EMBJ.ORG FOR YOUNG DOCTORS

EUROMEDITERRANEAN BIOMEDICAL JOURNAL
2020,15 (09) 40–46
(*FORMERLY: CAPSULA EBURNEA*)

Original article

ASSOCIATION OF RS2069502 CYCLIN-DEPENDENT KINASE 4 GENE WITH BREAST CANCER

Dalya Shakir Al-owaidi, Moaed E. Algazally, Alaa Sadeq Alawaad

Department of Biochemistry - College of Medicine, Babylon University, Hillah, Iraq

ARTICLE INFO

Article history:
Received 28 January 2020
Revised 22 March 2020
Accepted 06 April 2020

Keywords:
Breast Cancer, Molecular Classification, Cyclin-Dependent Kinase 4, Retinoblastoma protein, Elongation Transcription Factor 2, cyclin-dependent kinase inhibitor 2a.

ABSTRACT

The goal of this project was to evaluate the association of candidate genetic variant rs2069502 of the CDK4 gene with breast cancer and the effect of variant genotypes on the serum concentration of CDK4 enzyme and consequently, on the occurrence of breast cancer and breast cancer subtypes in the studied population. A total of 80 breast cancer patients were divided into 4 subtypes, Luminal A, Luminal B, Her2/neu enriched and TPN, while 80 healthy individuals were enrolled as controls. Our results revealed that there were no significant differences between breast cancer patients and control groups and among breast cancer subtypes in the serum concentration of CDK4 (p-value >0.05). An allelic and genotypic association of rs2069502 with breast cancer showed that there were no significant allele frequency differences, in both alleles A and G, between breast cancer patients and the control group(p-value > 0.05). Association of rs2069502 genotypes with breast cancer and under different inheritance models revealed that there was no significant association between rs2069502 genotypes and breast cancer (p-value> 0.05). The association of rs2069502 genotypes with CDK4 serum concentration showed the presence of a significant difference between A/A and A/G, A/A and G/G in the CDK4 level (p-value < 0.05). But A/G and G/G did not show any significant difference in the CDK4 level. The rs2069502 CDK4 gene did not show any significant association with breast cancer except, in the genotyping frequency among breast cancer subtypes.

1. Introduction

Breast cancer is the most common disease in women that lead to mortality worldwide[1]. Breast cancer begins in the lobules or in the milk ducts [2]. Breast cancer is the first most common cause of death among women in the Babylon province of Iraq. The number of breast cancer patients admitted to the Babylon Oncology center between 2017 and 2018 was 242 and 187 respectively[3].Breast Cancer is considered the major cause of death among women between the ages of 40 and 59[4].

The cell cycle is a complex process that includes cell growth and proliferation, as well as the regulation of DNA repair. During the cell cycle there are several regulatory proteins involved in the G1, S, G2 and M phases of mitosis, leading to the synthesis of 2 daughter cells.

Cell cycle phases have central proteins that include cyclin-dependent kinases CDKs, part of the family of serine/threonine kinases, and the cyclin proteins, together they form a complex functioning as a kinase checkpoint that regulates the progression of the cell during its cycle [5]. The cyclin-CDK complexes regulate the progression of the cell cycle that leads to (G0) the resting state, (G1) growth phase, (S) phase of DNA replication, and finally to the (M)phase when cell division occurs. Cell cycle will be interrupted when abnormalities in one of the phases occur, leading to initiate a signal until the problem is solved [6]. In mammalian cells, there are at least 7 CDKs [7]. During G1, an important target of the CDKs is the retinoblastoma protein (RB), and the G1 cyclin-CDK complexes phosphorylate RB on multiple residues[8]. Hypophosphorylated RB binds the E2F transcription factor, making it unavailable for transcription. Once the cyclin-CDKs phosphorylate RB, which releases E2F for the transcription of proteins necessary for the cell cycle progression [9].

* *Corresponding author*: Dalya Shakir, *dalyashakir@yahoo.com*
DOI: 10.3269/1970-5492.2020.15.9
 ISSN: 2279-7165 - Available on-line at www.embj.org

The action of growth factors on cells during G0 and G1. During the G1 phase of the cell cycle, the restriction point occurs when the cells no longer respond to dragging growth factors [10]. Growth factors stimulate the cells to enter into the cell cycle from G0. The removal of growth factors at the early stages of G1 will result in cells returning to G0. However, in the later stages of G1 when the cells have passed through the restriction point, despite the removal of the growth factor, the cells will continue to progress to the S phase. Therefore, the restriction point can be defined as the time after which the cell is committed to entering into the cell cycle. The restriction point is regulated largely by RB [11].

Cell division protein kinase 4, or human cyclin-dependent kinase 4, is an enzyme encoded by the CDK4 gene. CDK4 is a catalytic subunit of the protein kinase complex (CDK4-Cyclin D complex) that is important for G1 progression through the cell cycle, therefore the activity of CDK4 is limited to the G1-S phase.CDK4 activity is controlled by D-type cyclins as regulatory subunits, and $p16^{INK4a}$ as a CDK inhibitor. CDK4 with its partner CDK6 are responsible for the phosphorylation of the retinoblastoma protein RB. The cyclin D-CDK4 complex, especially the ser/thr-kinase component of that complex, inhibits RB1 retinoblastoma as a member of the RB protein family by phosphorylation, and regulates the transition of G1/S during the cell cycle. Once RB1is phosphorylated, the dissociation of the transcription factor E2F from the RB1/E2F complexes occurs ,targeting the subsequent gene transcription. These genes are responsible for the progression through the G1 phase, figure (1) explains the roles of CDKs in the cell cycle (Figure 1).

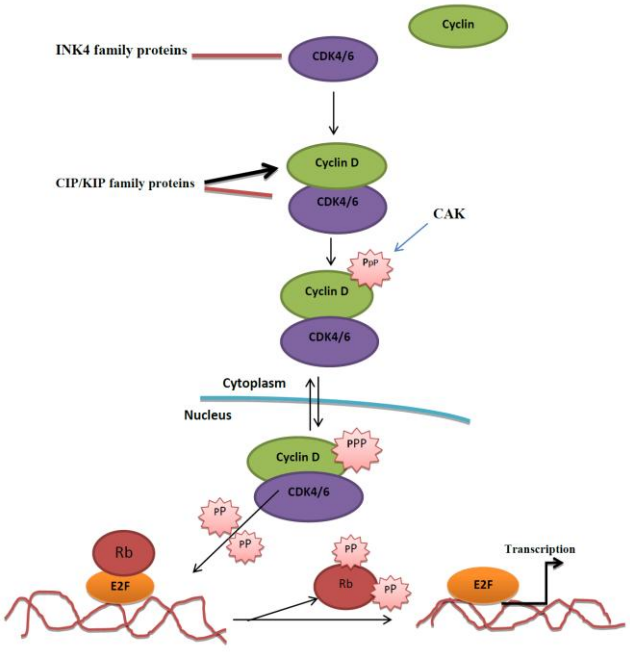

Figure 1. Role of CDK4 and CDK6 in Cell Cycle.

Mutations in the CDK4 gene, D-type cyclins, $p16^{INK4a}$ and RB are associated with numerous types of cancer, including breast cancer[12]. Our aim was to establish the correlation between rs2069502 CDK4 genotypes and breast cancer in Iraqi women.

2. Material and Methods

The subjects who participated in this study were 80 breast cancer patients between the ages of 30-80. The subjects were divided into 4 groups according to the molecular classification of breast cancer, using an immunohistochemistry technique for the expression of estrogen receptor (ER), progesterone receptor (PR), and Her2-enriched proteins:

-Luminal A: 37 female patients with breast cancer

-Luminal B: 19 female patients with breast cancer

-Her2\neu+ enriched: 15 female patients with breast cancer

-Triple-negative: 9 female patients with breast cancer

All patients of these groups did not receive any type of treatment (hormonal or chemotherapy), which means the collection of blood samples was done pre-dose and without using any medication to exclude the effect of these drugs on the biochemical and genetic results.

The control group enrolled in this study included 80 healthy females examined at the consulting clinic of breast cancer early detection in the Hilla teaching Hospital.

The control group matched the breast cancer patients' group in age. The mean value and SD of age for breast cancer patients and controls were (51.89±12.18), (49.2±11.49) respectively. No significant differences in BMI were found among patient subtypes (p-value > 0.05) and the mean values; standard deviations were Luminal A (31.33±4.03), Luminal B (29.84±5.05), Her2-enriched (28.31±6.68) and TPN (28.91±5.51).

Ethical approval

The project proposal and sampling method were approved by the Research Ethics Committee of the college of medicine at the Babylon University. Also, the project received the permission for ethical research in the Babylon Center of Oncology/Marjan medical city on 15/4/2018.

Sampling

Venous blood samples were collected from control and breast cancer patients;8 ml of blood was drawn from each subject by vein puncture, 3ml was put into EDTA purple tube and stored in deep freeze (-20˚C), to be used later for the genetic testing. The remaining 5 ml of blood were dispensed in separating gel tubes, stored at room temperature for 30 min allowing the blood to clot, and then centrifuged at 2000g for approximately 15 min. The serum was placed into a small Eppendorf tube and stored in deep freeze (-20℃), which was later used for biochemical testing of CDK4 enzyme levels using the ELISA technique.

Determination of Cyclin-Dependent Kinase CDK4 Concentration

Sandwich ELISA kits by Sunlong (China) were used in this study. Known concentrations of Human CDK-4 Standard and its corresponding reading OD were plotted on the log scale (x-axis) and the log scale (y-axis) respectively (Figure 2). The concentration of Human CDK-4 in the sample was determined by plotting the sample's OD on the Y-axis. The actual concentration was calculated by multiplying for the dilution factor.

Genetic marker Selection

The SNP: rs2069502 genetic marker was selected based on previous studies[13][14][15] and by using several databases and software such as (NCBI, ensemble, HUGE navigator, GWASdb).

PCR Primer Design

For the targeted genomic region we used the protocol described by[16], summarized as a flow chart (Table1). The product length was set to 100-200 bp, which was suitable for HRM analysis. The specificity of each primer pair was tested by blasting the primers against the human genome (RefSeq reprehensive genome). Furthermore, each primer was tested for its ability to form a secondary structure and dimer using the Oligcalc online software. 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 using the Umelt online software.

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self-complementarity	Self-3' complementarity
Forward primer	GCTACCAGATGGCACTTACAC	Plus	21	14	34	58.72	52.38	4.00	0.00
Reverse primer	GACTGAACACATGAAGCACATGA	Minus	23	135	113	59.50	43.48	4.00	2.00
Product length	122								

* refers to the reverse primer sequences
** refers to the forward primer sequences

Table 1. Accepted primers sets for the selected markers rs2069502

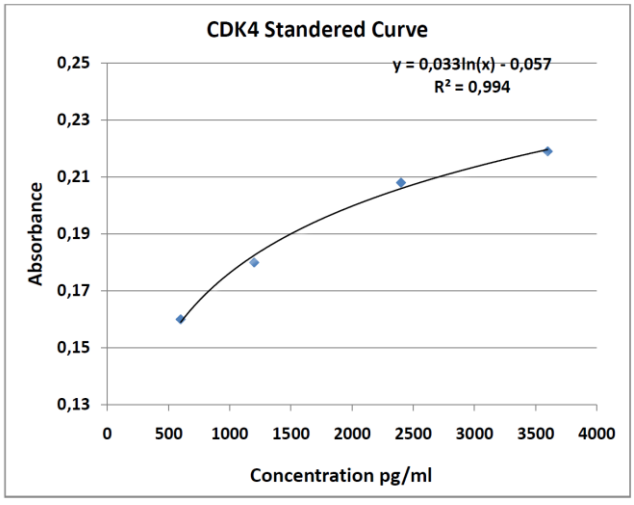

Figure 2. Human CDK-4 Standard Curve

DNA Extraction, spectrophotometry, and Electrophoresis

We followed the standard protocol for DNA isolation[17]and implemented a modification of the original procedure described by Gross-Bellard *et al* [18]. DNA was extracted. DNA quantity and quality were measured by nano-drop, using the scanning ability of a 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 less than 1.8 and /or 260/230 ratio less than 2, re-extraction was performed on the sample. The integrity and molecular weight of extracted DNA were determined using agarose gel electrophoresis[19].

Real-time PCR

The reaction mixture and amplification profile were optimized by applying different primer concentrations and different annealing temperatures, producing the most efficient and specific amplicon that yielded a uniform and clear melting curve.

HRMA Analysis

One µl of 50XEva green stain was added to real-time products and then subjected to high resolution melting analysis (HRMA), the HRMA profile is listed in Table 2. The HRMA data were analyzed using Novallele (Canon Biomedical USA). The HRMA analysis revealed that our samples segregated into 3 well-defined clusters.

Genotyping of Genetic Marker rs206950

Two random samples from each cluster produced by HRMA were analyzed using conventional PCR and then sequenced according to the company manual (Macrogen, South Korea). After sequencing, the HRMA analysis of rs2069502 CDK4 genotypes were explained in figure 3**.** A chromatogram confirmed the 3 different HRMA patterns, showing an obvious form of zygosity status in the 3 groups of investigated samples. The observed variant exhibited three different distributions in the analyzed samples, normal homozygous status (AA),mutant heterozygous status (AG) and homozygous status (GG), as seen in figure4.

Statistical Analysis

The general statistical parameters such as mean, standard deviation, percentage, and descriptive plots were carried out using Microsoft® Excel 2010 software. The phenotypic Odds ratio was calculated, according to Altman,[20]using medcalc.net software. While phenotypic means and standard deviation were compared by student t-test using the IPM®SPSS® software. The genetic association parameters were calculated using SNPStats® online software, while genotypes association was calculated using Fisher's exact test with the Quickcalcs software from GraphPad ®.

No	Stage	
1	Ramp	72-95 C°
2	Step	0.02 C°
3	Hold	3 sec

Table 2. High-Resolution Melting Analysis HRMA Profile

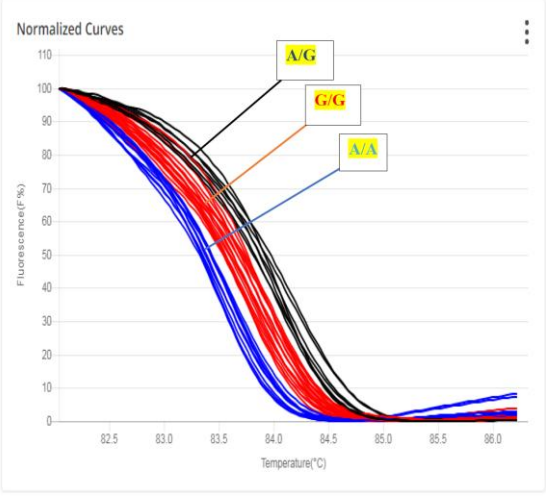

Figure 3. Normalized Curve of HRMA analysis of rs2069502 CDK4 genotypes

3. Results

Our results revealed that there were no significant differences between breast cancer patients and the control groups as far as CDK4 concentrations, expressed as pg/ml [(3956.7±5494.08), (3133.14±1031.56) mean±SD respectively, p-value > 0.05]. Also, there were no significant difference in CDK4 concentration between breast cancer patients subtypes(p-value > 0.05),the means and standard deviation of CDK4 (pg/ml) for breast cancer patients subtypes were Luminal A (2993.91±1273.46), Luminal B (3285.15±1238.0), Her2-enriched (3154.90±986.10) and TPN (2938.55±1554.0).

Our result revealed that there was no significant difference in both alleles A and allele G frequencies between breast cancer patients and control group (p-value >0.05). Allele frequency for patients and control are listed in table 3.The result from Hardy-Weinberg equilibrium model revealed that the genotype frequency in both control and breast cancer patient groups were (p-value =0.640) and (p-value =0.17) respectively, confirming the principle. Table 4 lists the genotype frequency of the 2 groups and the test result for Hardy-Weinberg equilibrium model.

An association of rs2069502 with breast cancer and under different models of inheritance was further tested, revealing that there was no significant association between rs2069502 genotypes and breast cancer, and the results of the genotypic association are listed in the table 5. The results in table 6 show a significant difference in the levels CDK4 between A/A and A/G genotypes, and significant difference in the CDK4 level between A/A and G/G genotypes (p-value < 0.05). These results show that homozygote A/A was associated with a higher serum concentration of CDK4. While A/G and G/G did not show any association with serum level of CDK4 (p-value >0.05). Figure 5 illustrates the comparison between rs2069502 genotypes in serum CDK4 concentration.

	control		Patients		OR (95% CI)	P-value [a]
Allele	Count	Proportion	Count	Proportion		
G	100	0.62	91	0.57	0.791 (0.506-1.238)	0.305
A	60	0.38	69	0.43	1.264 (0.808-1.977)	

a: Pearson's goodness-of-fit chi-square (degree of freedom = 1).
a: Two-tailed p-value of Fisher's Exact Test.

Table 3. Alleles frequency and alleles association of rs2069502

cdk4 exact test for Hardy-Weinberg equilibrium (n=160)						
	G/G	A/G	A/A	G	A	P-value
All subjects	59	73	28	191	129	0.51
control	30	40	10	100	60	0.64
Patients	29	33	18	91	69	0.17

Table 4. rs2069502 genotypes frequency and the P-values of exact test for the deviation from Hardy-Weinberg (HW) equilibrium

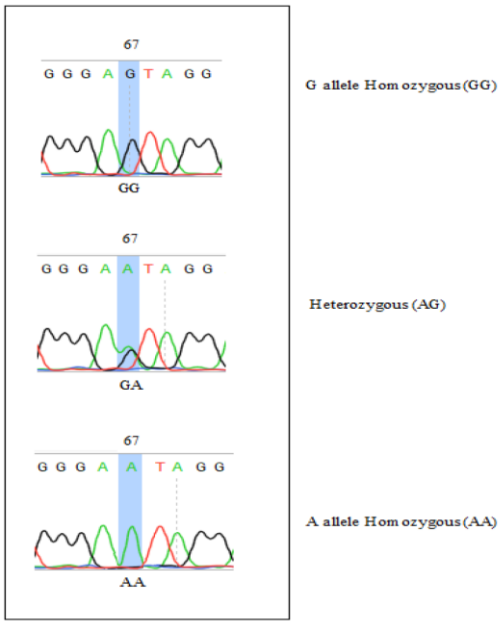

Figure 4. The Pattern of The Observed Substitution Mutation Within the DNA Chromatogram of The Targeted 138 bp Amplicons within the CDK4 gene. The observed substitution mutation is highlighted according to its position in the PCR products.

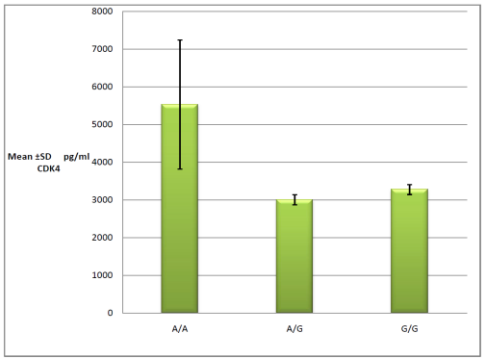

Figure 5. Comparison Between rs2069502 Genotypes in Serum CD4 Concentration.

Model	Genotype	control	case	OR (95% CI)	P-value a
Codominant	G/G	30 (37.5%)	29 (36.2%)	1.00	0.22
	A/G	40 (50%)	33 (41.2%)	0.85 (0.43-1.70)	
	A/A	10 (12.5%)	18 (22.5%)	1.86 (0.74-4.70)	
Dominant	G/G	30 (37.5%)	29 (36.2%)	1.00	0.87
	A/G-A/A	50 (62.5%)	51 (63.8%)	1.06 (0.56-2.01)	
Recessive	G/G-A/G	70 (87.5%)	62 (77.5%)	1.00	0.094
	A/A	10 (12.5%)	18 (22.5%)	2.03 (0.87-4.73)	
Over dominant	G/G-A/A	40 (50%)	47 (58.8%)	1.00	0.27
	A/G	40 (50%)	33 (41.2%)	0.70 (0.38-1.31)	

a: Two-tailed p-value of Fisher's Exact Test

Table 5. Association of rs2069502 genotypes with breast cancer under different models of inheritance

(I) cdk4	N	Mean± SD of CDK4 Concentration pg/ml	(J) cdk4	Mean Difference (I-J) of CDK4 Concentration pg/ml	P value
A/A	28	5528.97±9044.26	A/G	2525.88099*	.004*
			G/G	2255.23013*	.012*
A/G	73	3003.09±1143.25	A/A	-2525.88099-*	.004*
			G/G	-270.65087-	.691
G/G	59	3273.74±992.63	A/A	-2255.23013-*	.012*
			A/G	270.65087	.691
Total	160	3544.92±3961.93			

*The mean difference is significant at the 0.05 level.

Table 6. Association of rs2069502 Genotypes and CDK4 serum level.

4. Discussion and Conclusions

In this study the average age of breast cancer patients was 51.89 ±12.18. Women that reach menopause at the age of 55 have two times the risk for developing breast cancer compared with woman that reach menopause at the age of 45 [21].

An increased threat of developing breast cancer has been regularly connected with early age at menarche and late age at menopause when the period of exposure to high concentrations of endogenous estrogens was increased. It is estimated that premenopausal and postmenopausal breast cancer risk will be reduced by 7% and 3% respectively for every year of menstrual cycle delay, especially after the age of 12 [22].

Estrogen production in postmenopausal women is constantly compared to cyclic premenopausal women.

Consequently, the exposure of breast tissue (epithelium and stroma) to estrogen is continuous in postmenopausal women, leading to an increase in the hazards of mutations in rapidly proliferating breast tissues[23][24].

Also, in this study no major changes in age were shown among breast cancer patients, classified according to IHC. The IHC in the molecular classification assessment is extremely useful and should be requested as a part of routine diagnosis and for treating patients with breast carcinoma. Luminal A patients often have low-grade tumors and a good prognosis. Luminal B, HER2, and TPN types are widely recognized to have poorer survival rates and tumors with higher grades. At diagnosis, patient age was an important prognostic factor, young age at diagnosis was correlated with a worse prognosis.

Breast cancer in younger women had a reduced mRNA expression of ER and PR, and an increased expression of HER2.

WHO proposed the classification of BMI as [25]:

1. Overweight grade1 25.0–29.9 kg/m^2
2. Overweight grade 2 30.0–39.9 kg/m^2
3. Overweight grade 3 40.0 kg/m^2

Our results show that the means of BMI in breast cancer patient subtypes occur between grade 1 and 2 overweight. Obesity is the most significant factor for developing breast cancer in postmenopausal women[26]. Advanced age leads to the increased synthesis of estrone from androstenedione, and would be associated with an increase in adipose tissue activity [27].

We measured the actual concentration of CDK4,and the results revealed that there was no significant differences in CDK4 concentration between breast cancer patients and control groups, and among breast cancer patient subtypes.

Some studies reported that the enzymatic activity of CDK4 may be elevated in the breast cancer patients, leading to the continuous cell proliferation of breast tissue and promoting the occurrence of breast cancer phenotype.

Therefore, the enzymatic activity and enzyme kinetics should be considered in future studies of breast cancer in Iraq. In addition, the measurement of enzymes using another technique, such as gene expression profiling, should be used instead of measuring concentration using the Elisa technique.

We genotyped a highly polymorphic SNP rs2069502, and after a mutational analysis of selected polymorphism, the results showed that no significant allele frequency difference was found in both alleles A and G of rs2069502 between breast cancer patients and the control group. Kristy ED and *et al.* were reported in their British population-based study that there was no evidence of association for rs2069502 of the CDK4 gene with breast cancer [28]. An association of rs2069502 genotypes with breast cancer and under different models of inheritance, revealed that there was no significant association between rs2069502 genotypes and breast cancer, but A/A genotype showed slight distribution differences between breast cancer patients and the control group, where the genotyping frequency was 18 and 10 respectively. Also, p-value = 0.094 is close enough to the level of significance of 0.05, which perhaps could be proven by using a cohort of more than 80 patients, meaning that a large-scale study should be done to confirm our results.

Genetic alterations and mutations in more than half of human tumors occur in the components of the RB/CDK4/cyclin D/p16^{INK4a} pathway [29]. CDK4 mutation leads to loss of the binding ability of CDK4 to p16^{INK4a}, inducing the selectivity of tumor growth and increase the activation of CDK4 in cancer [30]. The CDK4 gene is amplified and over expressed in breast carcinoma [31]. Furthermore, in advanced tumors inducing senescence to prevent tumor progression by targeting CDK4 alleles was very important, therapeutic strategies of the pharmacological importance of CDK4 will be highlighted [31]. As presented in this study, the CDK4 gene had a substantial role in cellular proliferation and may have an important role in many types of cancer. Furthermore, many discrepancies were found in studies about the pathological role of the CDK4 gene and may be attributed to the ethnic and environmental factors.

Our results revealed the presence of a significant difference in the serum CDK4 level between A/A and G/G, A/A and A/G genotypes. Meaning that homozygotes A/A was associated with a higher serum level of CDK4.In some studies, an association between gene expression of the CDK4 gene and high CDK4 protein levels was reported in glioblastoma multiforme and breast carcinoma [32]. Han-X. A. and *et.al* reported that the immunohistochemical analysis of the tumor tissue specimens of 15 types of breast cancer with CDK4 gene amplification, and their results showed of over expression of the gene and increased levels of the gene product [33].

In this study, we tried to identify and investigate the effects of rs2069502 genotypes on the functional level of the CDK4 protein. This information may help when CDK inhibitors were used for future target identification. Many studies have assessed the CDK4 roles in cell growth, proliferation, and cancer[34]. CDK4 was considered as a key regulator of adipocyte function, controlling cell proliferation through the activity of E2F transcription factors [35].

We could conclude that homozygote A/A of rs2069502 CDK4 gene was associated with a higher serum level of CDK4 in breast cancer patients. A large-scale study in the Iraqi region is needed to confirm our results.

References

1. Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J cancer. 2010;127(12):2893–917.
2. S. B. Edge et al. Cancer staging manual. Am. Jt. Comm. Cancer (AJCC). 7th ed. New York Springer, 73, p. 74 (2010).
3. Iraq Ministory of Health .Annual stastical report. 2016.
4. Morgan JG. Position Paper of the American council on science and health on the risk factors of breast cancer. The Breast. 2013;4(3):177–197.
5. Alberts B, Bray D, Lewis J, Raff M, Roberts KW. The Cytoskeleton. Mol Biol Cell.Garl Publ New York. 1983:549–607.
6. Malumbres M, Barbacid M. Mammalian cyclin-dependent kinases. Trends Biochem Sci. 2005;30(11):630–641.
7. Pines J. Cyclins and cyclin-dependent kinases: theme and variations. In: Advances in cancer research. Elsevier; 181, p. 212 (1995).
8. Kato JY, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. 1993;7:331.
9. Arroyo M, Raychaudhuri P. Retinoblastoma-repression of E2F-dependent transcription depends on the ability of the retinoblastoma protein to interact with E2F and is abrogated by the adenovirus E1A oncoprotein. Nucleic Acids Res. 1992;20(22):5947–5954.
10. Barth H, Kinzel V. Epidermal growth factor rapidly impairs activation of p34cdc2 protein kinase in HeLa cells at the G2-M boundary. J Cell Physiol. 1995;162(1):44–51.
11. Zetterberg A, Larsson O, Wiman KG. What is the restriction point?. Curr Opin Cell Biol. 1995;7(6):835–842.
12. Entrez Gene .CDK4 cyclin-dependent kinase 4.. Available online from: https://www.ncbi.nlm.nih.gov/gene/ [Last accessed: 20th of December 2019]
13. Cortes A et al. Resequencing and fine-mapping of the chromosome 12q13-14 locus associated with multiple sclerosis refines the number of implicated genes. Hum Mol Genet. 2013;22(11):2283–2292.
14. Arroyo-Berdugo Y et al. Involvement of ANXA5 and ILKAP in susceptibility to malignant melanoma. PLoS One. 2014;9(4).
15. Shah AU, Mahjabeen I, Kayani MA. Genetic polymorphisms in cell cycle regulatory genes CCND1 and CDK4 are associated with susceptibility to breast cancer. J BU ON. 2015;20(4):985–993.
16. Hashim HO, Al-Saadi AH, Haider AH, Zaidan HK. Association of Uromodulin rs13333226 and Angiotensinogen rs699 genes variants with essential hypertension in Arab Iraqis of Babylon province. Res J Pharm Biol Chem Sci. 2015;6(6):589–601.
17. Repaske DR, Phillips JA, Kirby LT, Tze WJ, D'Ercole AJ, Battey J. Molecular analysis of autosomal dominant neurohypophyseal diabetes insipidus. J Clin Endocrinol Metab. 1990;70(3):752–757.
18. Gross-Bellard M, Oudet P, Chambon P. Isolation of high-molecular-weight DNA from mammalian cells. Eur J Biochem. 1973;36(1):32–38.
19. Sambrook J, Russell DW. Molecular cloning: A laboratory manual, the third edition. Cold spring harbor laboratory press, cold spring harbor, New York; 2001.
20. Altman DG. Analysis of survival times. Pract Stat Med Res. 1991;1.
21. Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev. 1993;15(1):36.
22. Saslow D et al. American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. CA Cancer J Clin. 2007;57(2):75–89.
23. Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis. 1998;19(1):1–27.
24. Pattarozzi A et al. 17β-Estradiol promotes breast cancer cell proliferation-inducing stromal cell-derived factor-1-mediated epidermal growth factor receptor transactivation: reversal by gefitinib pretreatment. Mol Pharmacol. 2008;73(1):191–202.
25. WHO. Obesity: preventing and managing the global epidemic: report of a WHO consultation on obesity, Geneva, 3-5 June 1997. Geneva: World Health Organization; 1998.
26. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res. 2003;9(6):1980–1989.
27. MacDonald PC, Edman CD, Hemsell DL, Porter JC, Siiteri PK. Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer. Am J Obstet Gynecol. 1978;130(4):448–455.
28. Driver KE et al. Association of single-nucleotide polymorphisms in the cell cycle genes with breast cancer in the British population. Carcinogenesis. 2008;29(2):333–341.
29. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta (BBA)-Reviews Cancer. 2002;1602(1):73–87.
30. Park S et al. Aberrant CDK4 amplification in refractory rhabdomyosarcoma as identified by genomic profiling. Sci Rep. 2014;4:3623.
31. Puyol M et al. A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. Cancer Cell. 2010;18(1):63–73.
32. Rollbrocker B, Waha A, Louis DN, Wiestler OD, von Deimling A. Amplification of the cyclin-dependent kinase 4 (CDK4) gene is associated with high cdk4 protein levels in glioblastoma multiforme. Acta Neuropathol. 1996;92(1):70–74.

33. An H-X, Beckmann MW, Reifenberger G, Bender HG, Niederacher D. Gene amplification and overexpression of CDK4 in sporadic breast carcinomas is associated with high tumor cell proliferation. Am J Pathol. 1999;154(1):113–118.
34. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev cancer. 2009;9(3):153.
35. Lagarrigue S et al. CDK4 is an essential insulin effector in adipocytes. J Clin Invest. 2016;126(1):335–348.
36. Koh J, Enders GH, Dynlacht BD, Harlow ED. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature. 1995;375(6531):506.