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Detection of Genetic Polymorphism of *HER2* Gene in *HER2* Positive Breast Cancer Women in Iraq

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Abstract

The human epidermal growth factor receptor-2 (*HER2*) gene plays a critical role in breast cancer development and progression. *HER2* overexpression characterizes a biologically and clinically aggressive breast cancer subtype. In this study, 60 samples from Iraqi women with breast cancer were collected and investigated for *HER2* protein in the tissue by immunohistochemistry. Also, 20 samples from healthy Iraqi women were used as a control. The results showed that 18 (30 %) patients expressed the *HER2* protein. A molecular study for single nucleotide polymorphism (SNP) was conducted on samples metastasizing to lymph nodes. DNA was extracted and polymerase chain reaction (PCR) was performed to amplify exon 17 and intron 17 of *HER2* gene. Sequencing of PCR product was achieved and two SNPs of *HER2* gene, one in exon 17 (Ile655Val) and another close to it in intron 17 (rs903506) were studied. In exon 17, SNP Ile655Val was found in 41% of patients, while in intron 17, the non-coding SNP rs903506 was found in 27% of patients. However, no polymorphism was found in the control group. The results may suggest that *HER2* gene can be used as a molecular marker for breast cancer.

Keywords: *HER2* SNP, *HER2* overexpression, *HER2* polymorphism, *HER2* gene.

الكشف عن التغيرات الوراثية لجين *HER2* لنساء عراقيات مصابات بسرطان الثدي نوات فحص موجب لبروتين *HER2*

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الخلاصة

يلعب جين مستقبل عامل نمو البشرة البشري -2 (*HER2*) دورًا مهمًا في تطور سرطان الثدي. حيث يتميز زياده تعبير بروتين *HER2* نوعًا فرعيًا من سرطان الثدي العدوانية بيولوجيًا وسريريًا. في هذه الدراسة، تم جمع 60 عينة من النساء العراقيات المصابات بسرطان الثدي وفحصها لمعرفة البروتين *HER2* في الأنسجة عن طريق الكيمياء الهيستولوجية المناعية. أظهرت النتائج أن 18 (30%) تعبر عن بروتين *HER2*. أجريت الدراسة الجزيئية

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على العينات التي يكون سرطان الثدي منتشر إلى العقد الليمفاوية والتي تم تحليلها من أجل الكشف عن تعدد أشكال النوكليوتيدات المفردة (SNP) على النحو التالي: تم استخلاص الحمض النووي وتم إجراء تفاعل البلمرة المتسلسل لتضخيم exon 17 و intron 17 من جين HER2 ، وتم إجراء Sequencing تسلسل لنتائج PCR ، تم دراسة تعدد أشكال النوكليوتيدات المفردة (SNP) لجين HER2 Ile655Val في exon 17 و SNP آخر (rs903506) بالقرب منه في intron 17 ، كما تم دراسة 20 عينة من النساء العراقيات الأصحاء كمجموعة سيطرة. في exon 17 ، تم العثور على SNP Ile655Val في 41% من النساء المصابات بسرطان الثدي بينما في intron 17 تم العثور على SNP غير المشفر (rs903506) في 27% من النساء المصابات بسرطان الثدي. و لا يوجد تعدد الأشكال في مجموعة السيطرة. قد تشير النتائج إلى أنه يمكن استخدام جين HER2 كعلامة مناعية وقد يكون كعلامة جزيئية للنساء المصابات بسرطان الثدي.

1. Introduction

Breast cancer is the most common type of female malignancy and the common cause of cancer-related deaths in women. It is still ranked as the top women killer [1, 2]. Accounting for approximately one-third of the registered female cancers, breast cancer has the first rank amongst other cancer types in incidence (23%) and death rate (14%) [3]. Among the population of Iraq, breast cancer is the main type of cancer, with a yearly incidence of 43% among all types of cancers [3, 4].

HER2 gene is located on chromosome 17q21. It is a proto-oncogene that acts through an intrinsic tyrosine kinase activity encoding a transmembrane glycoprotein that belongs to the epidermal growth factor receptors family [5]. *HER2* gene extension and overexpression are found in a variety of human cancers [6, 7]. The alteration is linked with an inferior general survival in patients with breast, endometrial, ovarian, gastric, and salivary gland carcinomas [8-10].

Potential source of heterogeneity in *HER2* protein expression diseases still the target, which therapeutic choices for *HER2* grade dichotomized as (positive and negative) with immunohistochemistry (IHC) state of *HER2* expression is different [11, 12]. With the beginning of the use of *HER2*-targeted therapeutics such as pertuzumab, lapatinib, emtansine and trastuzumab, patients with high *HER2* expression show important improvements in the outcome, once the therapy is maintained, particularly in chemotherapy combination [13].

Genetic variation, including mutations and gene polymorphisms, could affect not only cancer development, but also its progression and phenotypes [14,15]. *HER2* gene is a significant biomarker for investigating cancer cases, particularly . breast cancer. Research reported analyzing *HER2* Ile655Val polymorphism rs1801200) in exon 17 and rs903506 in intron 17 [16]. *HER2* gene is an essential biomarker in breast carcinoma. The Ile655Val polymorphism was positioned in the transmembrane domain that may affect abundance and protein integrity, as well as *HER2* gene activity [17].

Mutations were shown to be associated with the activation of the *HER2* pathway. Currently tyrosine kinase inhibitors lead to inhibit *HER2* in model systems with specific activating mutations [13,18]. The variation Ile655Val of *HER2* gene was investigated in several studies in association with breast cancer [19-21].

The initial reports could not so far successfully indicate positive association between Ile655Val variation and risk of breast cancer. [22- 25].

Conversely, another group of investigations in numerous studies demonstrated that, among younger women who have larger body mass and positive family history, the Val/Val genotype is related with increased breast cancer risk, in contrast to the Ile/Ile genotype [20, 26].

In the present study, we planned to estimate the polymorphisms in exon and intron 17 of *HER2* gene in breast cancer women. *HER2* was investigated as a biomarker that can aid in choosing the suitable strategy for breast cancer therapy. We also examined if the SNPs (Ile655Val and rs903506) are related with breast cancer. Therefore, we analyzed the SNPs in the target gene, compared them with the standards listed in the National Center for Biotechnology Information (NCBI) database and healthy individuals group, and observed their relation with breast cancer.

2. Methodology

2.1. Study groups and samples collection

Blood samples were collected from 60 women who were confirmed with breast cancer and referred to Al-Awram (Cancer Tumors) Hospital and Al-Yarmok Hospital in Baghdad during the period from the 1st of January, 2018 to the 1st of March, 2018. In addition, 20 blood samples were collected from healthy women as a control group, with a mean age of 45 year. Those women were without history of breast cancer and any other neoplastic disease. For both breast cancer and control groups, 5 ml of blood was collected in EDTA tubes and stored at -4 °C for the molecular study.

Also, formalin fixed paraffin embedded (FFPE) tissues were collected directly from patients, after obtaining their consent, and used for the immunohistochemistry study.

Risk factor data were also documented by a short structured questionnaire, including information on age, family history of breast cancer, menopause, and metastasis state.

2.2. Immunohistochemistry study

The expression of *HER2* in FFPEs of breast cancer tissues was measured by immunohistochemistry using *HER2* antibody kit (polyclonal rabbit anti-human *HER2* protein, Dako Danimark) as instructed by the manufacturer. Table 1 shows the scores recorded in the invasive component only.

The first step of the procedure was the application of peroxidase blocking solution and incubation for 5 min. The second step was the application of the primary antibody and incubation for 30 min. The third step was the application of HRP-labeled polymer and incubation for 30 min. Finally, the chromogenic substrate was applied and the mixture was incubated for 10 min. All of the steps were performed at room temperature according to the manufacturer's instructions (Dako, Danimark).

Table 1- Scores of *HER2* protein expression assessment and staining pattern

Score	HER2 protein expression assessment	Staining pattern
0	negative	No staining observed.
1+	negative	A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells exhibit incomplete membrane staining.
2+	Weakly positive	A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.
3+	Strong positive	A strong complete membrane staining is observed in more than 10% of the tumor cells.

2.3.Molecular study

DNA extraction

DNA was extracted from blood samples using QIAamp DNA blood mini kit (Qiagen, USA), as described by the manufacturer. Purity and concentration of extracted DNA were examined by using NanoDrop apparatus (Techne, UK).

Polymerase chain reaction and gel electrophoresis

The extracted DNA from samples was used as a template for PCR reactions. The PCR procedure was performed by utilizing Go Taq® Green PCR Master Mix (Promega, USA). Table 2 shows the sequence of forward and reverse primers for *HER2* gene, which were designed according to the target sequence in *HER2* gene for our study. Table 3 shows PCR material components and Table 4 describes the setup of PCR reaction program based on the melting temperature of primer and optimization.

Table 2- Primer sequence of *HER2* gene

HER2 gene primer	Sequence	Size
Forward	CAAGGAGTTTGGACTTTATTGTG	994 bp
Reverse	GGTTTGC GGGAGTCATATC	

Table 3- Optimal values of various steps of DNA amplification

Master mix components	stock	final	unit	Volume	
				1 sample	10 samples
Master mix	2	1	X	12.5	125
Forward primer	10	1	µM	1	10
Reverse primer	10		µM	1	10
Nuclease free water				8.5	85
DNA	10	10	ng/µl	2	
Total volume				25	
Aliquot per single rxn	23 µl of master mix per tube and add			2 µl of template	

Table 4- Reaction setup of PCR for HER2 gene amplification

Steps	°C	Time (m:s)	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	00:45	
Final extension	72	07:00	1
Hold	10	10:00	

For the detection of PCR product, agarose gel electrophoresis was performed by preparing 1.5% agarose gel and 1X Tris borate EDTA (TBE) buffer. Preparation of agarose and the detection procedure were performed according to Sambrook and Russell, 2001 [27]. The gel was run at a 5V/ cm for one hour and visualized by a ethidium bromide and UV trans-illuminator equipped with a digital camera.

2.4. DNA Sequencing

With the aim of examining the sequences of nucleotides and the incidence of SNPs, DNA sequencing was conducted at the national instrumentation center of environmental management (NICEM), Seoul National University, South Korea, using the ABI prism 3100 xl genetic analyzer (Applied Biosystems, USA). The sequencing results were compared with control group using BioEdid programme. The obtained sequences were aligned with normal sequences found at the NCBI website using BLAST software and examined for presence of SNPs and mutations.

3. Results and discussion

3.1. Patient characteristics

In this study, 60 samples were collected from women with breast cancer. The results in Table 5 revealed that with the patients had a mean age of 57 year. A proportion of 23% appeared with family history for the disease, 48% were menopause, and 62% suffered from metastasis to lymph nodes.

Table 5- Characteristics of samples collected from women with breast cancer

No. of Sample	Mean age	Family history		menopause		Metastasis to lymph nod	
		+ve	-ve	+ve	-ve	+ve	-ve
60	57	14	46	28	32	37	23
Percentage %		23% +ve		48% +ve		62% +ve	

3.2. Immunohistochemistry study for women with breast cancer

All the 60 samples of women with breast cancer were examined for expression of HER2 protein in (FFPE) tissues by using immunohistochemistry technique. The results showed a specific positive immune staining of HER2 in 18 (30%) women with breast cancer, as shown in Figure 1.

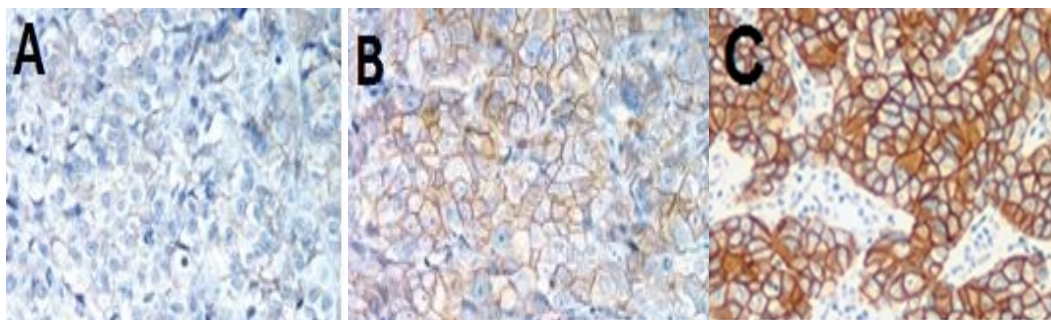


Figure 1-A case of infiltrative ductal carcinoma of the breast in a 50 years old female. Malignant cells show different expression scores of Her2/neu as: A: Score I (negative), B: Score II (Weak), C: score III (positive), using 400x and Hercep Testtm stain .

Also, it was noticed from the data that all samples with positive HER2 were from menopausal women with metastases to lymph nodes

HER2 overexpression was reported to be detected in about 20–30% of breast cancer patients , which is in line with our results which showed that 30 % of patients were HER2 positive [28, 29].

HER2 overexpression is characterized in a biologically and clinically aggressive breast cancer subtype. Then, the establishment of anti-HER2 targeted agents, survival rates of patients with HER2-positive metastatic breast cancer dramatically increased. Nowadays, the decision of the type of treatment protocol in metastatic breast cancer is depended on HER2 and hormone receptors status, so a currant recommended data for a number of biological heterogeneity sources could described positive HER2 metastatic breast cancer [30].

HER2 gene that codes for tyrosine kinase receptor is a powerful mediator of proliferation and cellular growth in normal and malignant epithelial cells. Gene amplification is observed in up to 25% of breast cancers (14.9), which is agree with our study which found high HER2 receptor in 30% of patients.

Somatic mutations in *HER2* gene may also play a role in breast carcinogenesis in tumors missing *HER2* gene amplification. Consequently, tumors with mutations may be resistant to reversible *HER2* inhibitors but sensitive to irreversible ones [31].

In around 25% of breast malignances, the cancer cells force an excess of the HER2 protein, which is produced by a mutation in the *HER2* gene. When the *HER2* gene mutates, it stimulates breast cell to grow and divide at an uncontrolled rate, resulting in tumor growth. Alteration in *HER2* gene was associated with the development of breast cancer. Indeed, 10–40% of breast cancer cases in women are caused by gene alteration [32].

3.3. Purity and concentration of DNA from women with breast cancer

DNA was successfully extracted from blood samples and the purity of extracted DNA varied from 1.8 to 2.0, while DNA concentration varied from 70 to 120 ng/ μ l.

3.4. Amplification of exon 17 and intron 17 of *HER2* gene from *HER2*-positive women with breast cancer

Thirty seven blood samples that showed metastases to lymph nodes were selected for the molecular study, while 20 blood sample from healthy women were used as control.

A DNA segment which included exon 17 and intron 17 in *HER2* receptor gene was identified by using PCR. The outcomes in Figure 2 show a positive reaction for this DNA segment which appeared as a band that is sized about 998 bp.

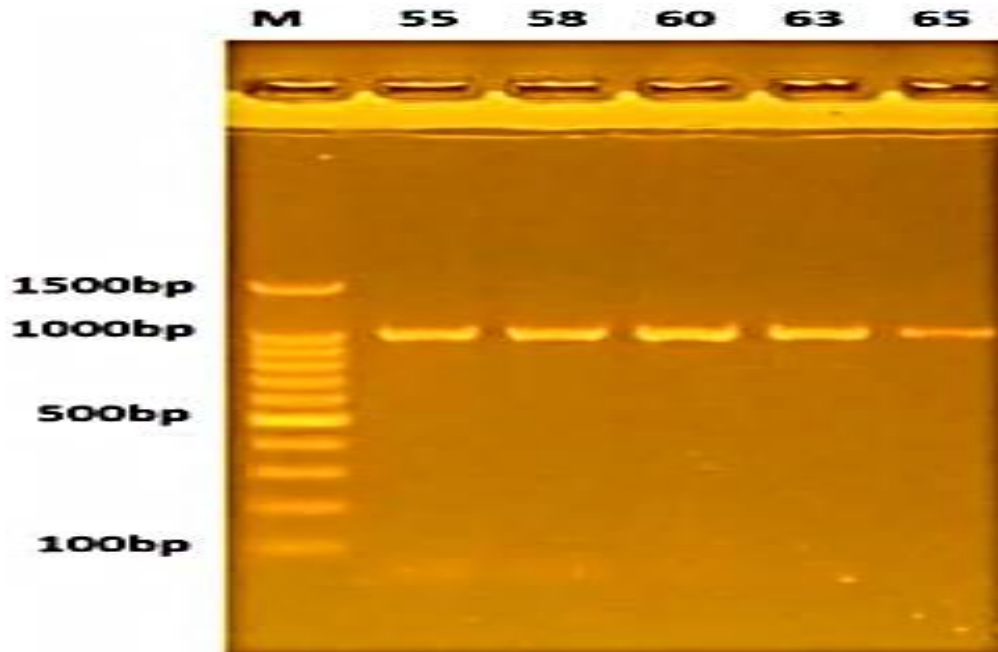


Figure 2-Amplification of DNA segment, including exon and intron 17, in *HER2* gene in blood of *HER2* positive women with breast cancer. The segment appears with a band of 998 bp. Lanes (55-65) indicate DNA of blood samples, Agarose 1.5%, 5V/cm for 1 hour, M: molecular ladder (100-1000 bp).

3.5. Sequencing of *HER2* gene in breast cancer women

The goal of the present study was to investigate two SNPs which are positioned at 175 bp apart; the first one is Ile655Val (rs1801200) positioned in exon 17 of the *HER2* gene and coding the transmembrane domain; the second one is A 23271 G polymorphism (rs903506) positioned in intron 17 and may show a key role in the onset and progression of breast malignancy [5].

The findings of the sequencing of 37 samples showed two groups; the first one is composed of 19 samples that demonstrate lymph nodes metastasis and *HER2* negativity, but do not have any single nucleotide polymorphism. The second group consists of 18 samples that have metastasis to lymph node and *HER2* positive. The results for exon 17 of *HER2* gene indicate one single nucleotide polymorphism (A is replaced by G) in 15 of all 37 (41%) samples with metastasis to lymph node and in 15 of 18 (83%) samples with lymph node metastasis and *HER2* positivity. This SNP is a substitution variation (non-synonymous), in which the amino acid Ile (isoleucine ATC) was altered to Val (valine GTC) at codon 655.

While intron 17 in *HER2* gene showed the presence of another SNP (rs903506), where G is replaced by A at position 37897962 in chromosome 17. This SNP was found in 10 (27%) of all 37 blood samples that have metastasis to lymph node and in (55%) of 18 samples that have

metastasis and HER2 positivity. Also, all 20 healthy women were without any genetic variation. The results shown in Figure 3 show data from breast cancer women matched with NCBI BLAST data, while Figure 4 explains differences between breast cancer women and control, as also shown in Table 6.

Table 6- Polymorphisms in exon and intron 17 of *HER2* gene in women with breast cancer.

No.	Position	All samples % (37 samples)	Her 2 % (18 samples)	SNP	Type	Amino acid change
rs1801200	exon 17	41% (15)	83% (15)	A replaced G	substitution (non-synonymous)	Ile altered to Val
rs903506	Intron 17	27% (10)	55% (10)	G replaced A	Intronic region	Intronic region



Figure -3 Alignment of exon and intron 17 of *HER2* gene of breast cancer women using a sequencer, as analysed by BLAST tool. Samples were compared with templates from NCBI center. “Query” number refers to the present data, whereas the “subjct” refers to the reference gene sequence in NCBI. Red color of nuclutides denotes exon 17 with rs1801200 polymorphism (A substitution by G at position 37879588), while black color of nuclutieds denotes intron 17 with rs903506 (G replaced by A at position 37897962) in chromosome 17.

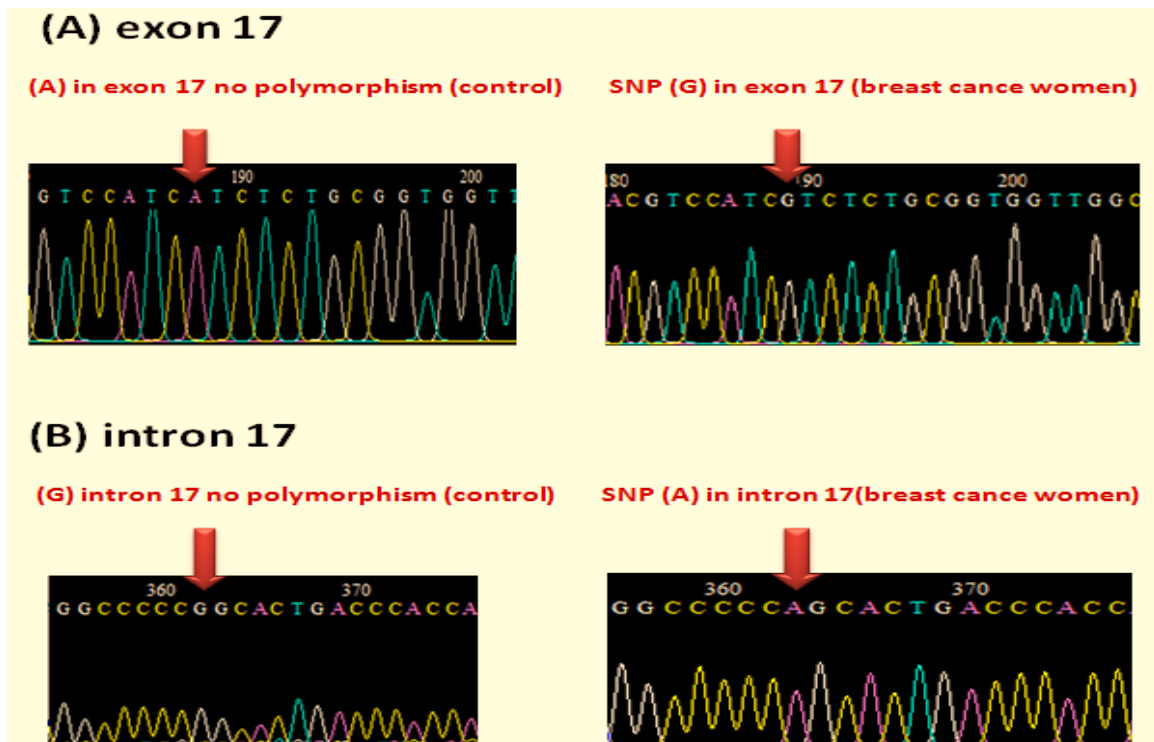


Figure 4-Nucleotide sequencing profile of exon and intron 17 of *HER2* gene breast cancer women. A: polymorphism in exon 17 rs1801200 (A substitution by G) and intron 17 rs903506 (G is replaced by A), as recorded in NCBI Finch TV software.

Gene overexpression and amplification of *HER2* was reported in breast cancer women and this alteration was related with a poor whole survival [33].

Recently, activating mutations were recognized in the extracellular and tyrosine kinase domains of *HER2* gene in breast cancer patients, mainly among those lacking amplification of *HER2* gene. Alterations and mutations established are linked with the activation of the *HER2* pathway. Presently, *HER2* inhibitors were found to inhibit *HER2* gene in model systems with specific activating mutations [34].

In different populations, many studies explained the association of genetic variation of *HER2* gene with breast carcinoma susceptibility. Alteration of *HER2* gene was related with developing breast cancer. Certainly, 10–40% of breast malignancy cases in women are caused by gene alteration [35]. These findings are in line with those of the current study which found an SNP in exon 17 of *HER2* gene (Ile655Val) in 41% of samples that were positive for *HER2* protein.

Recent development in the treatment of breast malignant cells focusing on the importance of multipurpose biomarkers for diagnostic and prognostic applications. Moreover, these markers are important in monitoring the response to anticancer drugs therapy. Numerous studies proved a strong association between breast cancer development and *HER2* gene polymorphism at Ile 655Val codon in the situation of overexpression of *HER2* protein. Indeed, the untreated cases with this presented abnormal protein expression have worse clinical results [20]

Furthermore, the Ile 655 Val polymorphism in *HER2* gene might be a prognostic factor in a neoadjuvant therapy set. The discovery of the *HER2* gene SNP Ile 655 Val in early diagnosed breast cancer women is helpful in scheming the best appropriate treatment, besides raising anticancer drugs efficacy [36].

A study by Fleishman showed that the existence of the amino acid isoleucine in codon 655 destabilizes the active *HER2* heterodimers formation, in the presence of protein overexpression. Also, the occurrence of the amino acid valine reduces the receptor activity, which slows endocytosis and quick receptor recycling [21]. However, another study explain hypothesis include GG (Val/Val) codes receptors with greater capacity dimerization also perhaps constitutive activation [37].

On the other hand, using tool of bioinformatics designed for estimating the effect of SNP on function of protein miscarried discovery of any functional influence of this polymorphism [38].

Furthermore, in this study, we found an intronic SNP in intron 17 of *HER2* gene in 27% of breast cancer women, which may affect gene regulation. Additionally, this study indicated that the non-coding region SNP is a potent regulatory polymorphism that contains promoter/upstream, downstream, and intron regions, which could affect transcription. While the intron and untranslated regions (that are transcribed to RNA) control transcription, stability, and RNA translation splicing, while the intergenic regions have an unidentified function [39, 40].

Furthermost of eukaryotic genes comprise introns that are co-transcriptionally split from the precursor by RNA splicing. The mechanisms regulating the pre-mRNA splicing are poorly understood. Recognition of exon and splicing involves the existence of basic splice sites (polypyrimidine tract, the splice sites and the branch point). The efficiency of this splicing process could be impacted by the presence of modulating or auxiliary elements in certain exons and introns [41].

SNPs show an important role in gene expression regulation in humans. Numerous SNPs have no special effects on the genes. In RNA sequence, single nucleotide variants can control alternative splicing or splicing efficiency that affecting the transcription factor (basal) and splicing machinery or regulating pre-mRNA splicing interaction with RNA [42,43].

In this study, it is uncertain whether this intronic SNP does a functional role by applying a direct effect on the expression of *HER2* gene or whether it is in linkage imbalance with another functional SNP, which needs more investigation.

4. Conclusions

In this study we analyzed SNPs in exon and intron 17 of *HER2* gene. One single nucleotide polymorphism, rs1801200, was found in exon 17, in which A is substituted by G. While in intron 17, the SNP rs903506 was found, in which G is replaced by A (41 % and 27%, respectively). These SNPs may affect breast cancer development and prognosis. *HER2* gene is considered as an important immunological marker and may be a molecular marker (by SNPs) for the diagnosis and decision of the suitable strategy for treatment and disease progression control. In the molecular aspect, there is a need for more developed bioinformatics tools and programs to study protein configuration and prove if variations are found in the functions of genes (proteins) that could affect cancer prognosis and development.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental, health, higher education and scientific research ministries in Iraq.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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