the present study, the paraffin-embedded tissues related to 50 CRC patients (with the mean age of 61.3 \pm 13) revealed that 30% of the population had undergone mutation in KRAS gene, while the remaining 70% had no mutation in this type of gene. Such a result is compatible with other international studies that have reported a 30-40% or, at times, a 50% mutation in KRAS codon for CRC (Amado et al., 2008; Shemirani et al., 2011). The incidence of mutation in NRAS gene in the population of Birjand with a 2% frequency was significantly lower than

the observed frequency for KRAS mutations. Totally, out of the 30% of the patients with mutation, 20% belonged to men and 10% to women. Therefore, in this study, the incidence of mutation was twice as high in men compared to women. Additionally, almost one hundred percent of the observed mutations were detected in patients over 50 years of age.

The multiplicity of studies conducted in various regions of Iran and the world as well as the diversity of the findings further illuminates the importance of investigating the given mutations in the incidence of cancer. In this regard, Dolatkah et al (2015), who explored the prevalent mutations in KRAS and BRAF genes of CRC patients in Tabriz, studied 30 tissues of these patients. The results of their study revealed that 6 patients (20%) experienced heterozygous mutation in KRAS gene in the tumoral tissue and the remaining 80% were normal (Roya Dolatkah et al., 2015). Another study (2012) on the population of Mashad (north eastern Iran) which investigated the mutation in codon 13 of KRAS gene in 54 cancer patients' paraffin blocks showed that 18.5% of the patients with this type of cancer had KRAS mutation (Lari et al., 1390). Edalat et al (2007) who aimed to explore the mutation in codon 12 of KRAS gene in 55 CRC patients also demonstrated that the prevalence of mutation in codon 12 of KRAS gene was about 65% (36 cases) (Edalat et al., 1385). Such a prevalence rate is higher than the ones reported in the majority of studies in Iran including the present one on the population of Birjand.

An investigation on the population of Tehran by Sobhani et al (2011) showed that 20.3% of the patients had mutation of codon 12 and 3.3% of them were shown to have codon 13 mutation (Sobhani et al., 1389). Following this study, a research by Shemirani et al (2011) in Tehran showed that in 95 biopsies (including 47 tumors and 48 polyps) of patients with cancer, there were 5 mutations in tumoral tissues related to codon 12 of KRAS gene (12.5%) and 1 mutation in codon 13 of this gene. Also, in polyp biopsies, 2 mutations were observed in codon 13 and 1 mutation in codon 12. In sum, 83.4% of mutations in the given study appeared in women and 16.6% in men (Shemirani et al., 2011). This finding is contrary to the figures related to the population of Birjand where the frequency of the mutation incidence in men was 20% and twice than in women.

To the best of our knowledge, no research has as yet investigated the frequency of the incidence of mutations in NRAS gene. In this regard, a study focusing on the population of Birjand to determine the frequency of the probable mutations in this gene would be the first presented statistics on a group from among the Iranian populations.

Wider scope studies are also available concerning these mutations at the global level. One of such studies is the investigation conducted by Chretien (2012) in France. The findings of this study revealed that 38% of people had mutations in KRAS gene out of which 82.4% happened in codon 12 and 17.6% occurred in codon 13 (Chretien et al., 2013). Totally, the frequency of the observed mutations for the population of France was higher for codon 12 than codon 13 which is fairly similar to our study.

A study on mutations of codon 13 of KRAS gene by Chen et al (2014) in China demonstrated that from among 214 paraffin-embedded tissues of cancer patients investigated, 25 cases had mutations in codon 13 and 71 cases showed mutations in codon 12 of KRAS gene (Chen et al., 2014). Two studies were also carried out on the populations of Greece and India in 2007 and 2012 respectively to investigate these mutations. In the 61 cases selected from the Greek population, the frequency of the mutation in the codon 12 of KRAS gene was 28.3% (Symvoulakis et al., 2007). Moreover, in the 100 CRC patients from India 23% and 2% of patients were respectively detected with mutations in KRAS and NRAS genes (Bagadi et al., 2012).

The importance of awareness of the prevalence of mutations in KRAS and NRAS genes lies in the fact that the carriers of mutation in the KRAS gene, despite paying high costs for treatment, are resistant to medicinal treatment and thus their chance for survival is decreased (Er et al., 2014). However, as mutation in KRAS gene can be diagnosed in the early stages of the disease (Edalat et al., 1385), this fact can be used to detect the susceptible individual quickly and to take the required treatment measures for increasing the patient's chance for survival. Statistics have already reported 3000 point mutations in KRAS gene (Shemirani et al., 2011). In the past twenty years, the average overall survival (OS) for patients with metastatic CRC have increased from 10-12 months to 24 months on the whole, a part of which was due to the new therapeutic agents approved (Wolpin and Bass, 2014).

Many studies have been conducted in this regard which include the investigation by Lievre et al (2006). For the first time, they showed that there is a significant relation between mutation in KRAS gene, response of the CRC patients to treatment by cetuximab, and survival in patients. In fact, out of the 30 patients studied in their study, 13 cases (43%) had KRAS gene mutation and were considerably faced with lack of response to treatment by cetuximab ($p=0.0003$). The overall survival of patients eligible for mutation was significantly lower than the patients without mutation in the study ($p=0.016$) (Lievre et al., 2006). Amado (2008) showed that from among 427 patients with metastatic CRC, the response rates to treatment by panitumumab in the groups with wild-type and mutant KRAS were 17% and 0% respectively, and the overall survival was higher in the wild-type group. Therefore, the effectiveness of treatment by panitumumab in CRC patients is limited to patients with wild-type KRAS tumor. Based on this, the status of KRAS should be considered in the selection of CRC patients for treatment using panitumumab (Amado et al., 2008). Hence, it can be said that resistance to treatment might be high among the population of Birjand making it necessary to examine patients with regard to KRAS gene prior to treatment.

Nonetheless, it has been shown that although about 40-60% of the CRC cancers had the wild-type KRAS, response rate to the medicinal treatment by cetuximab was 10% in them and the positive results of the treatment did not exceed 23% even in combination with chemotherapy (Herrerros-Villanueva et al., 2011; Er et al., 2014). The likely reason for this might be the fact that as very few cells

undergo mutation in the early stages of KRAS which are hardly diagnosable, the majority of the cells show the wild-type allele. Hence, despite the prescription of medication, the expected response is not achieved. Therefore, it is concluded that absence of KRAS gene mutation is not a guarantee to initiate treatment by panitumumab and cetuximab. In case the KRAS gene is normal, testing NRAS mutation is considered necessary (Er et al., 2014).

An investigation of biomarkers affecting the prediction of response to therapeutic agents in CRC are the bases of many clinical studies. Furthermore, the functional differences of the KRAS and NRAS genes in the normal and mutant states in the incidence, metastasis and response to treatment in multiple types of cancers, particularly CRC, have made the detection of these genes highly significant in oncology. Hence, as regards the observed frequency for the NRAS and KRAS mutations, especially mutation of the codons 12 and 13 of KRAS gene in the present study on the one hand, and as for the importance of the effect of these mutations on the lack of response to the treatment in CRC patients on the other, quick and inexpensive methods like PCR and RFLP can be used, instead of the costly direct sequencing, for the minimal diagnosis of these limited and important mutations in patients referring to oncology clinics.

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