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Polymorphism of FGFR2 gene in breast cancer of Iraqi patients

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ABSTRACT

The receptor tyrosine kinase Fibroblast Growth Factor Receptor type 2 (FGFR2) is required for the development of several tissues, including the breast. SNPs in FGFR2 have recently attracted attention because they constitute a high-risk haplotype linked to an elevated risk of breast cancer. The high risk SNP haplotype has a greater affinity for the transcription factor RUNX2, according to this study. Analysis and quantification of the FGFR2 transcript revealed no difference in splicing with genotype. It's unknown if a single polymorphism is responsible or if numerous SNPs are involved.

Key words: polymorphism, FGFR2 gene, breast cancer, Iraqi patient.

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INTRODUCTION

The tyrosine kinase receptor fibroblast growth factor receptor type 2 (FGFR2) is involved in a number of cell signaling pathways that contribute to cell development and differentiation [1]. FGFR2 is necessary for the development of a variety of tissues, including the breast and kidney [2,3]. LADD syndrome and syndromic craniosynostosis are rare monogenic disorders caused by mutations in the FGFR2 gene. SNPs in FGFR2's intron 2, such as rs2981582, have lately received attention because they form a high-risk haplotype associated to an increased risk of breast cancer [5, 6]. Both oestrogen receptor positive (ER+ve) and oestrogen receptor negative (ER-ve) cancers are associated to the FGFR2 risk haplotype, with a stronger association to ER+ve tumors, has a stronger relation to ER-positive cancers In a number of breast and cancer-related circumstances, FGFR2 has been found to be nuclear localized [7, 8]. The FGFR1 receptor has been shown to be actively trafficked to the nucleus, where it may function as a transcription factor that coordinates gene expression and promotes cellular differentiation [9]. FGFR2 has also been detected in mammary gland terminal end buds throughout development [8, indicating that nuclear localization is critical for breast development. Despite the fact that FGFR2 has a function in breast cancer and that FGFR2 polymorphisms are linked to breast cancer [5,6], It's unclear how these polymorphisms cause breast cancer. In a recent investigation of SNPs in the FGFR2 linkage disequilibrium block, including rs2981582 [5], a putative oestrogen receptor binding site and an extra variation in a POU transcription factor binding domain were found. One study [10] looked at the impact of FGFR2 genotype on transcription factor binding and mRNA production. According to this study, the high-risk SNP haplotype had a stronger affinity for RUNX2. However, analysis and quantification of the FGFR2 transcript revealed no differences in splicing based on genotype, suggesting that the minor (greater risk) haplotype boosted FGFR2 transcription. Further investigation into the FGFR2 gene region indicated that the polymorphism rs2981578 is to blame for the RUNX2 binding effect. Other

polymorphisms in the same linkage disequilibrium block, such as rs7895676 in the C/EBP binding area, have been linked to transcription factor binding [5, 11]. One effect of SNPs in the linkage disequilibrium block in breast cancer cell lines appears to be changes in local histone acetylation [12]. It's unclear if the influence is due to a single polymorphism or a combination of polymorphisms. Several studies looking at the expression of the FGFR2 protein in breast cancer tend to disprove the hypothesis that FGFR2 overexpression represents a stage of tumor progression. Early studies identified FGFR2 expression in just a limited number of breast cancers (4 percent to 12%) [13, 14]. A more recent study that used a variety of techniques to investigate gene expression, protein levels, and genomic changes involving FGFR2 in breast cancer discovered that FGFR2 levels in tumor tissue are lower than in adjacent normal breast ducts, and that this could be attributed to LOH or methylation involving the FGFR2 locus in some cases. The influence of genotype on gene and protein expression was not investigated in this study. the aim of the study research the polymorphism of FGFR2 gene in breast cancer of Iraqi patient.[15]

Primer design

The sequences of the designer primers utilized in this work are recorded (Table 1).

Tab. 1. Designer primers used in this study to determine polymorphism of region intron2, 3`untranslated region of FGFR2 gene

Primer name	Sequences
FGFR2 rs2981582-F	5`-CGGACAAGATGAACCACTAAA-3`
FGFR2 rs2981582-R	5`-CCAAGAGGCAGTTCCATAATC-3`
FGFR2 rs1219648-F	5`-GGCTATTCAGAGGCTAAGTTC-3`
FGFR2 rs1219648-R	5`-GCACATACACACACCATAGATA-3`
FGFR2-F 3UTR	5`-CTGAGGTGTCCGTTAATGTATAG-3`
FGFR2-R3 3UTR	5`-ACAGGATCAGAGAGGTTAAGT-3`

Primer preparation

Macrogen Company provided these primers in lyophilized shape. As a inventory answer, lyophilized primers had been dissolved in nuclease-loose water to a final concentration of 100pmol/l. A operating solution of those primers become generated with the aid of including 10l of primer stock solution (stored at -20 C) to 90l of nuclease-free water to acquire a 10pmol/l running primer solution

Primer optimization

To conclude the choicest toughening temperature for those preliminaries, the DNA layout was intensified utilizing a similar groundwork pair (Forward) and (Reverse) at assorted strengthening temperatures, including 55, 58, 60, 63, and sixty five°C, as previously portrayed. Every groundwork (10pmol) become added to 1 degree of GoTaq Green Master Mix (2X); six volumes of nuclease-loosened water; and two volumes of layout DNA have been utilized for each PCR intensification. A PCR cycler (Thermal Cycler, BioRad, USA) was utilized to do the intensification, with the accompanying temperature application: denatured at 94°C for 4 min, went with the guide of 30 patterns of denaturation at 94°C for 30 sec; toughening at 55, 58, 60, 63, or 65°C for 30 sec; and expansion at 72°C for 30 sec. A leftover expansion hatching of seven mins at 72 levels Celsius was embedded, trailed by a brief brooding at four degrees Celsius to convey the strategies to an end (Table 2,3).

Response setup and thermal cycling protocol

Tab.2. component of PCR reaction

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	12.5
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease Free Water					7.5
DNA		ng/µl		ng/µl	3
Total volume					25
Aliquot per single rxn	22	µl of Master mix per tube and add 3 µl of			Template

PCR programe

Tab. 3. PCR cycles for amplification

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60 or 65	00:30	
Extension	72	00:30	
Final extension	72	07:00	1

Agarose-Gel-Electrophoresis

•To affirm the presence of intensification following PCR enhancement, agarose gel electrophoresis changed into used to divide the results of the response. The PCR impacts were totally dependent on the removed DNA standards

Preparation of agarose

One hundred milliliters of 1X TAE were poured into a flask.

• Agarose was added to the buffer in the amount of 1.5 gm (for a 1.5% concentration).

• The arrangement became acquainted with a heat up (the utilization of a microwave) till all of the gel particles have been totally broken down.

• 1 mL of Ethidium Bromide (10 mg/mL) becomes acquainted with the agarose strategy to make it lackluster.

• The agarose become mixed to verify that it becomes very much mixed and that there had been no air pockets.

• The response becomes permitted to chill to 50-60°C sooner than being utilized.

PCR product loading

For the PCR product, 5 mL was directly injected into the wells. Electrical power was turned on for 60 minutes at a voltage of 100 volts and a current of 50 milliamps. DNA developments from the terrible Cathode shaft to the worthwhile Anode post. The gel imaging device changed into used to picture the groups of Ethidium bromide stained groups in the gel.

Standard Sequencing

The PCR stock were shipped off Macrogen Corporation – Korea for Sanger sequencing on an ABI3730XL electronic DNA sequencer, which became utilized for the interaction. Following receipt of the results via email, geneious software was used to conduct an analysis of them[16]

Statistical Analysis

The statistical analysis of this study have been done by SPSS version 24.2 and Microsoft excel has also used. The numerical data have represented as mean ± Standard error (S.E.) , t-student test has been used to compare two numerical data. ANOVA test used for numerical data more than two. Chi-square has used for two categorical parameters.

Odds ratio and their corresponding fisher value for the genetic polymorphism have been calculated by using the program Wini-pipi.

RESULTS AND DISCUSSION

DNA extraction

In order to assess the integrity of the DNA, the eluted samples have been tested by the nano-drop instrument and the results showed acceptable concentration ranged between 20- 70 mg/ml. then the samples have been run through agarose gel as shown in (Figure 1). The results of gel electrophoresis shown a clear sharp band for all samples.

Fig. 1. 1% agarose gel electrophoresis of DNA eluted samples subjected to 5 mv/cm, for 90 minutes

Polymerase chain reaction (PCR)

Amplification region of intron2 of FGFR2:

In order to reveal the genetic polymorphism of the FGFR2 gene. A region within the gene has been targeted by the PCR by a set of primer, and after the completion of PCR and to test the accuracy of the PCR the products have been ran through gel electrophoresis as shown in the (Figure 2). The results showed a clear and sharp band of 896bp length of DNA.

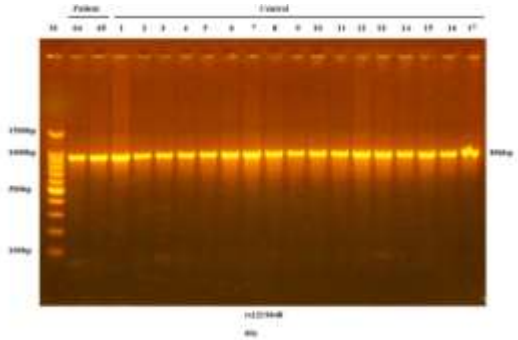

Fig. 2. Results of the amplification of rs1219648 of Human samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker

Amplification region of intron 2 of FGFR2:

A second region of the FGFR2 gene amplified successfully as the results of gel electrophoresis for the PCR products showed sharp band that length 993bp (Figure 3).

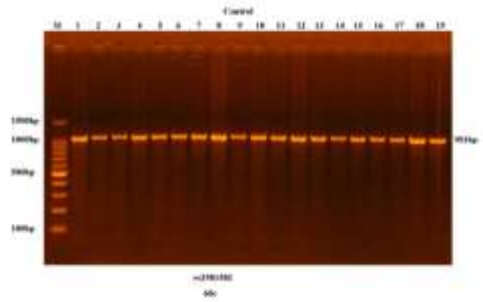

Fig. 3. Results of the amplification of rs2981582 of Human samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 7-44 resemble 993bp PCR products

Amplification 3` untranslated region of FGFR2 gene:

A successsful PCR process have been done for the third targeted region of the FGFR2 gene as the products of the PCR showed a clear band after ran through gel electrophoresis, the results of the gel electrophoresis are shown in the (Figure 4).

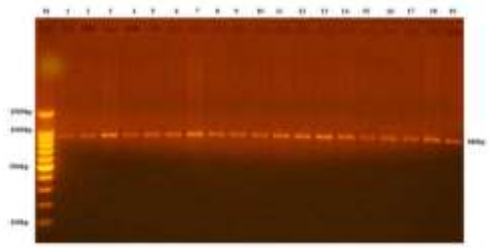

Fig. 4. Results of the amplification of 3` untranslated region of FGFR2 of Human samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 7-44 resemble 880bp PCR products

Then the PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

Genetic polymorphism of the FGFR gene

Genetic polymorphism of rs1219648 FGFR2 :

The genetic polymorphism rs1219648 which is located in the second intronic region of the gene FGFR2. The results in (Table 4). showed higher frequency of TC genotype in both patients and control with an odds= 0.88, followed by CC genotype which showed an odds=0.82. in another hand the TT genotype showed etiological odd ratio equal to 1.48.

Tab. 4. Genotypes and allele frequencies of SNP (rs1219648) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
TT	13 (21.6)	27 (45)	0.01	0.34	0.15 to 0.75
TC	28 (46.6)	19 (31.6)	0.134	1.89	0.90 to 3.95
CC	19 (31.7)	14 (23.3)	0.414	1.52	0.68 to 3.39
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% CI**
T	54	73	0.02	0.53	0.32 to 0.88

This study failed to find a significant relation between breast cancer and the frequency of rs1219648 SNP. The results of this study disagreed with a previous study that showed a significant association with

the rs1219648 and breast cancer risk (per-allele OR=1.22, 95%CI: 1.06-1.41; p-value=0.007). furthermore, In a study involving 1225 participants, Rebbeck reported a significant interaction between the use of estrogen plus progestogen therapy and rs1219648 on breast cancer risk (p-value=0.01). Estrogen plus progestogen therapy users who were wildtype at this locus were at an increased risk of disease compared to never users with the same genotype (OR=2.63; 95%CI: 1.46-4.76) (Rebbeck et al. 2009).

Genetic polymorphism of rs2981575 FGFR2

The frequency the SNP rs2981575 which is located in the third intronic region of the gene FGFR2 and the statistical values are showed in the (Table 5). The results showed higher frequency of the heterozygous genotype CT in both patients and control (40 and 49%, respectively) but the difference was non-significant, followed by the CC genotype which also showed non-significant difference between the patients and control (30% and 34%, respectively. P= 0.673, odd=0.83). In another hand the genotype TT showed a significant higher frequency in patients than in control and this relation showed etiologic odds equal to 1.39.

Tab. 5. genotypes and allele frequencies of SNP (rs2981575) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
CC	16	33	0.003	0.3	0.14 to 0.64
CT	30	20	0.095	2	0.96 to 4.16
TT	14	7	0.148	2.3	0.86 to 6.15
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% CI**
C	62	86	<0.002	0.42	0.25 to 0.72
T	58	34			

The results showed a great impact of the SNP with disease occurrence and the mutant genotype has a significant defence impact against the disease. This results agreed with a previous study done by and find that rs2981575 had the strongest association with breast cancer risk (per allele HR = 1.28, 95% CI 1.18-1.39) additionally this SNP was the only locus to reach genome-wide statistical significance. The results also agree with another study that done recently and have shown the SNP is significantly associated with risk of premenopausal breast cancer in the overall group and in the Her2-negative subgroup [18].

Genetic polymorphism of rs45631583 FGFR2:

The three genotypes of the SNP rs4563158 are shown in the (Table 6). The wild genotype showed no differences in frequencies of patients and control (5%, p=1.00). The genotype CT recorded higher frequency in control than in patients (29% and 27%, respectively, P=0.826, odds= 0.89). The genotype TT showed slightly higher frequency in patients than in control (68% and 66%, respectively. P=0.883, odds=1.11).

Tab. 6. Genotypes and allele frequencies of SNP (rs45631583) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
CC	3	21	0.001	0.1	0.03 to 0.35
CT	13	12	1	1.11	0.46 to 2.65
TT	44	27	0.03	3.36	1.57 to 7.18
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% CI**
C	19	54	0.001	0.23	0.13 to 0.42
T	101	55			

Genetic polymorphism of rs75798664 FGFR2:

The genotyping results of the SNP rs75798664 are represented in (Table 7). The results showed slightly higher frequency of CC genotype in control samples than in patients (98% and 92%, respectively. Odds=0.481). While the genotype CT recorded higher frequency in patients than in control (5% and 2%, respectively. Odds= 2.07). While none of the patients or control showed the TT genotype. After many research, this study considered as the first study that investigating the genotype of the SNP rs75798664 in Iraq. This frequency expected as the frequency of other countries

that have been submitted in the NCBI (rs75798664 RefSNP Report - dbSNP - NCBI n.d.).

Tab. 7. Genotypes and allele frequencies of SNP (rs75798664) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
CC	58 (92%)	40 (98%)	0.647	0.48	0.05 to 4.69
CT	3 (5%)	1 (2%)	0.647	2.07	0.21 to 20.08
TT	0	0	-	-	-
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% Cl**
C	119	81	0.65	0.49	.05 to 4.73

Genetic polymorphism of rs2981582 FGFR2:

The results of allele frequencies for the SNP rs2981582 are summarized in (Table 8). The results showed higher frequency CT genotype in both patients and control (44% and 43%, respectively, p=1). The CC genotype showed the lower frequencies in both patients and control (24% and 19%, respectively). The TT genotype showed a significant higher frequency in control than patients (36% and 28%, respectively. P=0.05).

Tab. 8. Genotypes and allele frequencies of SNP (rs2981582) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
CC	12 (20)	27 (45)	0.003	0.31	0.14 to 0.68
CT	28 (47)	18 (30)	0.091	2.04	0.97 to 4.29
TT	18 (30)	15 (25)	0.683	1.29	0.58 to 2.85
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% Cl**
C	52	72	0.026	0.54	0.32 to 0.91
T	64	48			

SNPs in intron 2 of the FGFR2 gene, such as rs2981582, have a role in multifactorial breast cancer risk. Increased mRNA transcription and altered transcription factor binding have been linked to the high risk polymorphism haplotype in the FGFR2 gene, although the impact on FGFR2 protein production is unclear.

For FGFR2 rs2981582 variations, a prior meta-study comprised 31 papers with 54,677 cases and 80,418 controls. In every genetic model, the link between the FGFR2 rs2981582 polymorphism and the prevalence of BC was substantial in the overall population. FGFR2 rs2981582 was also strongly related with BC risk in both Asians and Caucasians in ethnicity-specific analyses.

Genetic polymorphism of rs3135724 FGFR2:

Allele and genotypes frequencies of the SNP rs3135724 are shown in the (Table 9). The genotype AA showed a similar frequencies in both patients and control (9%) and the genotype GA showed higher frequency in control than in patients (64% and 57%, respectively, P=0.681) while the genotype GG showed higher frequency in patients than in control (34% and 27%, respectively, P=0.515).

Tab 9. Genotypes and allele frequencies of SNP (rs3135724) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
GG	20 (33)	32 (53)	0.042	0.44	0.21 to 0.91
GA	35 (58)	24 (40)	0.067	2.1	1.02 to 4.32
AA	5 (8)	4 (7)	1,00	1.27	0.33 to 4.93
Allele frequency (%)					
Allele	patients	control	P-value	Odds Ratio	95% Cl
G	75	88	0.097	0.61	0.35 to 1.05
A	45	32			

The results of this study failed to prove any correlation between the disease occurrence and genotypes of the SNP. For the best of our knowledge this is one of the first studies to correlate the occurrence of the disease with frequency of SNPs genotype.

The SNP (rs3135724) have been firstly reported on 2016 by a Cohort study which aimed to The prospective SIGNAL/PHARE cohort's primary goal was to find polymorphisms linked to particular molecular subtypes of breast cancer. Their premise is that genetic variations linked to molecular subtypes will provide new information about illness etiology and might lead to new advances in disease prevention

and therapy. Because their major goal was to build a clinical cohort, they concentrated on gathering data on histopathology and therapies, as well as patient follow-up.

Genetic polymorphism of rs2162540 FGFR2:

After revealing of the genotypes by RFLP results of the SNP rs2162540 are shown in (Table 10). The genotype GG showed non-significant higher frequency in control than in patients (44% and 36%, respectively, odd=0.407). the heterozygous genotype GA showed higher frequency in patients than in control (44% and 36%, respectively. Odd=0.539). the genotype AA showed the lower frequencies in both patients and control (21% and 20%, respectively. Odds= 1.12).

Tab.10. Genotypes and allele frequencies of SNP (rs2162540) in patients and control subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
GG	20 (33)	37 (62)	0.022	0.43	0.21 to 0.88
GA	27 (45)	15 (25)	0.035	2.45	1.14 to 5.29
AA	13 (22)	8 (13)	0.337	1.8	0.69 to 4.68
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% Cl**
G	67	89	0.004	0.44	0.26 to 0.76

It has been shown that mutations of FGFRs were able to facilitate tumor growth by driving cell proliferation and survival, but also could suppress tumor growth. Consistently, studies of mouse models have revealed that FGFR2 not only serves as an oncogenic gene, but also acts as a tumor suppressor in a certain intracellular environment.

The results for this study disagreed with a meta-study that showed a significant higher frequency in the AA genotype in breast cancer patients in North Carolina. But the results agrees with a recent study done in China .

Genetic polymorphism of rs2860200 FGFR2:

The genotype frequency of the SNP rs2860200 are summarized in (Table 11). The samples of both patients and control showed only the genotype AA (100%). For the best of our knowledge this study considered as the first study that focused on this SNP. Although, in the NCBI website this SNP also showed similar results of this study (0%) in many other countries (Korean, Japan, Qatar, Vitnam, Siberia). (rs2860200 RefSNP Report - dbSNP - NCBI n.d.) [17,18,19].

Tab 11. Genotypes and allele frequencies of SNP (rs2860200) in patients and contro subjects

Genotype	Patients	control	P-value	odds ratio	95% C.I.
AA	62	41	1	1	1
AG	0	0	-	-	-
GG	0	0	-	-	-
Allele frequency (%)					
Allele	**patients**	**control**	**P-value**	**Odds Ratio**	**95% Cl**
A	124	82			
G	0	0			

CONCLUSION

The present results show high polymorphismis in the genotyp of fgfr2

Thats lead to good early in brad wide women and early treatment.

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