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Detection of *K-ras* Codons (12\13 and 61) Genetic Variations in Iraqi

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Abstract: Breast cancer is the most common type of malignancy recorded in the cancer registries of almost all countries within the Eastern Mediterranean Region and most prevalent malignancy in women in Western countries currently accounting for one third of all female cancers. *K-ras* is proto-oncogene is a Kirsten ras oncogene, *K-ras*, is a protein that in humans is encoded by the *K-ras* gene located on the short arm of chromosome 12 (12p12.1). Women who are not carriers of *BRCA* genes may have a *K-ras*-variant. Paraffin-Embedded (FFPE) were used to detection of the *K-ras* genetic aberrations in breast cancer Iraqi patients and their consent were taken, to determine *K-ras* gene ability to predict prognosis, by extracting the DNA from the samples and amplified the area of interest by using the modern techniques for genetic analysis like HRM (High resolution melting) and Sequencing by using specific designed primers. The aim of this study to assessments of *K-ras* patients with breast cancer and find the correlation between the *K-ras* mutations and breast cancer. In this study *K-ras* gene mutations can be present in breast cancer patients representing somatic mutations. However, they should be confirmed as a germ-line mutation in patients. I recommend establishing *K-ras* mutations in breast cancer patients as a routine study in specialized centres by amplification and sequencing and detection more variation especially in *BRCA* negative breast cancer patients.

Key word: Breast cancer, *K-ras* gene, Gene, Iraq, Mutation. Codon 12-13, Codon 61.

التحري عن التغيرات الوراثية لجين *K-ras* في الكودونات 13/12 و 61 لدى مرضى سرطان الثدي العراقيين

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الخلاصة: سرطان الثدي من اكثر السرطانات المسجلة في معظم بلدان الشرق الاوسط وكذلك دول الغرب حيث يشكل الثلث بين السرطانات التي تصيب النساء .يشفر لبروتين الـ *K-ras* في الانسان من قبل جين الـ *K-ras* والواقع على الكروموسوم 21 الذراع القصير. (12p12.1) 2122 تزداد احتمالية حصول طفرات في الـ *K-ras* لمرضى سرطان الثدي للمرضى الذين لا يحملون الطفرات في جين الـ *BRCA* في هذه الدراسة تم عزل ال DNA من البلوكات الشمعية للمرضى حيث تم فحص الـ DNA باستخدام تقنية الـ HRMوالهدف هو دراسة *الـ K-ras* في مرضى سرطان الثدي والطفرات الحاصلة فيه، والتوصية في اعتماد دراسة جين الـ *K-ras* في المراكز التشخيصية المتخصصة لمرضى سرطان الثدي وخاصة من هم لا يملكون طفرات في جين الـ *BRCA* .

Introduction

In 1982 Chang and Der, two postdoctoral fellows working in Geoffrey Cooper's laboratory, discovered Kristen Rat Sarcoma Virus and Murine Sarcoma Virus; retroviral oncogenes related to rodent sarcoma virus genes (1). K-ras is proto-oncogene is a Kirsten ras oncogene, K-RAS protein that in humans is encoded by the K-RAS gene (2-3). This oncogene located on the short arm of chromosome 12 (12p12.1). The gene is a member of the Ras family of small guanine nucleotide–binding proteins, first identified as a cellular homolog of a transforming gene in the Kirsten rat sarcoma virus (4). K-RAS protein contains 188 amino acid residues with a molecular mass of 21.6 kD and participates in intracellular signal transduction (2). K-RAS protein remains inactive until it binds to Guanosine-5'-triphosphate (GTP). The switch from an inactive to an active form is regulated by intracellular signals. Once the GTP is bound to the K-RAS protein, K-RAS undergoes conformational changes that involve two regions of the protein, thus activating it. These two important regions are known as Switch 1 (amino acids 30–38) and Switch 2 (aminoacids

59–67), which form an effector loop, controlling the specificity of the binding of this GTPase to its effector molecules (5). It appears that ras gene mutations can be found in a variety of tumor types, although the incidence varies greatly. The highest incidence is found in adenocarcinomas of the pancreas (90%), the colon (50%) and in thyroid tumors (50%). For some tumor types a relationship may exist between the presence of a ras mutation and clinical or histopathological features of the tumor (6). Breast cancer is of startlingly high incidence (approaching 1 in 9 women), but unfortunately current therapies for the disease are inadequate once it has metastasized. The disease is characterized by excessive morbidity and mortality. Normal as well as malignant growth is regulated by endocrine hormones and by local tissue Subject

Materials and Methods

Twenty two of breast cancer patients paraffin blocks since three years ago, in addition to seventeen benign tumours (fibroadenoma) was chosen as a control because it carries the same risk of breast cancer as of normal breast, the cases were collected. All the cases diagnosed by specialist histo-pathologist. FFPE of patients was attending to the Teaching Laboratories /Medical City and Al-Yarmouk Teaching Laboratories / Baghdad were collected with their clinical and pathological data. All B.C patients were with infiltrative ductal carcinoma, stage two and three.

factors, such as polypeptide growth factors. Breast carcinomas seem to progress as hyperplastic ductal or lobular epithelial growth, acquiring progressive genetic changes (including those of oncogenes and tumor suppressor genes) leading to clonal outgrowths of progressively malignant cells (7). High resolution melt (HRM) analysis represents an efficient method to evaluate K-RAS mutations that consist of a variety of nucleotide substitutions at different positions. Applications of HRM have been described for the identification of germ line and somatic mutations. Due to its high sensitivity, HRM is considered a valid approach to detect a minimal fraction of mutated cells in cancer tissues. This level of detection is, in many cases, not achievable by direct sequencing (8).

DNA was extracted from FFPE sections using QIAmp DNA mini kit (50) from Qiagen in Molecular Oncology Unit lab. In GSTS Pathology Guy's and St. Thomas' NHS Foundation Trust / London. DNA yield was measured using NanoDrop ND-1000 spectrophotometer in which 1µl of nuclease free water is used first as blank, then 1µl of the patient genomic DNA is loaded in order to be measured, stored and then can be used for genetic tests. High Resolution Melting (HRM) step and the primer designing were done in the Molecular Oncology Unit lab. In GSTS Pathology Guy's and St. Thomas' NHS Foundation Trust/ London for K-ras codons (1213, 61) table (1) figures (1) and (2).

Table (1) Size of primer sequence for KRAS

Codon No.	Primer Sequance	Product size	Tm/°C	GC%
1213	F-TATAAGGCCTGCTGAAAATGACTGAA-3' R- GGTTTCTCTGACCATTTTCATGA	211	65 60.3	37.0 39.1
61	F- CCAGACTGTGTTTCTCCCTTC R-CTAAATCATTTGAAGATATTCACCA-5'	198	58.8	52.4

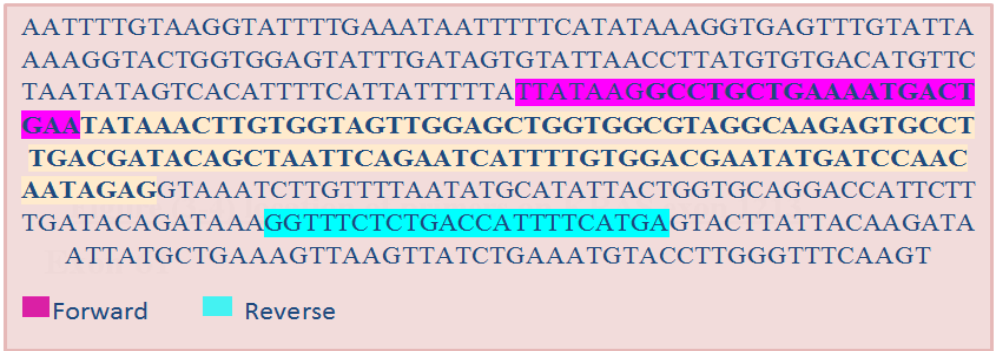

Figure (1): location of primer sequence for KRAS codon 1213

Figure (2): location of primer sequence for KRAS codon 61

The reaction mix preparation for HRM reaction must be done under sterile condition, the quantity of reagent per reaction table (2). The HRM reaction mixes for each amplicon was prepared as per the volumes according to the HRM template as in table (3).

- 16µl of the reaction mixes was aliquot in the assigned wells.
- 4µl of H2O was added to each H2O well.
- The plate was taken to the Nucleic Acids clean laboratory.
- 4µl of DNA was added to the relevant well on the plate. According to the template layout. Starting with the Negative controls (N1/N2)

- The wells were sealed except the positive control ones.
- The plates were taken to the Equipment Laboratory and placed it in the designated hood.
- 4µl of positive controls was added to the relevant wells.
- The wells were sealed.
- Centrifuged for 3 minutes.
- The plate was taken to the ABI 7900HT machine and use the High resolution melting program was used table (4).
- Purification of HRM Products achieved by usingCharge Switch PCR Clean-Up Kit.
- The data was analysed by using HRM V2.0 software.
- Run the product on the gel.

Table (2): HRM template of the master mixes preparation for KRAS

Agents	K-ras1213 1x/ µl	K-ras 61 1x/ µl
Buffer	2	2
MgCl2 (25mmol)	1.28	1.28
dNTPs	0.35	0.35
Polymerase enzyme	0.25	0.25
Water	10.5	10.5
Dye	1	1
Primer Mix	0.62	0.62
DNA	4	4
Total reaction volume	20	20

Table (3) HRM template prepared as in the example

	1	2	3	4	5	6	7	8	9	10	11	12
A	H2O	N1(wt)	1	2	3	4	5	6	7	8	9	pos1 1213
B	H2O	N2(wt)	1	2	3	4	5	6	7	8	9	pos1 1213
C	H2O	N1(wt)	1	2	3	4	5	6	7	8	9	pos2 1213
D	H2O	N2(wt)	1	2	3	4	5	6	7	8	9	pos2 1213
E	H2O	N1(wt)	1	2	3	4	5	6	7	8	9	pos1 61
F	H2O	N2(wt)	1	2	3	4	5	6	7	8	9	pos1 61
G	H2O	N1(wt)	1	2	3	4	5	6	7	8	9	pos2 61
H	H2O	N2(wt)	1	2	3	4	5	6	7	8	9	pos2 61

Table (4) HRM amplification program

HRM Program		
Stage	**Temp/Time**	**No. of cycles**
1	95ºC/10min	
2	95ºC/15 sec	10
	67ºC/1min	
3	95ºC/15 sec	5
	62.5ºC/1min	
4	95ºC/15 sec	35
	71ºC/3min	
	85.5ºC/20sec	
	62.5ºC/1min	
5	95ºC/15 sec	
	50ºC/1min	
	95ºC/15 sec	

Sequencing reaction was done by using Dye terminator cycle sequencing (ABI) for the HRM products, and the component of sequencing reaction mix preparation table (5). The reaction program was stored in thermo cycler for this reaction table(6). Clean-up of sequencing reaction products by using

Agencourt Clean SEQ kit (Agencourt ® CLEANSEQ ® Dye Terminater Removal from Beckman Coulter/USA). Transferred 35µl of the clear sample into a 96 well plate for loading on the 3730 sequencer. The data were examined by using the Mutation Surveyor software. After cycling, the samples proceeded to the next purification or were stored in the freezer overnight. Clean Up of Sequencing reaction Products by Agencourt CleanSEQ kit.

Table (5): Sequencing master mix components

REAGENT	Volume (µl)
	X1
Ready Mix (from kit)	0.7
5X Buffer (from kit)	2
10µM primer	1
molecular grade water	2.8
DNA template (cleaned PCR product)	3.5
Total reaction volume	10

Table (6): Sequencing reaction Program

Temperature oC	Time (seconds)	Cycles
96	1min	X1
96	10s	X15
60	1min 20s	
96	10 s	X5
60	1min 35s	
96	10s	X5
60	2min5s	

Results and Discussion

The exon two codon (12/13) and three codons (61) of K-ras were studied by amplification and sequencing. A missense mutation (g.175G>C, A59P) was detected in codon 61 in one breast cancer patient FFPE sample (4.55%) table (7), it did not appeared in fibroadenoma samples. The difference was not significant P>0.05, suggestion that it is irrelevant to breast cancer development. This mutation was previously reported by (9, 10 and 11) they also referred to it non-significant P>0.01. The relationship of K-ras gene mutations with the disease stage table (8) revealed that the unique mutation appeared with stage three tumour and the difference was significant P<0.01. There was no similar relationship in the literature. All the reading of the mutations were done in the labs of oncology unit at Guy's & St. Thomas hospital (12).

Table (7): K-RAS mutations in FFPE samples and Fibroadenoma

K-RAS	Tissue samples mutation	Fib Ad No.		Fibroadenoma	
		No	%	No	%
12/13	-----	0	0	0	0
61	g. 175G>C, A59P	1	4.55	0	0

Table (8): Relationship between K-ras mutations (codons 12/13 ,61) and disease stage

K-ras Mutation	Stage I	Stage II	StageIII	Stage IV	Percentage (%)
g. 175G>C, A59P			+		25.00
Percentage (%)	0.00 %	0.00 %	100.00 %	0.00 %	---

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