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ORIGINAL RESEARCH ARTICLE

Genetic Association of rs2237572 Cyclin-Dependent Kinase 6 Gene with Breast Cancer in Iraq

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Abstract This case–control study is aimed to evaluate serum concentration of Cyclin-Dependent Kinase 6 (CDK6) and the genetic association between rs2237572 *CDK6 gene* and breast cancer (BC) in Iraq. To attain this goal, 80 patients with BC as cases and 80 healthy individuals as controls were included. Further, BC patients were sorted according to the molecular classification into four subtypes of Luminal A, Luminal B, Her2/neu enriched and TPN. Serum concentration of CDK6 enzyme, allelic and genotypic frequencies of rs2237572 *CDK6*, and the occurrence of BC phenotype and its subtypes in the studied population were investigated. ELISA technique was used to perform the biochemical testing, while the molecular analysis was achieved by real-time PCR, high resolution melting analysis, conventional PCR, as well as sequencing analysis. The results revealed no significant difference in serum concentration of CDK6 enzyme between patients and healthy controls ($p > 0.05$). Also, no significant differences were shown between BC patients subtypes ($p > 0.05$). The rs2237572 *CDK6* genotypes were associated with the BC and affirmed that allele C was inherited as a recessive risk factor. Moreover, a highly significant difference between patients' subtypes in the genotypic frequency of rs2237572 ($p < 0.01$) was noted. Furthermore, the association of rs2237572 genotypes and CDK6 serum concentration in BC patients showed a considered significant difference between C/C and T/T, C/C and T/C and the CDK6 level ($p < 0.05$). Nevertheless, T/T and T/C did not show any significant difference with the CDK6 level. Hence, it was concluded that the rs2237572 of *CDK6* gene is significantly correlated with BC.

Keywords Breast cancer · Cell cycle · Cyclin D · Cyclin-dependent kinase 6 · rs2237572

Introduction

Most tumors can carry numerous abnormalities that deregulate hundreds of genes, which can occur at chromosomal, gene replication, transcription, and epigenetic levels [1]. Breast cancer (BC) is the most frequent cancer among women and the second commonest cancer worldwide [2]. It represents a multifactorial disease due to the genetic, epigenetic and transcriptional changes involving multiple genes and proteins. These factors are related to clinical prognosis and medical treatment. In order to resolve the complexities of underlying carcinogenesis mechanisms, a broad approach is needed [3]. However, the development of molecular biology at the level of genetic mutations and gene expression may be helpful in the orientation of treatments [1].

Cyclin D-kinase 6 (CDK6), also called human cell division protein kinase 6, is an enzyme encoded by the *CDK6* gene. It is regulated by cyclins, more specifically by Cyclin D and Cyclin-dependent kinase inhibitor proteins. The protein encoded by *CDK6* gene is a member of the

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cyclin-dependent kinases, (CDKs) family, which includes CDK4 [4]. CDK6 is a vital factor in mediating G1/S transition and is linked to tumor progression as well [5]. Moreover, it is considered an important factor for tumor inhibition during clinical treatment [6]. Furthermore, CDK6 and CDK4 play key roles in mammalian cell proliferation. And, they are integrated with D-type cyclin to form enzymatically active holoenzyme complexes that drive cell cycle into S phase of DNA synthesis [7]. However, imbalance of the cyclin D and CDKs pathway in cancer cells could result in diversion away from a pathway to senescence and toward a more proliferative phenotype [8].

Although BC has multiple genetic variants, it also can be related to geographic areas and ethnic groups [9]. Variations in the *CDK6* gene highlight the importance of studying the genetic susceptibility [10]. Still, limited reports were conducted in Babylon to elucidate how such variations could affect patients with BC. In this respect, we sought to investigate how the rs2237572 genotypes of *CDK6* are associate with BC and how they affect the serum level of CDK6 enzyme in those patients.

Materials and Methods

Study Subjects and Test Samples

This study was conducted at the Babylon University, College of Medicine, Department of Clinical Biochemistry and its affiliated laboratories during the period extended between April to December 2019. After giving informed consents, 80 participants with BC their age range between 30 and 80 years and 80 healthy individuals were included. BC patients were divided according to the molecular classification based on immunohistochemistry findings that depends on the expression of ER, PR, and Her2-enriched proteins, into four groups as follows:

- Luminal A: 37 patients.
- Luminal B: 19 patients.
- Her2/neu+ enriched: 15 patients.
- Triple-negative: 9 patients.

All patients were not giving any treatment (hormonal or chemotherapy), that means the collection of blood samples were done a pre-dose in order to exclude the effect of these drugs on biochemical and genetic results. The physicians of Consulting Clinic for Early Detection of Breast Cancer in Hilla Teaching Hospital checked out healthy controls. Eight milliliters of venous blood were collected from each participant (patients and controls), 3 ml were moved into EDTA tubes and stored at − 20 °C (deep freeze) in order to be used later in genetic analysis. Into disposable separating gel tubes, the remained 5 ml were pushed slowly and allowed to clot at room temperature for 30 min. Then, clotted blood samples were centrifuged at $2000\times g$ for approximately 15 min and the serum was separated onto small Eppendorf tubes and kept in a deep freezer (− 20 °C) to be used for biochemical investigation of CDK6 enzyme by ELISA technique.

Determination of CDK6 in Serum

Sandwich-ELISA kit (Sunlong, China) was used to determine CDK6 level in serum. Known concentrations of the human CDK6 standard and its corresponding OD read were plotted on the log scale (x-axis) and the log scale (y-axis) respectively (Fig. 1). The concentration of human CDK6 in the sample was determined by plotting the sample's OD on the y-axis and then by multiplying by the dilution factor, the original concentration was calculated. More than 20% of the samples were performed in duplicates.

Genetic Marker Selection rs2237572

The SNP was selected according to previous study by Cai et al. [11], due to its high frequency and wide linkage disequilibrium region by employing several databases and software such as (NCBI, ensemble, HUGE navigator, and GWASdb). The effect of this SNP on CDK6 enzyme is unknown because it is an intronic variant.

PCR Primers' Design

For the targeted genomic region, we employed the protocol described by Hashim et al. [12]. The sequence was retrieved from the NCBI database (assembly GRCh38.p7, annotation release 108). It was downloaded as a FASTA format file, which farther processed in NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design a specific primer pair for the targeted region, the primers explained in Table 1. The product length was set to 100–200 bp to be suitable for High Resolution Melt

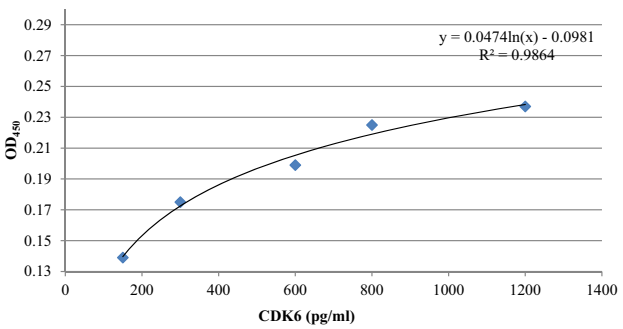

Fig. 1 Human CDK6 standard curve

Table 1 Primer sets for the selected marker rs2237572

Sequence (5′→3′)	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TAGAGACGATCAGGGACACC	Plus	20	33	57.95	55.00
Reverse primer	AGGTTCTAATTTGGTCGAGTGA	Minus	22	179	57.12	40.91
Product length	147					

(HRM) analysis. The same software was tested the specificity of each primer pair by blasting the primers against the human genome (RefSeq reprehensive genome). Furthermore, each primer was tested for its ability to form secondary structure and dimers by the aid of OligoCalc online software (https://biotools.nubic.northwestern.edu/). The amplicon sequences of each primer pair were retrieved from the NCBI sequence viewer (V 3.30.0) as a FAST format, the amplicon melting curve was simulated by Umelt online software (https://dna-utah.org/umelt/umelt.html).

DNA Extraction, Purification, and Electrophoresis

The DNA was isolated by using the regular protocol described by Repaske et al. [13] and the modifications described by Gross-Bellard et al. [14]. The DNA quantity and quality were measured by nano-drop, by employing the scanning ability of diode array from 200 to 320 nm wavelength and by calculating the 260/280 and 260/230 ratios. If the 260/280 ratio of the sample was showed lesser than 1.8 and/or 260/230 ratio lesser than 2, the re-extraction of the sample was made. The integrity and molecular weight of extracted DNA was determined by agarose gel electrophoresis [15].

Real-Time PCR

The reaction mixture and amplification profile were optimized by implying different primers concentration and different annealing temperatures to produce most efficient and specific amplicon that produces a uniform and clear melting curve.

High Resolution Melt Analysis (HRMA)

Eva green stain (Cyntol, Russia) was added to PCR product and then subjected to HRMA. The HRMA data were analyzed by using Novallele (Canon Biomedical USA). It revealed that our samples segregated into three well-defined clusters.

Genotyping of Genetic Marker rs2237572

Two random samples from each cluster produced by HRMA were subjected to conventional PCR and then they sequenced according to the company manual (Macrogen, South Korea). After sequencing, the resulting HRMA of rs2237572 *CDK6* genotypes were presented in Fig. 2. An electrocardiogram had confirmed these three different HRMA patterns by showing an obvious form of zygosity status in the three groups of investigated samples, the observed variant had exhibited three different distributions in the analyzed samples, normal homozygous status (TT), mutant heterozygous status (TC) and homozygous status (CC), (Fig. 3).

Ethical Approval

This study has approved by the Research Ethics Committee of Babylon Medical College and Hilla Teaching Hospital. The informed consents were obtained from all participants before carrying out the practical part of this study. All the protocols of this study were in accordance with the Declaration of Helsinki.

Statistical Analysis

All the numerical data were expressed as mean ± SD. Descriptive statistics and graphs were carried out using the Microsoft Excel 2010. The phenotypes odds ratio was calculated according to Altman [16] by the aid of (medcalc.net.) software, while the phenotypes mean ± SD were compared using student *t* test and One Way ANOVA with SPSS 21 (SPSS Inc., Chicago, Ill., USA). Genetic

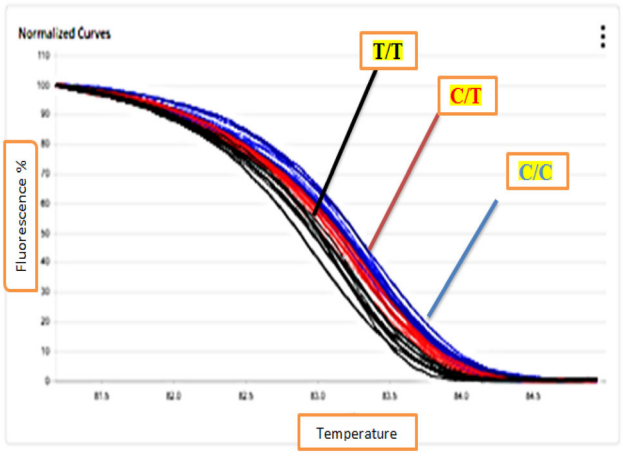

Fig. 2 Normalized curve of HRM analysis of rs2237572 CDK6 genotypes

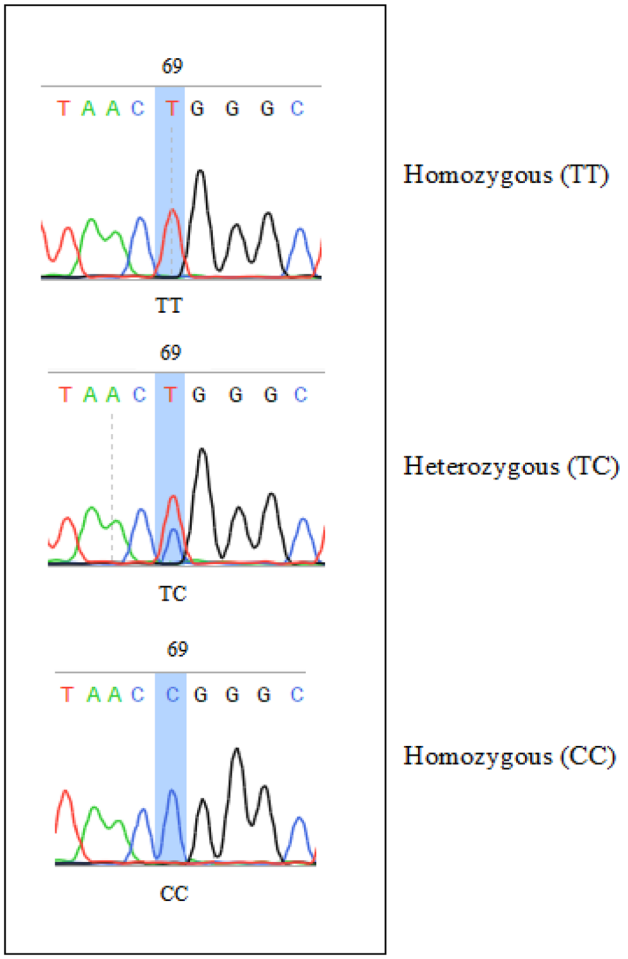

Fig. 3 The pattern of the observed substitution mutation within the DNA chromatogram of the targeted 147 bp amplicons within the *CDK6* gene

association parameters were carried out with the aid of SNPStats online software, except genotypes association which was carried out by Fisher's exact test with the aid of Quickcalcs software from GraphPad.

Results

Control group matched the BC patients group in age ($p > 0.05$), mean values and SD of age for patients and controls were 51.89 ± 12.18 and 49.2 ± 11.49 years respectively. In addition, there were no significant differences in the age among BC subtypes ($p > 0.05$), means and SD were Luminal A = 53.59 ± 12.67 years, Luminal B = 48.52 ± 12.06 years, Her2-enriched = 52.06 ± 12.19 years, and TPN = 49.77 ± 13.17 years.

No significant difference was observed between patients and healthy controls in serum concentration of CDK6 ($p > 0.05$), means and SD were 2700.29 ± 4438.49 and 1788.46 ± 4094.06 pg/ml, respectively (Table 2). Also, no differences in mean values of CDK6 concentration were shown among BC subtypes ($p > 0.05$), Luminal A = 2141.90 ± 3717.15, Luminal B = 1529.04 ± 2780.91, Her2-enriched = 2838.99 ± 4177.82, and TPN = 3800.47 ± 6355.36 (Table 3, Fig. 4).

An allelic and genotypic association of rs2237572 with BC revealed no significant allele frequency in both alleles T and C between patients and the healthy individuals. Allelic frequency for patients and control (Table 4). The genotype frequency in control and patients groups follows the Hardy–Weinberg Equilibrium (HWE, $p = 0.055$ and $p = 0.24$, respectively). Table 5 presents the genotype frequency of both groups and the exact test result for HWE.

The association of rs2237572 genotypes with BC under different modes of inheritance was further tested, the result exhibited that the C allele is a risk factor for BC and inherited as a recessive risk factor. The CC genotype has a related odd ratio compared to (T/T + CT) of 3.08 (1.14–8.35), while T allele represents a dominate protective genetic factor conferring lesser BC susceptibility for the carrier individuals (Table 6).

Notable difference between four BC subtypes in the genotyping frequency (GF) of *rs2237572* was statistically detected ($p < 0.05$). In Luminal A, T/C revealed a higher GF about 37.8%; Luminal B, T/C revealed a higher GF about 63.1%; Her2-enriched, T/T revealed a higher GF accounted about 86.7%; and TPN, T/C revealed a higher GF about 55.6% (Table 7).

The Luminal A had the highest C/C GF of rs2237572, while the Her2-enriched had the highest T/T GF of rs2237572 and Luminal B had highest T/C GF of rs2237572 (Fig. 5).

Association of rs2237572 genotypes and CDK6 serum concentration is detailed in (Table 8). A statistically considered difference in the CDK6 level between C/C and C/T, C/C and T/T of rs2237572 was found ($p < 0.05$). Except, no significant difference was seen between C/T and T/T in the CDK6 level ($p > 0.05$).

Our result revealed that the different genotypes did not affect the age of disease onset.

Table 2 CDK6 concentration for patients and control groups

Group	N	CDK6 (pg/ml)	*p* value*
BC patients	80	2700.29 ± 4438.49	0.17
Control	80	1788.46 ± 4094.06	

*p value of student t test

Table 3 CDK6 concentration among patients of the four BC subtypes

Group	N	CDK6 (pg/ml, mean ± SD)	Comparison group	Mean CDK6 (pg/ml)	*p* value
Luminal A	37	2141.90 ± 3717.15	Luminal B	612.86	0.587
			Her2-enriched	697.08	0.569
			TPN	1658.56	0.266
Luminal B	19	1529.04 ± 2780.91	Luminal A	612.86	0.587
			Her2-enriched	1309.94	0.344
			TPN	2271.42	0.163
Her2-enriched	15	2838.99 ± 4177.82	Luminal A	697.08	0.569
			Luminal B	1309.94	0.344
			TPN	961.48	0.568
TPN	9	3800.47 ± 6355.36	Luminal A	1658.56	0.266
			Luminal B	2271.42	0.163
			Her2-enriched	961.48	0.568
1481.8903	80	2313.64 ± 3963.33			

*p value of ANOVA test

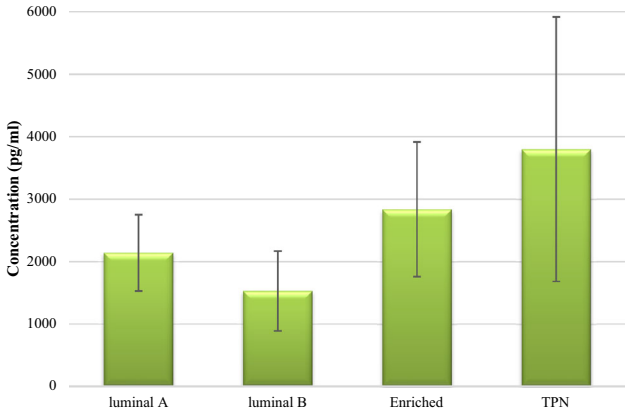

Fig. 4 CDK6 concentration among BC subtypes ($p > 0.05$)

Table 5 Association rs2237572 genotypes frequency and the *p* values of exact test for the deviation from Hardy–Weinberg (HW) equilibrium

	T/T	C/T	C/C	T	C	*p* value*
CDK6 exact test for Hardy–Weinberg equilibrium (n = 160)						
All subjects	60	78	22	198	122	0.74
Control	29	45	6	103	57	0.055
Case	31	33	16	95	65	0.24

*p values of exact test for the deviation from Hardy–Weinberg (HW) equilibrium test

Discussion

Our results showed that the average age of BC patients' is 51.89 ± 12.18 years. The rate of estrogen receptor (ER) synthesis increases along with age but the rate progesterone receptor (PR) synthesis remains constant in all age groups [17]. Moreover, the estrogen synthesis in premenopausal women takes place in the ovary while it occurs in the peripheral tissues in postmenopausal women due to conversion of androgens to estrogen by aromatase and is the most important source for estrogens in the tissue of breast [18, 19]. Estrogen production is constant in postmenopausal women in contrast to cyclic premenopausal women which in turn leads to continuous exposure of breast tissues to estrogen [19]. As a consequent, this continuous exposure to high levels of estrogen could interpret the increased occurrence of BC [20].

Serum CDK6 concentration revealed no significant changes between both, BC patients and controls. More, the significant changes were also absent between BC subgroups. Furthermore, our results showed no significant allele frequency in both allele C and T of rs2237572 between patients and healthy controls. Association of rs2237572 genotypes with BC under different models of

Table 4 Alleles frequency and alleles association of rs2237572

Allele	Control		Case		OR (95% CI)	*p* value*
	Count	Proportion	Count	Proportion		
T	103	0.64	95	0.59	0.809 (0.515–1.271)	0.35717
C	57	0.36	65	0.41	1.236 (0.787–1.943)	

*Pearson's goodness-of-fit chi-square ($df = 1$)

Table 6 Association of rs2237572 genotypes with BC under different models of inheritance

Model	Genotype	Control	Case	OR (95% CI)	*p* value*
Codominant	T/T	29 (36.2%)	31 (38.8%)	1.00	0.036
	C/T	45 (56.2%)	33 (41.2%)	0.69 (0.35–1.35)	
	C/C	6 (7.5%)	16 (20%)	2.49 (0.86–7.25)	
Dominant	T/T	29 (36.2%)	31 (38.8%)	1.00	0.74
	C/T-C/C	51 (63.8%)	49 (61.2%)	0.90 (0.47–1.71)	
Recessive	T/T-C/T	74 (92.5%)	64 (80%)	1.00	0.02
	C/C	6 (7.5%)	16 (20%)	3.08 (1.14–8.35)	
Over dominant	T/T-C/C	35 (43.8%)	47 (58.8%)	1.00	0.057

*p value of tow-tailed Fisher exact test

Table 7 Genotypes frequency of rs2237572 of *CDK6* gene in BC groups

Group	CDK6			Total	*p value
	C/C (%)	T/C (%)	T/T (%)		
luminal A	12 (32.4)	14 (37.8)	11 (29.7)	37	0.001
luminal B	3 (15.8)	12 (63.1)	4 (21.1)	19	
Enriched	0 (0)	2 (13.3)	13 (86.7)	15	
TPN	1 (11.1)	5 (55.6)	3 (33.3)	9	
Total	16	33	31	80	

*p value of Exact chi-square test

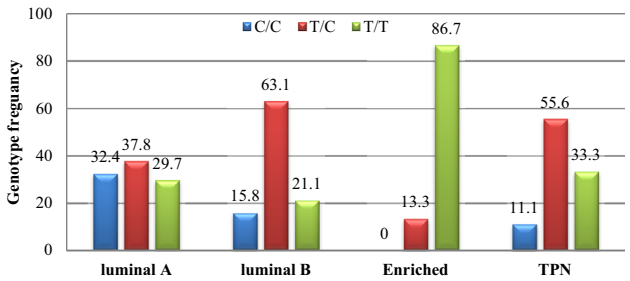

Fig. 5 Comparison of genotypes frequency of rs2237572 among BC subtypes

inheritance showed that C allele is a risk factor for BC and inherited as a recessive pathological factor, while T allele represents a dominate protective genetic factor conferring lesser BC susceptibility for the carrier individuals. A British population-based study by Driver et al. [21] found no significant ($p > 0.05$) association of rs2237570 *CDK6* gene with BC. Aierken et al. [22] were demonstrated the relationship between cervical cancer susceptibility in females from China population and the 3′UTR variants in the *CDK6* gene. Their results revealed abnormal expression of *CDK6* in cervical cancer patients and rs8179, rs42032, and rs42033 of *CDK6* gene were significantly associated with the cervical cancer risk among Chinese females [22].

In this study, a significant difference in the CDK6 serum level was observed between (C/C and T/T), (C/C and C/T) of rs2237572 genotypes. When the alleles C was tested under different models of inheritance, the result showed that the C allele represents as a recessive allele and inherited as a risk factor for BC and CC genotype has a related odds ratio compared to (T/T + CT) of 3.08 (1.14–8.35). That's mean allele C was associated with a higher serum level of CDK6 therefore, allele C was recessive allele that leads to an elevated level of CDK6. While (C\T and T/T) didn't show any association with serum level of CDK6. Mendrzyk et al. [23] found that the

Table 8 Association of rs2237572 genotypes with CDK6 serum level

Genotype	N	CDK6 (pg/ml)	CDK6	CDK6 (pg/ml)	*p value*
C/C	22	4500.77 ± 5742.34	C/T	2679.04728	0.009
			T/T	2534.28602	0.017
C/T	78	1821.72 ± 4105.79	C/C	2679.04728	0.009
			T/T	144.76127	0.842
T/T	60	1966.48 ± 3665.34	C/C	2534.28602	0.017
			C/T	144.76127	0.842
Total	160	2244.38 ± 4280.80			

*p value of ANOVA test

CDK6 gene and genomic amplification were associated with increased CDK6 protein levels [23]. In human tumors, the deregulation of *CDK6* and elevated cyclin activity were associated with the uncontrolled proliferation of cells [24]. In addition, mutations of the *p16INK4a* gene have been associated with a higher risk for developing cancer through uncontrolled CDK4/6-mediated proliferation [25].

Also, our results revealed that different genotypes of rs2237572 did not affect the age of BC onset. Ma et al. [26] were found no significant association in age (> 60 to ≤ 60) among (rs42723, rs22853323 and rs42377) *CDK6* genotypes.

One study by Sherr et al. [27] proposed that the enzymatic activity CDK6 may be elevated in the BC patient and that leads to continuous cell proliferation of breast tissues and help in the occurrence of BC phenotype. Therefore, the enzymatic activity and enzymes kinetics of (CDK4/CDK6) enzymes should take priority in future researches of BC in Iraq. In addition, the measurement of both enzymes by other techniques such as gene expression profiling instead of ELISA technique, perhaps explains the cell cycle disorders in both BC and its subtypes.

Conclusion

No significant difference between patients and healthy controls and between BC patients subtypes in serum concentration of CDK6. Significant association between rs2237572 *CDK6* polymorphism and CDK6 serum concentration was concluded. The C allele of rs2237572 genotype represents a recessive pathological allele and inherited as a risk factor for BC, while the T allele of rs2237572 genotype represents a dominant protective genetic factor conferring lesser BC susceptibility for the carrier individuals.

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Author Contributions SA carried out the molecular genetic studies, sequence alignment, immunoassays and drafted the manuscript. EA participated in the study design and coordination and helped to draft the manuscript. SA conceived of the study and participated in the study design. All the authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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