K-RAS GENE MUTATION IN IRANIAN WOMEN WITH BREAST CANCER

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Abstract- TheK-ras mutations play different role in diagnosis, prognosis and treatment efficacy in different types of cancer. Breast cancer is one of the most common cancers in Iranian women therefore the study was designed for the analysis of K-ras mutation and their clinical pathological significance in breast cancer. In K-ras codon 12 and 13 of exon 2 that had an important role in various types of cancer were studied, in irani women suffering from triple negative invasive ductal carcinoma of the breast. Circulatory DNA was obtained from the blood plasma of 50 breast cancerous patients with triple negative invasive ductal carcinoma and 20 normal non-cancerous blood plasma samples, K-ras mutational status was determine in the purified DNA sample by ARMS PCR, Nested PCR using wiled type and mutational specific primer for Gly12Ala, Gly12Asp, Gly12Arg, Gly12Cys, Gly12Ser, Gly12Val and Gly13Asp. The results were confirmed by DNA Sanger
Sequencing. The results of our study find out that there is no mutations in any of the seven studied SNPs of K-ras in triple negative breast cancer as well as some important SNP in of exon 2, codons 10, 11, 15, 18, 19 and 22.The low frequency of K-ras mutation in irani women of triple negative breast cancer little can be concluded about the malignant potential of atumor containing an activated K-ras gene.

Index Terms- Breast Cancer, K-ras mutation, ARMS PCR, Sequencing

I. INTRODUCTION

The three members of ras family K-, N-, and *H-ras* that have 85% amino acid sequence similarities are found mutated in about 30% of human tumors. Out of total mutations in *ras* gene family about 85% is found in *K-ras* followed by 15% in *N-ras* and about 1% in *H-ras* [1].

The *K-ras*gene encodes a 188 amino acids protein that has inherent catalytic activity. Post-translation modification of this protein facilitates its localization to the cell membrane. The *K-ras* genes are found on chromosome 12 and are mostly mutated at 12, 13 or 61 codon of exon 2 and affect GTPase activity which results in constitutive activation of *RAS* signaling [2]. Point mutation in codon 12 and 13 cause increase affinity between ras and GTP so ras remains in ON state while point mutation in codon 61 inactivates the ras autocatalytic GTPas function thus preventing at conformation to convert to OFF state so ras signaling remain in activated state [3]- [4].

The most common 6 mutation in codon 12 of *K-ras* is glycine (GGT) to alanine (GCT), glycine (GGT) to arginine (CGT), glycine (GGT) to serine (AGT), glycine (GGT) to valine (GTT), glycine (GGT) to cysteine (TGT) and glycine (GGT) to aspartic acid (GAT) [5] - [6]. In codon 13 the glycine (GGC) is mutated to aspartic acid (GAC) (Dudley et al., 2015b) [6]. In general the *K-ras*, tandem mutation in Glycine of codon 12 is 86% and Glycine of codon 13 is 13% so accounts for about 99% of the mutations detected in *K-RAS* [7]. *K-ras* mutations are most frequently detected in colorectal tumors, lung carcinomas and in pancreatic carcinomas than *N-ras*, where *H-ras* mutation is associated with tumors of the skin, head and neck and *N-ras* mutations are common in hematopoietic malignancies [8] – [9]. The *K-ras* mutation play different role in diagnosis, prognosis and treatment efficacy in different types of cancer.*K-ras* mutations are found in one third of colorectal cancer, regarded as driver of tumor progression and known as a critical determinant of the therapeutic response of CRC.

The present study was designed to analyze *K-ras* mutations and their clinico pathological significance, in Irani women suffering from breast cancer. In *K-ras* codon 12 and 13 that had an important role in various types of cancer were studied, in triple negative invasive ductal carcinoma of the breast. The SNP in codon 12 and 13, including three SNP in 1st base G >(T, A, C) three SNP in 2nd base G> (T, A, C) of codon 12 and one SNP of the 2nd base G> A of codon 13 were investigated.
We detect *K-ras* mutations in blood sample because the circulating tumor DNA in blood is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer [10].

II. MATERIALS AND METHODS

A. Sample collection

A total of 70 blood samples consisting of 50 blood sample of triple negative invasive ductal carcinoma

and 20 normal non-cancerous blood samples were collected from Imam Khomeini Hospital Tehran and fardis lab Alborz Iran respectively.

B. DNA extraction

The QIAamp Circulating Nucleic Acid Kit (55114) was used for the extraction of circulating DNA from plasma acording to standered protocole provided by the manufacturer. The DNA extraction was carried out from 5 ml of plasma.

C. Primers designing for PCR

The primer was design using online primer design program for Tetra primer ARMS-PCR http://primer1.soton.ac.uk/primer1.html accessed 31/01/2014 .The primers were designed for our desired seven SNP The list of the primer and their amplicon size ~ is given in the (Table1).

D. PCR Amplification

The TianGen PCR master mix of Tiangen Biotech (Beijing) Co, Ltd was used for the PCR reactions. Each PCR reaction was made according to the provided protocol. 12.5 µl of 2x master mix, 1 µg of template DNA, 10 µM of each forward and reverse primer was added to the reaction tube. The total volume of the reaction tube was made 25 µl by adding distilled water. The PCR conditions were: initial denaturation for 3 minutes at 94°C, 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, elongation for 1 minute at 72°C and final elongation at 72 °C for 5 minutes. The products were evaluated by 1% Agarose gel electrophoresis for 45 minutes at 100V.

E. ARMS-PCR Reaction

Allele Specific-PCR was used to find out our desired seven SNP in codon 12 and 13 of exon 2. Two outer primers and two internal primers (one normal and one mutated) instead of two primer in normal PCR. The two internal primers were used to find out mutation in our desired location. The amounts of the reagent were remained the same as the first PCR with only changes in dilution of outer primer (1:15), the thermal cycling condition were kept the same as first PCR reaction.

F. Nested PCR

The product of first PCR reaction was used as the template DNA for a second round of PCR with primers designed to amplify a smaller DNA region within the primary amplicon. Combinations of normal and mutated nested primer along with outer forward and outer reverse were used. The second round of PCR Reaction was performed at 2 µl of first PCR product under the same condition as first round of PCR reaction.

G. DNA Sequencing

PCR product were purified and sent to the DNA sequencing facility to determine whether sequencing could detect *K-ras* mutations in the breast cancer specimens. The DNA sequencing was carried out by Sanger sequencing using ABI PRISM 3730xl Genetic analyzer developed by Applied Biosystems, USA. The BigDye® terminator v3.1 cycler sequencing kit was used. The DNA sequences were analyzed using online databases and sequence analysis tool.

III. RESULTS

The PCR amplification reactions were performed for total 50 cancerous samples under the best optimized condition. The PCR was run for the entire sample and the result was checked by 1% agarose gel electrophoresis. All the sample result was positive i.e. our desired expected bands of 343 base pair were observed in the entire sample. The PCR reactions were run with tetra Primer for the entire breast cancer specimen under study. The results were analyzed on 1% agarose gel electrophoresis. In the entire breast cancer specimens we get only the band for the wild type of allele which means no mutation found in any of the seven SNP. The result of the ARMS PCR ~ are summarized in the (Table 3) ~ and presented in the (Figure 1). We run nested PCR amplification reaction to chick internal normal and mutated primer individually in order to be sure that our ARMS PCR results concede with nested PCR result and we get the same result as ARMS-PCR. The PCR product were sequence in both forward and reverse direction by DNA sequences and checked against references stored at online database 'Gene bank' by a BLAST analysis. Mutations were identified by comparison between the online NCBI reference sequences NC_000012.12 of *K-ras* genes on the database and the sequences obtained from each breast cancer specimen. We found no evidence of *K-ras* somatic mutation in breast cancer patients by sequencing. We cannot exclude the possibility of errors in DNA sequencing result therefore we repeat the DNA sequencing for 2 samples but the results remain the same with no mutation.

The important *K-ras* mutation in exon 2 including codon 10 (GGA) code for Glycine, codon 11 (GCT) code for Alanine, codon 15 (GGC) code for Glycine, codon 18 (GCC) code for Alanine, codon 19 (TTG) code for Leucine and codon 22 (CAG) code for Glutamine were also checked from DNA sequencing result. We did not find any mutations in exon 2, codons 10, 11, 15, 18, 19 and 22.

IV. DISCUSSIONS

Understanding the complex role of *K-ras* mutations in malignancies may be helpful in development of new treatment strategies and the move toward personalized cancer therapy.

In codon 12 the 6 under study SNP, substitutes wild type amino acid glycine to different amino acid and

affect the activity of *K-ras* gene. When glycine 12 of *K-ras* is replaced with any amino acid, except proline, causes the biochemical activation of *K-ras* by the reduction of its intrinsic GTPase activity [11].In the present study of triple negative invasive ductal carcinoma of breast, we find out no mutation in any of the seven studied SNPs. The nucleotide sequence of codon 12 and 13 were found wild type mean GGT and GGC respectively. The DNA sequencing result has also confirmed that there is no mutation in some other important *K-ras* exon 2 mutations like codon 10, 11, 15, 18, 19 and 22. All these codons appeared normal in our results. The result of our study coincides with most of the previous studied *K-ras* mutations in breast cancer. *K-ras* mutations were not reported as frequent mutations in breast cancer and found about 5% in all breast carcinomas [12]. Hoadley and co-workers also found no common mutations in *BRAF*, *HRAS* and *K-ras* in breast cancer, when they sequence 96 breast cancer samples for *K-ras* as control and mentioned infrequent mutation of *K-ras* in breast cancer [13].The present study support the previous investigations conducted on 35 [14], 65 [15] and 107 [16]population size of triple-negative tumors, stating *K-ras* and BRAF mutations as rather rare events in breast cancer. The study conducted for the of *EGFR* and *K-ras* gene mutations in breast cancer from 143 Chinese female patients with breast cancer including 2 patients in stage 0, 31 patients of stage I, 76 patients of stage II, 33 patients of stage III and 1 patient of stage IV, the number of invasive ductal carcinomas patients were 132 (92.3%), their results were almost similar with our finding as they found no evidence of oncogenic mutations in both *EGFR* and *K-ras* in these breast cancer patients [17]. The result of our study contradict the study conducted by [18] of 116 breast cancer patients, identified, 8% *K-ras* mutation frequency in breast cancer, the reason may be studying different population area, different tissue FFPE, with different sample size in contrast to our 50 sample size of triple negative invasive ductal carcinoma of the breast in Iranian women and the sampling was blood.

Our results are also not in agreement with the study of [19]where they found one case (5 %) out of 20 to have a *K-ras* G12V mutation in micro papillary carcinoma of the breast. The reason may be their studied 20 patients of micro papillary carcinoma of the breast which is the typical type of invasive ductal carcinoma and the difference is not so different from our result.

This study evaluated the molecular constitutions of the *K-ras* gene in irani women suffering from breast cancer. The triple negative breast cancer were analyze for the assumption of, predictive, diagnostic, prognostic and regarding the efficacy of targeted therapy. The low frequency of *K-ras* mutation in irani women of triple negative breast cancer little can be concluded about the malignant potential of atumor containing an activated *K-ras* gene. This is the first ever study to have confirmed the lack of *K-ras* mutation in Irani women suffering with breast cancer.

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Table 1: Combinations of primer and their amplicon size

Primers name	Primer sequence	Amplicon size	Primer status
Forward outer Reverse outer	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	343	Primary
Forward inner 34G allele Reverse outer	5′-CTGAATATAAACTTGTGGTAGTTGGAGCTG-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	158 bp	Normal
Forward outer Reverse inner 34T allele	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-GCACTCTTGCCTACGCCACA-3′	235 bp	Mutated
Forward outer Reverse inner 34A allele	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-GCACTCTTGCCTACGCCACT-3′	235 bp	Mutated
Forward outer Reverse inner 34C allele	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-GCACTCTTGCCTACGCCACG-3′	235 bp	Mutated
Forward outer Reverse inner 35G	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-AGGCACTCTTGCCTACGCCAC-3′	234	normal
Forward inner 35T allele Reverse outer	5′-ACTTGTGGTAGTTGGAGCTGT-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	158	mutated
Forward inner 35A allele Reverse outer	5′-ACTTGTGGTAGTTGGAGCTGA-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	158	mutated
Forward inner for 35C allele Reverse outer	5′-ACTTGTGGTAGTTGGAGCTGC-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	158	mutated
Forward inner 38A allele Reverse outer	5′-GTGGTAGTTGGAGCTGGTGA-3′ 5′-ATCAAAGAATGGTCCTGCACCAGT-3′	176	mutated
Forward outer Reverse inner 38G allele	5′-GTCTGCAGTCAACTGGAATTTTCATG-3′ 5′-GTCAAGGCACTCTTGCCTACGC-3	241	normal

Abbreviations: G, Guanosine; T, Thymine; A, Adenine; C, Cytosine; bp, Base Pair

Table 3: Tetra Primer ARMS PCR Result

1st base codon 12 base			2nd base of codon 12			2nd base of codon 13		
Base	Amplicon size	result	Base	Amplicon size	result	Base	Amplicon size	Result
Outer primer	343	+	Outer primer	343	+	Outer primer	343	+
Internal G	158	+	Internal G	234	+	Internal G	241	+
Internal T	235	-	Internal T	158	-	Internal A	176	-
Internal A	235	-	Internal A	158	-			
Internal C			Internal C					

Table 4 Sequencing result of seven SNP

Base change	Result
Codon 12	
G-A	
Glycine (GGT)→Serine (AGT)	Wild type
Glycine (GGT)→Aspartate (GAT	Wild type
G-T	
Glycine (GGT)→Cysteine (TGT)	Wild type
Glycine (GGT)→Valine (GGT)	Wild type
G-C	
Glycine (GGT)→Arginine (CGT)	Wild type
Glycine (GGT)→Alanine (GCT)	Wild type
Codon 13	
G-A	
Glycine (GGC) Aspartate (GAC)	Wild type

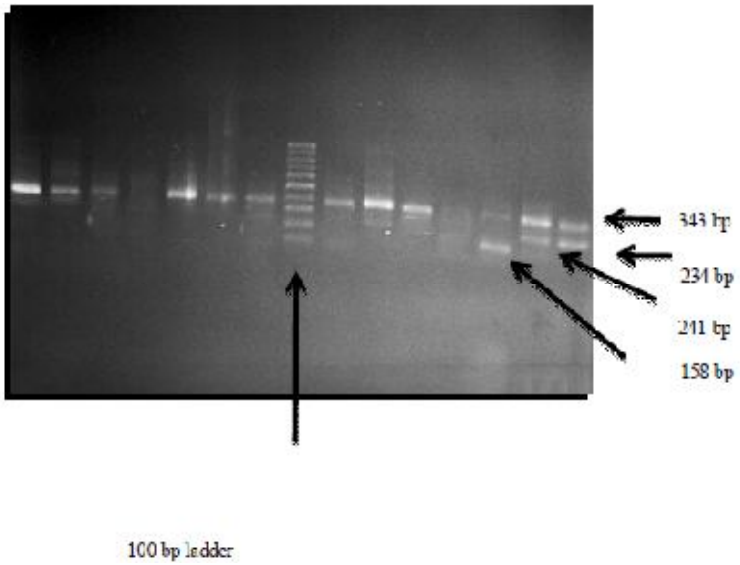


Figure 1:
The ARMS PCR result with two outer and one internal primer

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