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Association of Single Nucleotide Polymorphism (rs 2108429654A/T) in *PI3KCA* Gene with Risk of Breast Cancer in Iraqi Women

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Abstract

The catalytic subunit The catalytic subunit (P110α) of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), which is encoded by *PI3KCA* gene located in chromosome 3, has been found to be upregulated significantly in patients with breast cancer. The research aims to study single nucleotide polymorphisms (SNP rs2108429654) located in exon 20. This SNP (rs2108429654) was genotyped in 70 women with breast cancer (50 postmenopausal and 20 premenopausal) and 40 control women using the DNA sequencing technique (Sanger method). According to statistical analysis of hormone receptors, prostaglandin hormone (PR), estrogen hormone (ER), and human epidermal growth factor receptor 2 hormones (HER-2) with menopausal status in patients with breast cancer show there were no significant differences. Isolated DNA is used to perform polymerase chain reaction (PCR) followed by DNA sequencing. The gene expression of *PIK3CA* was also determined by converting mRNA into cDNA via a reverse transcription mechanism, which will be used later in qPCR. The results of genotypic analysis of rs (2108429654) for AA genotype (OR= 1.0), AT genotype (OR= 0.1500, p= 0.0021), and TT genotype (OR= 0.2344, p= 0.0039) were significant associations with increased risk of BC. The median gene expression of *PIK3CA*was (32.52) in patients increased significantly (p= 0.001) compared to control women (30.97) with expression fold for patients (3.525). All genotypes (AA, TT, and AT) of the SNP rs 2108429654 represent a high level of gene expression in patients compared to controls.

Key words: *PI3KCA* gene, SNPs, DNA sequencing, Gene expression.

العلاقة بين تعدد اشكال النيوكلوتيدات المفردة (rs (2108429654 في الجين PI3KCA وخطر الإصابة بسرطان الثدي لدى النساء العراقيات

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الخلاصة

Phosphatidylinositol−4,5−bisphosphate 3−kinase catalytic subunit (*PIK3CA)* هو جين موجود على الكروموسوم الثالث . بينت الدراسات الحديثة ان البروتين p110 هو الجزء المحفز للمسار (PI3K) ويشفر عنه بواسطة الجين *(PIK3CA)* ويلعب دور مهم لدى النساء المصابات بسرطان الثدي. الهدف من هذا البحث هو دراسة تعدد الاشكال الوراثية المفردة (SNP rs2108429654) الموجود في الاكسون 20. وتم اجراء البحث الجيني على 70 عينة لنساء عراقيات مصابات بسرطان الثدي (50 نموذج لنساء بعد سن الياس و20 نموذج لنساء قبل سن الياس) و40 نموذج لنساء سليمات بواسطة تقنية تسلسل الحامض النووي (DNA sequencing tec.). لا يوجد فرق معنوي بين مستقبلات الهرمونات المدروسة وحالة انقطاع الطمث بالاعتماد على نتائج التحليل الاحصائي. DNA المستخلص تم استخدامه لأجراء تفاعل البلمرة المتسلسل (PCR) وبعد ذلك تم استخدام ناتج التفاعل في تفاعل تسلسل DNA. يتم قياس التعبير الجيني للجين (*PIK3CA*) عن طريق تحويل mRNAالى cDNA بواسطة تقنية الاستنساخ العكسي ليتم استخدامه لاحقا في qPCR. كانت نتائج التحليل الجيني للـ(rs2108429654) للالايلات متغيرة الزايكوت AT (OR= 0.1500، *p*= 0.0021) والليلات المتطفرة TT (OR=0.2344، *P*= 0.0039) والليلات المتماثلة AA(OR=1.0) حيث كانت مرتبطة بزيادة خطورة الإصابة بسرطان الثدي. كان الوسيط للتعبير الجيني للجين في النساء المصابات مساوي لـ (32.52) ويزداد ارتباطه بسبب قيمة *P* مساوية لـ (0.001) عند مقارنته بنتيجة النساء السليمات (30.97) بينما كانت نتيجة expression fold(3.525). وكانت نتيجة التحليل الجيني للأنماط الوراثية الثلاثة (AA, TT, AT) الخاصة بتعدد الاشكال الوراثية المفردة (SNP rs 2108429654) مرتفعة لدى النساء المصابات بسرطان الثدي عند مقارنتها بالنساء السليمات.

1. Introduction

In general, cancer is considered a heterogeneous multifactorial disorder. There are many causative factors linked with breast cancer (BC), like levels of hormones such as estrogen and progesterone, mutations, polymorphisms, and other daily routines like breastfeeding, physical action, and kind of nutrition. More than 2 million women are diagnosed with BC around the world, and about 10% of cases are hereditary [1]. Breast cancer has been raised largely through the past two decades. According to the Iraqi Cancer Registry (ICR) (2016), an elevated percentage of cancer belongs to the BC among women [2]. The results of conducted studies that were carried out on Iraqi patients reveal the percentage of females with BC was (33.81%) of the overall status of cancer. The ratio of BC in various regions, such as, Jordan, Kuwait and Bahrain,was lower when matched with other Arab countries. Many causes may raise the danger of BC, like medical history, being overweight, and an incompetent conception of the riskiness of the status. Many sign agents have been helped to predict clinical consequences for patients with BC [3]. In the 1980s, Phosphoinositide 3-kinases (PI3Ks)/AKT pathways were discovered, which comprise a lipid kinase family and many biological assignments mediated by (PI3Ks) like cell survival, proliferation, and differentiation [4,5].PI3K/AKT pathway activity is mediated via extracellular growth factors and hormones. Serine/threonine kinase AKT is activated if PI3K/AKT is dysregulated and thereby initiates many types of cancer due to modifying a wide range of downstream proteins, which encourage uncontrolled tumor growth [6]. PI3Ks are heterodimer lipid kinases built up by a catalytic subunit (p110) and the adaptor or the regulatory subunit (p85) variant parts encoded by different detached genes and different splicing. The most prevalent isoform is *PIK3CA,* which is dysregulated in breast cancer. PI3K is activated by the kinase domain of P110 that is encoded by the *PIK3CA* gene through the phosphorylation of different downstream signalling proteins [7, 8]. Mutation in the *PIK3CA* signaling pathway has oncogenic properties in different types of malignant, especially BC tumorigenesis. Previous studies suggested about (20-30)% of mutations in this pathway are tumorigenesis [9]. Several types of BC occur due to missense mutations, the major hot spot oncogenic

mutations located in exon 9 and exon 20 that encode for kinase and the helical domain of enzymes, resulting in an overexpression of p110 protein [10].

Cancers are developed due to variations in DNA that lead to molecular aberrations in the cell cycle. This mutation can be divided into pathogenic or non-pathogenic depending on the role of the abnormal gene, and it can increase cancer prognosis. Gene can be altered either through oncogenes activation or via tumour suppressor gene silencing. The PI3K pathway is associated with different pathways like apoptosis, cell signalling, and cell differentiation. PI3K is an important pathway, that precipitates in signal transduction by receptor tyrosine kinases, or G-protein coupled receptors [11]. This aimed to research study the SNP (rs 2108429654) of the *PIK3CA* gene in a group of women with BC, intending to determine their role in susceptibility to disease; in addition, analysis of gene expression of *PIK3CA* to evaluate their association with SNP genotype.

2. Materials and methods.

Ethical statement.

The ethics committee of the College of Science, University of Baghdad, confirmed this project (Ref: CSEC/1222/0143). Every patient enrolled in this study has a written approval letter to collaborate in this study and use their blood samples for molecular assay.

Patients and Controls.

The total number of all samples was 110, divided into two groups. Group one represents the patient group consisting of 70 blood samples collected from women with BC, their age range from (25-60) years, who were diagnosed and histologically confirmed at the Al-Amal National Hospital for Cancer Patients, City of Medicine, Ministry of Health, Baghdad, Iraq according to the criteria of the Iraqi Ministry of Health. While the control group consisted of 40 random samples, whose ages ranged from (25-60) years, they were apparently healthy women from different places in Baghdad.

Blood sample collection.

Whole blood has been collected in sterile ethylene-di-amine-tetra- acetic acid (EDTA) tubes for DNA isolation. Mixing 600 µl of blood specimens with 200-µl of traizol in Eppendorf tubes to isolate mRNA for gene expression experiments. Information about patients, and hormones is collected from their information paper after diagnosis and confirmed by a specialized doctor.

SNP selection and genotyping.

The DNA sequence of *PIK3CA* was downloaded from the National Center of Biotechnology Information (NCBI) and explored to identify SNPs. This SNP (rs2108429654) was chosen based on the result of DNA sequencing, which represents the most repeated SNP in patients and healthy women. Geneious prime program was used to analyze DNA sequencing results and identify the most repeated SNPs.

All primers utilized in this study were designed by the primer 3 plus program, which exists freely online depending on the gene sequence from the NCBI. Macrogene Company (Korea) synthesized and lyophilized primers for all experiments. The sequence of primers that are utilized for the amplification of the *PIK3CA* gene by PCR are; forward primer (`5-CATTTGCTCCAAACTGACCA-3`), reverse primer (`5-TGAGCTTTCATTTTCTCAGTTATCTTTTC-3`).

To study the relationships between SNPs of exon 20 in *PI3KCA* and BC in women a partial a sequence was selected to amplify. *AccuPower*® PCR PreMix kit (Bioneer, Korea) was used,

and the reaction was carried out according to the manufacturer's protocol. The final volume of PCR reactions was 25 microliters. The components of the reaction were as follows: 1 µl of forward primers, 1 µl reverse primer, 6.5 µl of nuclease - free water, 4 µl of template, 12.5 µl *AcuuPower PCR premix.* The conditions of PCR reactions were initial denaturation (95 ºC for 5 min., 1 cycle), denaturation (95 ºC for 30 Sec.), annealing (57±5 ºC for 30 Sec.), extension (72 ºC for 30 Sec.) for 30 cycles. Final extension (72 ºC for 7 min., 1 cycle).

Quantitative Real -Time PCR (qPCR) has estimated the levels of *PIK3CA* gene expression. It is an appropriate, sensitive technique to estimate the constant state of the mRNA level. The sequences for primers used for gene expression of *PIK3CA* gene by qRT-PCR. were forward primer (`5-TTGAAGTGGGTTTTTGACTGC-3`), reverse primer (`5-TCTCATTTGTGACTGCTTCCA-3`), while the primers sequences of housekeeping gene *GAPDH- (*Glyceraldehyde 3-phosphate dehydrogenase) were forward primer (`5-TGCCACCCAGAAGACTGTGG-3`), reverse primer (`5-TTCAGCTCAGGGATGACCTT-3`).RT-qPCR SYBR Green assay was used to confirm a quantitative real-time for the *PI3KCA* gene and the *GAPDH* gene was used as a housekeeping gene to equalize the level of the *PI3KCA* gene's mRNA. *AccuPower® GreenStar™* qPCR PreMix kit (Bioneer, Korea) was used to estimate the expression level, fold, and threshold cycle (Ct) of *PI3KCA* and *GAPDH* genes. The conditions of RT- qPCR reactions were as follows initial duration (95 ºC for 3 min., 1 cycle), duration (95 ºC for 15 Sec., 14 cycles), annealing (55 ºC for 45 Sec., 40 cycles) extension (72 ºC for 60 Sec., 40 cycles) .

Statistical analysis.

IBM SPSS Statistics 26 program was applied to the statistical analysis data of our study to reveal the action of various agents on study parameters (0.05 and 0.01 probability). The web tool was used to calculate Hardy-Weinberg equilibrium. Odds ratios (ORs) with a 95% confidence interval (CI) were calculated to detect the ability of the relationship between studied SNP and BC. Pearson correlation analysis was used to analyze the association degrees between variables. A two-followed *p*-value less than 0.05 ($p<0.05$) was rated significant. Qualitative data were presented as numbers and percentages. Characterization of quantitative data employs range (minimum and maximum), mean, standard deviation, and median.The calculations of fold expression of target gene (*PI3KCA)* against housekeeping gene *(GAPDH)* according to livak equation (12)

ΔCt =CT (gene) – CT (HKG)

The expression ratio was calculated according to the formula:

$2^{-\Delta CT}$ = Normalized expression ratio.

$\Delta\Delta Ct=\Delta Ct$ (patient) - ΔCt (control).

Lastly, the fold-change value in gene expression was calculated as below:

Fold change =$2^{-\Delta\Delta Ct}$ Normalized expression ratio.

3. Results and discussion.

In this research, 110 blood samples were collected from women to evaluate the association of the SNP (rs2108429654) in *PIK3CA* gene with BC, determine the level of gene expression of PIK3CA, and evaluate the correlation of PIK3CA with BC in females.

This SNP was chosen because it was the most repeated SNP in patients and control samples depending on DNA sequencing products. These 110 samples were divided into two groups. The first group include70 samples representing women with BC, while the other groups include 40 samples representing apparently healthy women.

Statistical analysis of the SNP (rs2108429654) revealed that it is highly significant for patients while it is not significant for control samples as p-values represent (<0.0001) (0.0618) respectively. Genotype frequency (%) for patients and control samples have

significant differences for hetero-genotype (AT) and mutant genotype (TT) with p- values (0.0021), (0.0039), respectively, allele frequency (%) significant for this SNP with p-value (0.0004). Table (1) represents all data about genotype frequency (%) and allele frequency (%).

Table 1: Association analysis and Hardy-Weinberg analysis of *PIK3CA* single nucleotide polymorphism (rs 2108429654) in women with BC.

Genotype Frequency (%) *rs2108429654*						
Genotypes	**Controls n.=40**	**Patients n=70**	***P*-value**	**Chi-square**	**Odds Ratio**	**95% Cl**
AA (W)	6 (15%)	32 (46%)	----	----	1.0	----
AT (H)	10 (25%)	8 (11%)	0.0021 **	9.464	0.1500	0.04807 to 0.5164
TT (M)	24 (60%)	30 (43%)	0.0039 **	8.334	0.2344	0.08443 to 0.6746
Chi-square	5.566 NS	41.64 **				
***p* Value**	0.0618	<0.0001				
Allele frequency (%)						
Alleles	**n= 80**	**n= 140**	***P*- value**	**Chi-square**	**Odds Ratio**	**95% Cl**
A (W)	0.3 (22)	0.51 (72)	0.0004 **	12.57	0.3477	0.1929 to 0.6389
T (M)	0.7 (58)	0.49 (66)				

NS=Non-significant, * significant at p value ≤ 0.05, ** significant at p value ≤ 0.01

Table 2 revealed the relationships between patients and controls depending on menopausal and family history of BC. According to statistical analysis, there is no significant difference between patients and controls for these parameters.

Table 2: Associations between controls and patients depending on menopausal status and family history.

Menopausal Statues	Control (N, %)	Patients (N, %)	Chi-Square	Sig.	*p* Value
Meno.	18 (45%)	29 (35%)	0.5640	NS	0.4526
Post.	22 (55%)	41 (65%)			
Total	40 (100%)	70 (100%)			
Family History	Control (N, %)	Patients (N, %)	Chi-Square	Sig.	*p* Value
Yes	18 (45%)	35 (50%)	0.1335	NS	0.7148
No	22 (55%)	35 (50%)			
Total	40 (100%)	70 (100%)			

NS=Non-significant, * significant at p value ≤ 0.05, ** significant at p value ≤ 0.01

The relationship between hormones, receptors (prostaglandin hormone, estrogen hormone, and human epidermal growth factor receptor 2 hormones), and menopausal status in patients with BC is illustrated in Table 3. The results showed there were no significant differences between menopausal status and hormones in patients with BC.

Table 3: Association between hormones receptors and menopausal statues.

ER	Meno. (N, %)	Post (N, %)	Chi-Square	Sig.	*p* Value
-ve	12 (36%)	22 (54%)	1.200	NS	0.2734
+ve	17 (64%)	19 (46%)			
Total	29 (100%)	41 (100%)			
PR	**Meno. (N, %)**	**Post (N, %)**	**Chi-Square**	**Sig.**	***p* Value**
-ve	11 (38%)	22 (54%)	2.349	NS	0.1254
+ve	18 (62%)	19 (46%)			
Total	29 (100%)	41 (100%)			
HER-2	**Meno. (N, %)**	**Post (N, %)**	**Chi-Square**	**Sig.**	***p* Value**
-ve	16 (57%)	25 (65%)	0.2637	NS	0.6076
+ve	13 (43%)	16 (35%)			
Total	29 (100%)	41 (100%)			

ER (Estrogen receptor), PR (progesterone Receptor), HER-2 (human epidermal growth factor receptor 2)

Statistical analysis of gene expression revealed that the expression fold of *PIK3CA* in patients was (3.21) while the expression fold for controls was = (1.00).

The expression level has been increased in females with BC when matched with healthy females, with highly significant differences in gene expression between patients and healthy women equal to (*p* value= 0.001). Table 4 represents the outcome of the statistical analysis of gene expression. Figure 1 represents the expression fold of *PIK3CA* in patients and healthy women.

Table 4: Determination of the gene expression of *PIK3CA* by q-RT-PCR in women with BC and controls**.**

Variants	Patients	Controls	p- value
Ct (Mean ± SD)	30.92±0.98	32.51±1.33	0.001
Δ Ct (Mean ± SD)	3.63 ± 2.14	4.12± 2.69	
Δ Δ Ct (Mean ± SD)	-0.53 ± 2.12	0 ± 0	
$2^{-\Delta\Delta ct}$ (Mean ± SD)	3.21 ± 3.5	1.00± 0	

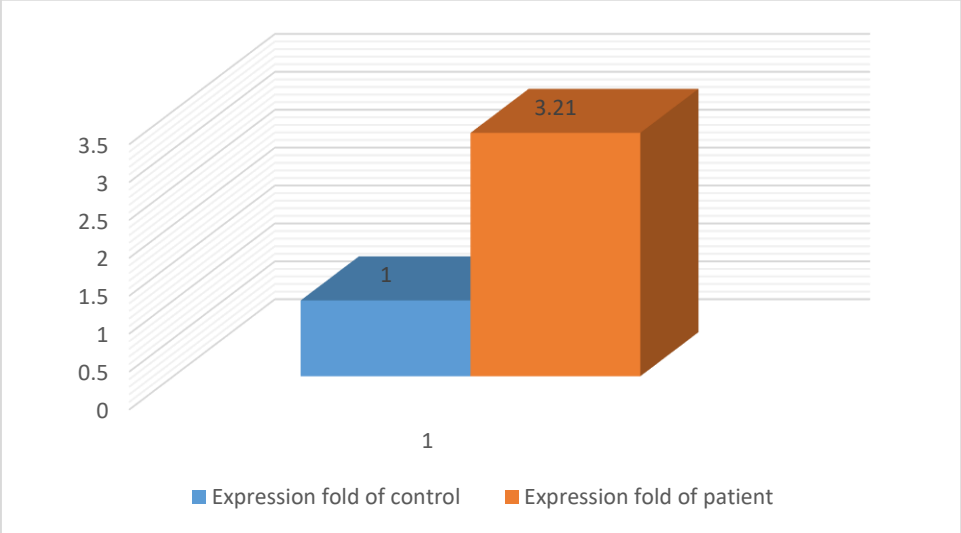

Figure 1: Expression folds of *PIK3CA* gene in women with BC and controls

SNP rs2108429654 represents higher levels of gene expression within all genotypes (AA, TT, AT) in patients than in control samples. Allele (A) represents the wild type, while allele (T) represents the mutated allele in this SNP (rs 2108429654 A/T).The results of gene expression level represented as mean ± SD for AA in patients (32.41 ± 0.23) while in controls (32.02 ± 0.25), the results of mean± SD for TT in patients (32.09± 0.25), and in controls (31.22± .024) and lastly the results of AT in patients (32.0.12) and controls (28.51± 0.94) Breast cancer may develop due to SNPs in the *PIK3C*A gene. The results also show that the SNPs in *PIK3CA* will increase gene expression in patients with breast cancer due to oncogenic activity.

The risk of BC may increase due to SNPs in other genes like Fibroblast Growth Factor Receptor Type 2(*FGFR2).* A study published by Salman *et al.,* showed the increase of gene expression in intron 2 of (FGFR2*)* in patients with BC. [13].

Research has been published that *PIK3CA* may activate PIP3 to stimulate phosphoinositide-dependent kinases (*PDK*1). After activation of *PDK*-1 by phosphorylation, AKT is activated, which is responsible for control survival, proliferation, metastasis, inhibition of cell apoptosis, and oncogenic transformation of tumor cells [14]. Mutations in this pathway have been found in almost all types of tumors in humans, even in breast cancer; hyperactivation of this pathway occurs due to different variations that represent about 60% of the tumors. Many hallmarks of cancer, like uncontrolled proliferation, genomic instability, and metabolic reprogramming in tumor cells, are linked with the deregulation of this pathway [15]. Deregulation of a catalytic subunit (p110), which is encoded by the *PIK3CA* gene, occurs due to oncogenic mutation in this gene that leads to hyper-activation of the PI3K pathway. These mutations are particularly recognized in BC; where it reaches up to 27% of women possess mutations in *PIK3CA*gene. According to previous studies, changes in *PIK3CA* gene sequence and expression play an important role in breast cancer [16].

Many previous types of research represent that hotspot mutation in *PIK3CA* increases the activity of lipid kinase when matched with wild type and enhances phosphorylation of AKT and, as a result, the transformation of regular epithelial cells of the breast to tumor cells via in vivo and in vitro studies [9].

Many studies reported that mutations in *PIK3CA* were mostly seen in tumors with expressed PTEN, ER, PR, and ERBB2 genes. Studies demonstrated that mutations in *PIK3CA* were more common in ER hormone receptor-positive and HER2-positive breast cancers [17]. In a recent study by Wu *et al.,* [18] it was represented that mutations of *PIK3CA* were positively associated with ER-positive, PR-positive, and low Ki67 labelling index and negatively associated with the triple-negative breast cancer subtype [18]. Mutations of PIK3CA were not associated with age at diagnosis, tumor stage, lymph node status, tumor size, or HER2 status [19]. Another study reported a higher frequency of mutations in SNPs could increase BC development by driving proliferation and survival; furthermore, these results represent a great - association of the SNP with disease occurrence, and the mutant genotype has a significant association against the disease[20]. PI3K is an important lipid kinase family that plays an important role in the arrangement of cellular roles like proliferation, differentiation, motility, cell growth, and intracellular trafficking. In addition, prior research has represented *PIK3CA* as an important factor in tumor cell permanence. PI3K/Akt/ mammalian goal of rapamycin (mTOR) is a major intracellular pathway that is extremely stimulated in BC. PI3K/Akt/ mTOR can be stimulated by different conductivity of hormones, nutrients, and growth factors [14].

The statistical analysis results of menopausal status agreed with Maruyama *et al.* study that showed no significant associations between *PIK3CA* mutations and menopausal status, tumor size, lymph node status, or histologic grade [21]. Many researchers studied the effect of menopausal status study the effect of fluctuating hormone levels before and after menopause that increase the gene expression patterns as investigated between premenopausal and postmenopausal BC patients [22].

These results represent particular genes and their function in a menopausal status-dependent pattern. It was noted that somatic mutations in *TP53* were observed in 47.6% of premenopausal breast cancer patients, while 38.1% manifest mutations in *PIK3CA* [23].

Another study has the same conclusions about menopausal status in a study performed on premenopausal breast cancer patients of Latin American descent, that both *TP53* and *PIK3CA* feature as the two usually mutated genes [24].

Another study represents the same results as our study in which there is no significant association between family history and mutations in *PIK3CA* in breast cancer between healthy women and patients [25]. The frequency for family history is equal in patients with a family history and those without. A previous study found that about 25% of women have a family history of breast cancer, and about only 10% of cases occur because of monogenic inheritance with autosomal dominant inheritance and high penetrance [26]. Menstruation at an early age is considered a major risk factor that increases the risk of BC in females [27].

Another reason sources of PI3K activity increased is that it is associated with both gain/amplification of gene copy number and somatic mutations of *PIK3CA*, which cause inhibition of apoptosis and develop into cancer. These events' amplification of genes and gain of gene copy is passable as a final effect in the development of tumors like in somatic mutations [28].

The hotspot mutation in *PIK3CA* is generally located in the helical domain and kinase domain. The helical domain carries out allosteric regulation of the kinase domain. The steps of activation as below helical domain draw a signal from the regulatory subunit (p85) by the SH2 domain and then regulate the activity of the kinase region. When mutations happen, the inhibitory impact of p85 will be desregulated, which puts the kinase domain in hyperactivity mode. Because exon twenty is located in the kinase region, mutations in this exon will cause an earning of function and, as a sequence, change, its capacity of transformation [11].

Expression fold was higher in patients than in control samples, and these results agreed with the Alowiri *et al.,* [29] study. Many researchers applied quantitative procedures via real-time PCR to estimate mRNA expressions of the objective gene (concerned*PIK3CA*). Previous research has shown that *PIK3CA* is an extremely mutated gene in women with BC [30, 31]. Most previous studies identified *PIK3CA* as a frequently mutated oncogene in BC. Mutations of *PIK3CA* were found in 8-40% of patients with BC [32].

Deregulation of PI3K track is associated with different types of cancer distinctive features, like genomic instability, uncontrolled proliferation, and metabolic reprogramming in tumor cells. Mutations in the catalytic subunit (p110α) that is encoded by the *PIK3CA* gene are known as powerful oncogenic mechanisms concerned with the hyperactivation of the PI3K pathway. Breast cancer may develop due to any variation in the *PIK3CA* sequence or gene expression [14]

4. Conclusion.

The results of the study represent that SNP (rs 2108429654), which is located in the *PIK3CA* gene, plays a critical role in BC development. It is considered a risk mutation affecting the binding protein located in the helical domain and kinase domain, drawing a signal from the regulatory subunit (p85) by the SH domain and then regulating the activity of the kinase region. Breast cancer may develop due to SNPs in the *PIK3CA* gene, which will increase gene expression in patients with breast cancer due to oncogenic activity.

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