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Alterations of KRAS Exon 2 Codon 12/13 Mutation Status in Prostatic Adenocarcinoma; Bioinformatics Aspects

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Abstract

Prostate cancer is one of the most common cancers which develops by mutations or/and other genetic alterations in specific genes. According to the recent studies there are predominant mutations occur in KRAS gene in different types of cancers. Missense mutations in codon 12 and codon 13 of KRAS proto-oncogene are main point mutations that happen in about 20% of diverse malignancies in human. The aim of this study is to find out the prevalence of two known main point mutations of KRAS gene (p.G12V/c.35G>T and p.G13D/c.38G>A) in addition to bioinformatics survey of its in Iranian patients with prostatic adenocarcinoma. A total of 35 prostatic adenocarcinoma fresh tissue samples that enriched in neoplastic cells, were obtained from the Cancer Institute of Iran. The presences of mutations at codons 12 and 13 of KRAS were investigated by direct Sanger sequencing. To investigate of the role of the mentioned mutations on structure and protein function, bioinformatics assessments were performed by using SWISS-MODEL server and the PSIPRED Protein Sequence Analysis software. KRAS mutations were detected in 2 of 35 patients (5.7%). Two cases carried the homozygote mutations on exon 2 in codon 12 (G12V) and codon 13 (G13D), respectively. Several prediction programs such as SIFT, Mutation Taster and polyphen-2 classified them as pathogenic. However, the bioinformatics survey shows that amino acid changes on codons 12/13 do not cause any probable disorder on protein structure, but effect on protein function. The result indicated that the protein function is modified. Based on the group of patients with prostate adenocarcinoma our study indicates that p.G12V/c.35G>T and p.G13D/c.38G>A mutations in codons 12 and 13 KRAS are most frequently occur in prostate carcinomas. The bioinformatics results from this study demonstrate that KRAS may have effects on pathogenesis of prostate cancer.

Keywords: Prostate cancer; KRAS; Mutation; Bioinformatics

Introduction

The occurrence of prostate cancer (PC) is increasing worldwide, with strong dissimilarity among different regions. It is one of the most common and leading causes of cancers deaths in many developed countries [1]. Also it is less common in developing countries, somewhat due to lack of nationwide screening program and not high quality cancer registration system [2,3], however, its incidence and mortality has been on the rise [4]. It is a major cause of morbidity and mortality in Iran [5,6].

Worldwide, prostate cancer is the second most frequently diagnosed cancer and the fifth leading cause of death from cancer in men according to the WHO GLOBOCAN database (2012). Regardless of the recent advent of anticancer agents, there is still no cure for the advanced stage of the disease. The “gold standard” treatment for metastatic prostate cancer is androgen deprivation therapy (ADT) [7].

Nevertheless, resistance to ADT known as castrate-resistant prostate cancer (CRPC) is a main problem in prostate cancer treatment [8]. Currently, the molecular mechanisms responsible for PC development, progression and hormone-independence are not clear yet. Several findings suggest that alterations of different pathways involving growth factor receptors play a role in this multistep process [9].

Alteration of RAS/RAF/MEK/MAPK pathway is important in many tumor types and mediates cellular responses to growth signals, differentiation, and programmed cell death [10]. Constitutive activation of this pathway because of mutation of

upstream targets such as KRAS and BRAF has been seen in various human cancers, including prostate carcinoma in human [11].

KRAS is a proto-oncogene, functions downstream of EGFR induced cell signaling. Mutations in RAS/RAF/MAPK pathways lead to resistance to anti-EGFR targeted therapies [12].

In general, rare and/or no structural point mutation was reported for proto-oncogene BRAF gene but the various type and rate of KRAS mutations were reported in prostate carcinoma [13]. The clinical pattern of prostate cancer vagaries remarkably over the past few years and a leading cause of death among men (10%) in Western countries.

The tumor differentiation has a profound effect on the expression of serum PSA but in some complicated cases PSA levels do not alone reflect tumor burden precisely. The clinical profiles such as; tumor stage, Gleason score (GS) and serum prostate specific antigen (PSA) levels are usually used to predict pathogenesis of tumors in patients with localized PC. Consistent screening of PSA shows a decline in the mortality frequency due to primary discovery and treatment but need support by new methods to improvement specification of tumor type, stage and etiological parameters during the therapy periods [14].

KRAS mutations are relatively frequent in carcinomas of pancreas and colon; often affecting codons 12/13in the gene [15,16] also, KRAS mutations were reported in prostate carcinoma in different populations [17-22]. KRAS mutations are associated with a decreased response to EGFR tyrosine kinase inhibitors (TKIs) in NSCLC [23].

In the current study it was aimed to investigate the prevalence and predictive significance of KRAS mutations in Iranian patients with prostate carcinomas.

Materials and Methods

Sample preparation

A total of 200 prostate samples were collected from the Cancer Institute of Iran, between 2010 and 2013. Out of 200 samples, 35 samples were prostatic adenocarcinoma fresh tissue samples that enriched in selected neoplastic cells. Other 165 samples were Benign Prostatic Hyperplasia (BPH).

All of the participants have given an informed written consent and the study protocol was approved by the Ethics Board of Tehran University of Medical Sciences which was in compliance with the Helsinki declaration.

PCR amplification and Sanger sequencing

The presences of mutations at codons 12 and 13 (of KRAS), were performed in 35 prostatic adenocarcinoma samples by direct Sanger sequencing.

Approximately, 25 mg fresh tumoral samples were used for genomic DNA isolation by the use of DNeasy blood and tissue kit (Qiagen, Valencia, CA).

In vitro amplifications were carried out in a 25 µl reaction volume containing genomic DNA (30–50 ng). The PCR primers were designed using Primer Premier 5.0 software.

For KRAS (exon 2), the primer sequences were:

F: 5`-AAGGTACTGGTGGAGTATTTGA-3`, R: 5`-CACAGAGAGTGAACATCATGGA-3`. Amplification was performed under the following conditions in ABI thermocycler (ABI, Life technologies, USA, LT), the profile consisted of an initial melting step of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final elongation step of 7 min at 72°C. PCR products were purified and sequenced with a BigDye TM Terminator v1.1 Cycle Sequencing kit and ABI 3500 x L Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In case of mutation detection, it was validated by a second PCR and sequencing with reverse primer. The sequencing results were analyzed by chromas software and compared with the reference sequences of the KRAS gene in the NCBI database.

Statistical analyses

Statistical analyses were performed using SPSS software.

Bioinformatics analysis

To investigate of the role of these two mutations on protein function and structure, bioinformatics assessments were done by using SWISS-MODEL server (http://swissmodel.expasy.org) and the PSIPRED Protein Sequence Analysis software (http://bioinf.cs.ucl.ac.uk/psipred). The variants were investigated *in silico* with PolyPhen-2, Mutation Taster and SIFT [1,23].

Results

A total of 35 male patients in the mean age of 68.97 ± 9.64 (53–90) years with prostate carcinomas were clinically diagnosed and treated. Preoperative mean serum PSA level was 24.76 (4–195). Clinico-pathological characteristics are summarized in **Table 1**.

Each tumor was graded and staged according to the Gleason system and the tumor-node-metastasis staging system, respectively [24,25]. The number and tumor stages that investigated in the current study were; 7 in stage 2, 19 in stage 3 and 9 patients in stage 4. Gleason score of patients was between 4 and 8. Histopathologic evaluation revealed 35 adenocarcinoma samples out of 200 prostatic samples and the remaining samples were BPH. Seven patients have low grade cancer with Gleason score 4 and 13 patients have high grade of Gleason score 7 and 8. Mutations of codons 12 and 13 in KRAS gene were found in 2 of the 35 prostate adenocarcinomas.

Case 22, carried homozygote mutation in codon 12 (G12V), was 90 year old man with 9.8 serum PSA level and his Gleason score and tumor stages were 7 and 3, respectively. Also, case 33, carried homozygote mutation in codon 13 (G13D), was 86 year old man that his serum PSA level, Gleason score and

tumor stages were 21, 5 and 3 respectively. *In silico* analysis using PolyPhen-2, SIFT and Mutation Taster predicted that the variants p.G12V/c.35G>T and p.G13D/c.38G>A in KRAS are pathogenic **(Table 2)**.

Table 1 Clinical characteristics of patients with prostate adenocarcinoma.

Case no.	Age	PSA level	Gleason score	T-stage
1	63	6.4	6	3
2	83	60.6	5	3a
3	61	8	7	3
4	61	14.8	5	3a
5	72	7.41	6	3b
6	68	32.8	7	3
7	69	8.9	6	4b
8	57	5.5	4	2
9	63	9	7	3c
10	65	16.26	6	3b
11	55	7.2	4	2a
12	74	10.3	7	3b
13	61	9.3	6	4b
14	76	4	4	2b
15	83	6.1	5	3a
16	72	8	5	3b
17	66	10.9	6	3a
18	76	74.3	7	4b
19	74	40.6	7	4b
20	63	10.5	5	3a
21	64	4.95	4	2
22	90	9.8	`7	3b
23	55	9.24	4	2
24	78	6	7	4b
25	80	78	6	4a
26	77	9.4	6	3a
27	61	69.8	8	4b
28	53	31.38	7	3a
29	61	6.22	4	2
30	82	45.7	7	3b
31	69	15.57	7	4
32	70	195	7	4b
33	86	21	5	3a
34	70	5.8	4	2c

35	56	8	5	3b

Table 2 The variants were studied *in silico* with PolyPhen-2, Mutation Taster and SIFT.

Change	Conserved	Plyphen-2	SIFT	Mutation Tester
p.G12V/c.35G>T	Yes	Damaging	Deleterious	Disease causing
			-0.01	-1
				amino acid sequence changed
				known disease mutation at this position (HGMD CM087372)
				splice site changes
p.G13D/c.38G>A	Yes	Damaging	Deleterious	Disease causing
			0	-0.999
			Listed as SNP	amino acid sequence changed

According to **Figures 1** and **2**, it was determined that the mutations did not fundamentally change the domains of KRAS protein and PSIPRED Protein Sequence Analysis software showed that the amino acid alterations do not change the helix and coil residues of protein but, regarding to bioinformatics results of Molecular Function Predictions Software, it was shown that the mutations have caused fundamental changes on protein function of KRAS protein also, the result indicated that the protein function is modified **(Table 3)**.

Table 3 Molecular function predictions.

	GTP binding		**GTPase activity**	
	(GO:0005525)		**(GO:0003924)**	
	Prob	SVM Reliability	Prob	SVM Reliability
Normal protein	0.862	H	0.854	H
p.G13Dc.38GA	0.884	H	0.887	H
p.G12V/c.35G>T	0.932	H	0.886	H
	Protein dimerization activity		**Metal ion binding**	
	(GO:0046983)	**GO:0046872**		
	Prob	SVM Reliability	Prob	SVM Reliability
Normal protein	0.503	L	0.702	L
p.G13Dc.38GA	0.548	L	0.703	L
p.G12V/c.35G>T	0.521	L	-	-
	Receptor binding		**Identical protein binding**	
	(GO:0005102)	**(GO:0042802)**		
	Prob	SVM Reliability	Prob	SVM Reliability
Normal protein	0.548	H	0.524	L
p.G13Dc.38GA	0.577	H	0.571	L
p.G12V/c.35G>T	-	-	0.553	L

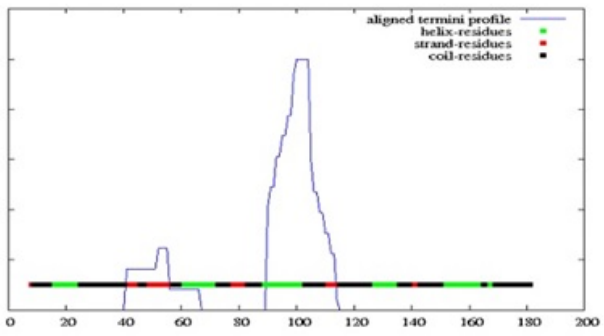

Figure 1A The PSIPRED software showed that the amino acid alterations do not change the helix and coil residues of protein B; this diagram shows that amino acid changes on codons 12/13 do not cause any probable disorder on protein structure.

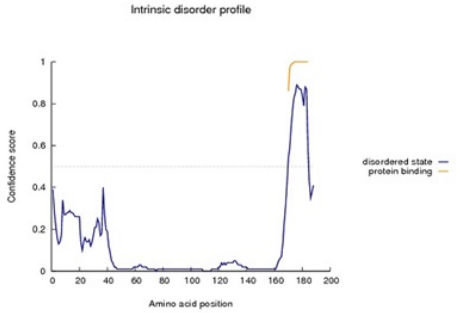

Figure 1B Amino acids in the input sequence are considered disordered when the blue line is above the grey dashed line, that is the confidence score is higher than 0.5. The orange line shows the confidence of disordered protein binding residue predictions.

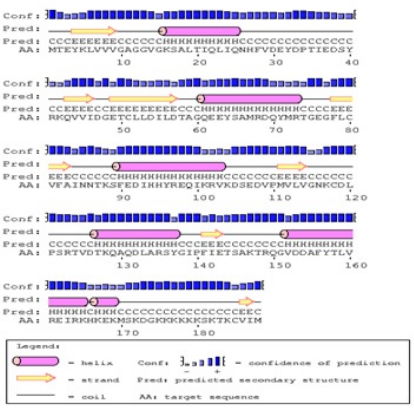

Figure 2A Secondary structure of normal protein.

Figure 2B Secondary structure of P.G13D mutant protein.

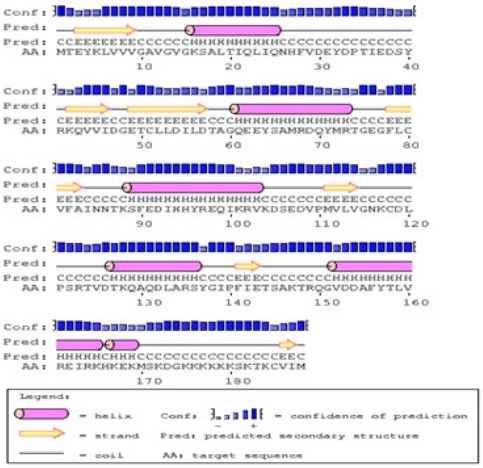

Figure 2C Secondary structure of P.G12V mutant protein.

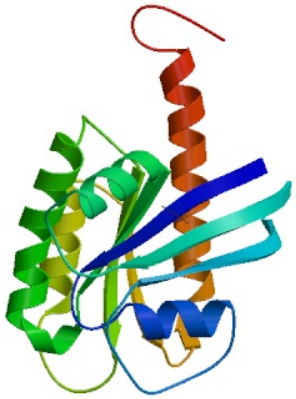

Figure 2D Secondary structure of Quaternary structure of normal and mutant protein.

Discussion

Carcinogenesis is a complex process and involves various genetic and epigenetic alterations. Structural point mutations in RAS proto-oncogene are common in human cancers including prostate cancer [17,19,21,22], and the mutation frequency varies, depending upon tumor types and stages [26,27].

In our study, KRAS mutation was detected in 2 of 35 patients (5.7%). Consistent with our study, findings from previous studies on different populations demonstrated that KRAS may have effects on pathogenesis of prostate cancer. KRAS mutation were found in 3% to 16% of Japanese patients [19], 7.3% of Korean patients [17], 40% of Turkish patients [21] and 2.3% to 9.1% of Chinese patients [19,22], it was lower in western countries patients though [20,28]. It is supposed that during tumor progression, accumulative alterations leading to increase of RAS mutant allele [29]. Therefore, KRAS expression suppression decreases prostate cancer cells proliferation and migration [30].

According to bioinformatics findings the p.G12V/c.35G>T and p.G13D/c.38G>A mutations do not alternate the secondary and quaternary structures of protein, but considering the several programs have predicted that these mutations are highly pathogenic, probably there are other reasons which change the function of the protein and ultimately make these mutations pathogenic. Although, The sequence alignment of KRAS protein with other spices shows that the implicated domains of KRAS protein is conserve **(Table 4)** but the mentioned mutations do not change the helix and coil residues of protein.

Table 4 The sequence alignment of KRAS protein with other spices.

Species	Match	Gene	Alignment
Human			12 MTEYKLVVVGAGGVGKSALTIQLI
Mutated (p.G12V/c.35G>T)	Not Conserved		12 MTEYKLVVVGAVGVGKSALTIQL
Mutated (p.G13D/c.38G>A)	Not Conserved		12 MTEYKLVVVGADGVGKSALTIQL
P. troglodytes	All Identical	ENSPTRG00000004775 http://oct2012.archive.ensembl.org/Pan_troglodytes/Gene/Summary?db=core;g=ENSPTRG00000004775	12 MTEYKLVVVGAGGVGKSALTIQL
M. mulatta	All Identical	ENSMMUG00000015381 http://oct2012.archive.ensembl.org/Macaca_mulatta/Gene/Summary?db=core;g=ENSMMUG00000015381	12 MTEYKLVVVGAGGVGKSALTIQL
F. catus	No Homologue		
M. musculus	All Identical	ENSMUSG00000030265 http://oct2012.archive.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000030265	12 MTEYKLVVVGAGGVGKSALTIQL
G. gallus	All Identical	ENSTRUG00000005329 http://oct2012.archive.ensembl.org/Takifugu_rubripes/Gene/Summary?db=core;g=ENSTRUG00000005329	12 MTEYKLVVVGAGGVGKSALTIQL
D. rerio	All Identical	FBgn0003205 http://oct2012.archive.ensembl.org/Drosophila_melanogaster/Gene/Summary?db=core;g=FBgn0003205	12 MTEYKLVVVGAGGVGKSALTIQL
C. elegans	All Identical	ZK792.6 http://oct2012.archive.ensembl.org/Caenorhabditis_elegans/Gene/Summary?db=core;g=ZK792.6	12 MTEYKLVVVGDGGVGKSALTIQL
X. tropicalis	All Identical	ENSXETG00000014935 http://oct2012.archive.ensembl.org/Xenopus_tropicalis/Gene/Summary?db=core;g=ENSXETG00000014935	12 MTEYKLVVVGAGGVGKSALTIQL

Noting these results and the role of KRAS gene in RAS/RAF/MEK/MAPK pathway and other pathways, there is a possible more noticeable role from this gene in pathogenesis of prostatic carcinoma. Also, by understanding the implicated

locations of mutations on protein function, it could be possible to design better drugs on targeted therapy in cancers related to KRAS mutations.

Web resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

Mutation Taster, http://www.mutationtaster.org/

Online Mendelian Inheritance in Man (OMIM), (http://www.omim.org)

PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.jcvi.org/

Ensembl Genome Browser (www.ensembl.org)

NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/NC)

SWISS-MODEL server (http://swissmodel.expasy.org)

PSIPRED Protein Sequence Analysis software (http://bioinf.cs.ucl.ac.uk/psipred)

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