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

Sahar M. Hussein1*, Fatimah A. Abdul Jabbar2, Hadeel Mohamed Khalaf1
1Department of Medical and Molecular Biology, College Biotechnology, University of Al-Nahrain, Baghdad, Iraq
2Department of Plant Biotechnology, College Biotechnology, University of Al-Nahrain, Baghdad, Iraq

Received: 15/3/2021 Accepted: 2/7/2021

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 لنداء عراقيات مرابطات بدرطان الثدي ذوات فحص موجب HER2

سحر مدحت حدين1*، فاطمه احمد عبد الجبار2 و هديل محمد خلف1
1قسم التقنيات الحيوية الجزيئية والطبية، كلية التقنيات الاحيائية، جامعة النهرين، بغداد، العراق
2قسم التقنيات الاحيائية النباتية، كلية التقنيات الاحيائية، جامعة النهرين، بغداد، العراق

الخلاصة

يلعب جين مستقبل عامل نمو البشرة البشري 2 (HER2) دورًا مهمًا في تطوير سرطان الثدي، حيث يتميز زيادة تعبير البروتين 2 نوعًا فريًا من سرطان الثدي الوراثي بيولوجيًا وسريريًا. في هذه الدراسة، تم جمع 60 عينة من النساء غير السرطانات المصابات بسرطان الثدي وفحصها لمعرفة البروتينات في الأنسجة الأمامية عن طريق الكيمياء الهيستولوجية المناضلة. أظهرت النتائج أن 18 (30%) تعبير عن البروتين HER2. أجريت الدراسة الجزيئية

*Email: saharhadad84@gmail.com
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 i 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

| Primer   | Sequence                  | Size  |
|----------|---------------------------|-------|
| Forward  | CAAGGAGTITGGACTTTATTGTG   | 994 bp|
| Reverse  | GGTITGCAGGGATGCTATAC      |       |

Table 3- Optimal values of various steps of DNA amplification

| Master mix components | stock | final | unit | Volume     |
|-----------------------|-------|-------|------|------------|
| 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 |
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 programe. 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
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](image_url)

**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 Test™ 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 current 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/µl.
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](image)

**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 3-3 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 “subject” refers to the reference gene sequence in NCBI. Red color of nucleotides denotes exon 17 with rs1801200 polymorphism (A substitution by G at position 37879588), while black color of nucleotides denotes intron 17 with rs903506 (G replaced by A at position 37897962) in chromosome 17.
Gene overexpression and amplification of \textit{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 \textit{HER2} gene in breast cancer patients, mainly among those lacking amplification of \textit{HER2} gene. Alterations and mutations established are linked with the activation of the \textit{HER2} pathway. Presently, \textit{HER2} inhibitors were found to inhibit \textit{HER2} gene in model systems with specific activating mutations [34].

In different populations, many studies explained the association of genetic variation of \textit{HER2} gene with breast carcinoma susceptibility. Alteration of \textit{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 \textit{HER2} gene (Ile655Val) in 41% of samples that were positive for \textit{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 \textit{HER2} gene polymorphism at Ile 655Val codon in the situation of overexpression of \textit{HER2} protein. Indeed, the untreated cases with this presented abnormal protein expression have worse clinical results [20].

Furthermore, the Ile 655 Val polymorphism in \textit{HER2} gene might be a prognostic factor in a neoadjuvant therapy set. The discovery of the \textit{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].

Figure 4-Nucleotide sequencing profile of exon and intron 17 of \textit{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.
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].

Furthermore 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.

FUNDING: Self-funding.
References

[1] Al-Jubouri, R. S., Al-Rawi, F. A. and Al-Khafaji, A. H. “Urokinase – type Plasminogen Activator System in Human Breast Cancer”. *Iraqi Journal of Science*, vol. 54, no. 4, pp. 1050-1056, 2013.

[2] 1- Abdul-Jabbar, R. A., Ali H. Ad’hiah, A. H. and Alrekabi, A. N. G. “An Evaluation of Some Risk Factors and ABO Blood Groups in Breast Cancer Patients”. *Iraqi Journal of Science*, vol. 54, no. 4, pp. 1036-1043, 2013.

[3] Shamsee, Z. R., Al-Saffar, A. Z., Al-Shanon, A. F. and Al-Obaid, J. R. “Cytotoxic and cell cycle arrest induction of pentacyclic triterpenoides separated from Lantana camara leaves against MCF-7 cell line in vitro”. *Molecular Biology Reports*, vol. 46, no. 1, pp. 381-390, 2019.

[4] Ministry of Health-Iraq-Iraqi Cancer Board. Acceptance of official cancer datasets from Iraq, 2017.

[5] 5- Kalle, I., Kharrat, N., Al-fadhly, S., Rebai, M., Khabir A., Boudawara, T. S., and Reba A. “HER2 Polymorphisms and Breast Cancer in Tunisian Women”. *Genetic Testing and Molecular Biomarkers*. Vol. 14, no. 1, pp. 29-35, 2010.

[6] Slamon, D. Eiermann, W., Robert, N., Pienkowski, T., Martin, M., Press, M., Mackey, J., Glaspy, J., Chan, A., Pawlicki, M., Pinter, T., Valero, V., Liu, M. C., Sauter, G., von Minckwitz, G., Visco, F., Bee, V., Buyse, M., Bendahmane, B., Tabah-Fisch, I., Lindsay, M. A., Riva, A. and Crown, J. “Breast Cancer International Research G: Adjuvant trastuzumab in HER2- positive breast cancer”. *The New England Journal of Medicine*, vol. 365, no. 14, pp. 1273-83, 2011.

[7] Baselga, J., Cortes, J., Kim, S. B., Im, S. A., Hegg, R., Im, Y. H., Roman, L., Pedrini, J. L., Pienkowski, T., Knott, A., Clark, E., Benyunes, M. C., Ross, G. and Swain, S. M. “Group CS: Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer”. *The New England Journal of Medicine*, vol. 366, no. 2, pp. 109-19, 2012.

[8] Banerji, S., Cibulskis, K., Rangel-Escareno, C., Brown, K. K., Carter, S. L., and Frederick, A. M. “Sequence analysis of mutations and translocations across breast cancer subtypes”. *Nature*, vol. 486, pp. 405-409, 2012.

[9] Stephens, P., Hunter, C., Bignell, G., Edkins, S., Davies, H. and Teague, J. “Lung cancer: intragenic ERBB2 kinase mutations in tumours”. *Nature*, vol. 431, no. 7088, pp. 525-6, 2004.

[10] Lee, J. W., Soung, Y. H., Seo, S. H., Kim, S. Y., Park, C. H., Wang, Y. P., Park, K., Nam, S. W., Park, W. S., Kim, S. H., Lee, J. Y., Yoo, N. J. and Lee, S. H. “Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas”. *Clinical Cancer Research*, vol. 12, no. 1, pp. 57-61, 2006.

[11] Normanno, N. De., Luca, A., Bianco, C. “Epidermal growth factor receptor (EGFR) signaling in cancer”. *Gene*, vol. 366, pp. 2-16, 2006.

[12] Griguolo, G., Brasó-Maristany, F., Pascual, T., Chic., N. Vidal, M., Adamo, B., “ERBB2 mRNA as predictor of response to anti-HER2-antibody-drug conjugates (ADC) in breast cancer (BC)”. *Annals of Oncology*, vol. 30, no. 3, pp. 1117, 2019.

[13] Bose, R., Kavuri, S. M., Searleman, A. C., Shen, W., Shen, D., Koboldt, D. C., Monsey, J., Goel, N., Aronson, A. B., Li, S., Ma, C. X., Ding, L., Mardis, E. R. and Ellis, M. J. “Activating HER2 mutations in HER2 gene amplification negative breast cancer”. *Cancer Discovery*, vol. 3, no. 2, pp. 224-37, 2013.

[14] Teng, Y., Tan, W., Thike, A., Cheok, P., Tse, G., Wong, N., Yip, G., Bay, B. and Tan, P. “Mutations in the epidermal growth factor receptor (EGFR) gene in triple negative breast cancer: possible implications for targeted therapy”. *Breast Cancer Research*, vol. 13, no. 2, pp. R35, 2011.

[15] Hussein, S. M., Wali, M. H., Kubba M. A., and Fleih, B. H. “Detection of genetic polymorphism in EGFR in triple negative breast cancer women from Iraq”. *Biochemical and Cellular Archives*, vol. 20, no. 1, pp. 1921-1925, 2020.

[16] Kharrat, N., Al’Fadhli, S. and Reba, A. “Screening and validation of dinucleotide repeats in intron1 of human EGFR gene and its paralog in Her2 gene”. *Journal of Receptors and Signal Transduction*, vol. 28, no. 5, pp. 475-483, 2008.

[17] Shimian, Q. U., Qiuyin, Cai, Q., Gao, Y. T., Lu, W., Cai, H., Su, Y., Wang, E. W., Shu, X. O. and Wei, Z. “ERBB2 genetic polymorphism and breast cancer risk in Chinese women: a
population-based case-control study”. *Breast Cancer Research and Treatment*, vol. 110, no. 1, pp. 169-176, 2008.

[18] Sparreboom, A., Michael, C. C., Milin, R. A. and William, D. F. “Herbal remedies in the United States: potential adverse interactions with anticancer agents”. *Journal of Clinical Oncology*, vol. 22, no. 12, pp. 2489-503, 2004.

[19] Papewalis, J., Nikiit, A.Y. and Rajewsky, M.F. “G to A polymorphism at amino acid codon 655 of the human erbB2=HER2 gene”. *Nucleic Acids Research*, vol. 19, no. 19, pp. 5452, 1991.

[20] Xie, D., Shu, XO., Deng, Z., Wen, WQ., Creek, KE., Dai, Q., Gao, YT., Jin, F. and Zheng, W. “Population-based, case-control study of HER2 genetic polymorphism and breast cancer risk”. *Journal of the National Cancer Institute*, vol. 92, no. 5, pp. 412-417, 2000.

[21] Fleishman, S. J., Schlessinger, J. and Ben-Tal, N. “A putative molecular-activation switch in the transmembrane domain of erbB2”. *National Academy of Sciences*, vol. 99, no. 25, pp. 15937-15940, 2002.

[22] Montgomery, K. G., Gertig, D. M., Baxter, S. W., Milne, R. L., Dite, G. S., McCredie, M. R., Giles, G.G., Southey, M. C., Hopper, J. L. and Campbell, I. G. “The HER2 I655V polymorphism and risk of breast cancer in women < age 40 years”. *Cancer Epidemiology, Biomarkers & Prevention*, vol. 12, no. 10, pp. 1109-1111, 2003.

[23] Kamali-Sarvestani, E., Talei, A. R. and Merat, A. “Ile to Val polymorphism at codon 655 of HER-2 gene and breast cancer risk in Iranian women”. *Cancer Letters*, vol. 215, no. 1, pp. 83-7, 2004.

[24] An, H. J., Kim, N. K., Oh, D., Kim, S. H., Min Jung Park, M. J., Moon Young Jung, M. Y., Kang, H., Kim, S. G., Lee, K. P. and Lee, K. S. “Her2 genotype and breast cancer progression in Korean women”. *Pathology International*, vol. 55, no. 2, pp. 48-52, 2005.

[25] Benusiglio, P. R., Pharoah, P. D., Smith, P. L., Lesueur, F., Conroy, D., Luben, R. N., Dew, G., Jordan, C., Dunning, A., Easton D. F. and Ponder B. A. J. “HapMap based study of the 17q21 ERBB2 amplicon in susceptibility to breast cancer”. *British Journal of Cancer*, vol. 95, pp. 1689-1695, 2006.

[26] Millikan R, Eaton A, Worley K, Biscocho, L., Hodgson, E., Huang, W., Geradts, J., Iacocca, M., Cowan, D., Conway, K. and Lynn Dressler, L. “HER2 codon 655 polymorphism and risk of breast cancer in African Americans and whites”. *Breast Cancer Research and Treatment*, vol. 79, no. 3, pp. 355-64, 2003.

[27] Sambrook, J. and Russell, D. *Molecularly cloning: from A Laboratory Manual Gold Spring*. New York : Harbor Laboratory Press, 2001.

[28] Slamon, D. J., Clark, G. M. and Wong, S. G. “Human breast cancer: correlation of relapse and survival with amplification of the HER-2 neu oncogene”. *Science*, vol. 235, no. 4785, pp. 177-82, 1987.

[29] Wen, W., Chen, W., Xiao, N., Bender, R., Ghazalpour, A., Tan, Z., Swensen, J., Mills, S., Basu, G., Gatalica, Z., and Press, M. “Mutations in the Kinase Domain of the HER2/ERBB2 Gene Identified in a Wide Variety of Human Cancers”. *The Journal of Molecular Diagnostics*, vol. 17, no. 5, pp. 487-495, 2015.

[30] Maria, V., Federica, M., Gaia, G. and Valentina, G. “Biomarkers for HER2-positive metastatic breast cancer: Beyond hormone receptors”. *Cancer Treatment Reviews*, vol. 88, pp. 102064, 2020.

[31] Bose, R., Kavuri, SM., Searleman, A. “Activating HER2 mutations in HER2 gene amplification negative breast cancer”. *Cancer Discovery*, vol. 3, no. 2, pp. 224-37, 2013.

[32] Ménard, S., Tagliaabue, E., Campiglio, M., Pupa, S. M. “Role of HER2 gene overexpression in breast carcinoma”. *Journal of Cellular Physiology*, vol. 182, no. 2, pp. 150-62, 2000.

[33] Gordon, M. A., Gundacker, H. M., Benedetti, J., Macdonald, J. S., Baranda, J. C., Levin, W. J., Blanke, C. D., Elatre, W., Weng, P., Zhou, J. Y., Lenz, H. J. “Press MF: Assessment of HER2 gene amplification in adenocarcinomas of the stomach or gastroesophageal junction in the INT0116/SWOG9008 clinical trial”. *Annals of Oncology*, vol. 24, no. 7, pp. 1754-1761, 2013.

[34] Bose, R., Kavuri, S. M., Searleman, A. C., Shen, W., Shen, D., Koboldt, D. C., Monsey, J., Goel, N., Aronson, A. B., Li, S., Ma, C. X., Ding, L., Mardis, E. R. and Ellis, M. J. “Activating HER2...
mutations in HER2 gene amplification negative breast cancer”. Cancer Discovery, vol. 3, no. 3, pp. 224-37, 2013.

[35] Ménard, S., Tagliaabue, E. and Campiglio, M. “Role of HER2 gene overexpression in breast carcinoma”. Journal of Cellular Physiology, vol. 182, no. 2, pp. 150-162, 2000.

[36] Budiarto, B. R. and Desriani. “Detection of HER2 Gene Polymorphism in Breast Cancer: PCR Optimization Study”. Scientia Pharmaceutica, vol. 84, no. 1, pp. 103-111, 2016.

[37] Pinto, D., Vasconcelos, A., Costa, S., Pereira, D., Rodrigues, H., Lopes, C. and Medeiros, R. “HER2 polymorphism and breast cancer risk in Portugal”. European Journal of Cancer Prevention, vol. 13, no. 3, pp. 177-181, 2004.

[38] Choura, M. and Rebai, A. “Applications of computational tools to predict functional SNPs effects in human ErbB genes”. Journal of Receptor and Signal Transduction Research, vol. 29, no. 5, pp. 286-91, 2009.

[39] Liao, P. Y., and Lee, K. H. “From SNPs to functional polymorphism: the Insight into biotechnology applications”. Biochemical Engineering Journal, vol. 49, no. 2, pp. 149-58, 2010.

[40] Sadee, W., Wang, D., Papp, A. C., Pinsonneault, J. K., Smith, R. M., Moyer, R. M. and Johnson, A. D. “Pharmacogenomics of the RNA world: structural RNA polymorphisms in drug therapy”. Clinical Pharmacology and Therapeutics, vol. 89, no. 3, pp. 355-65, 2011.

[41] McCullough, A. J. and Berget, S. M. “An intronic splicing enhancer binds U1 snRNPs to enhance splicing and select 5’ splice sites”. Molecular and Cellular Biology, vol. 20, no. 24, pp. 9225–923, 2000.

[42] Hull, J., Campino, S., Rowlands, K., Chan, M. S., Copley, R. R. and Taylor, M. “Identification of Common Genetic Variation That Modulates Alternative Splicing”. PLoS Genetics, vol. 3, no. 6, pp. e99, 2007.

[43] Heinz, E.L. “Tissue-specific genetic control of splicing: implications for the study of complex traits”. PLoS Biology, vol. 6, no. 12, pp. e1000001, 2008.