Association Study of *BIF-1* Gene Expression with Histopathological Characteristics and Hormone Receptors in Breast Cancer

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Abstract

Background

Breast cancer (BC) is a heterogeneous disease that has different clinical outcomes. Bax-interacting Factor-1 (*BIF-1*) is a member of the endophilin B family that produces the pro-apoptotic BCL2-Associated X (BAX) protein in response to apoptotic signals. Lack of *BIF-1* inhibits the intrinsic pathway of apoptosis and increases the risk of tumor genesis. The aim of the present study was to investigate the relationship between hormone receptors (ER, PR, HER2) status and different levels of *BIF-1* gene expression in breast cancer patients.

Methods

BIF-1 gene expression was evaluated in 50 breast cancer tumors and 50 normal breast mammary tissues using SYBR Green Real Time RT-PCR technique. Multivariate and univariate analyses were used to evaluate the relationship between the prognostic significance of the *BIF-1* gene using SPSS software. In this study, *BIF-1* was selected as a candidate for a molecular biomarker and its expression status in breast cancer patients with hormone receptors (ER, RR, HER2) compared to patients without these hormone receptors.

Results

The study showed that the relative expression of *BIF-1* gene in tissues of patients with hormone receptor in breast cancer compared to those without hormone receptor were not statistically significant. The expression levels of *BIF-1* gene in different groups were evaluated for hormone receptor status. No significant relationship was found between *BIF-1* gene expression and hormone receptors (ER, PR and HER2) ($p > 0.05$).

Conclusion

BIF-1 gene expression may be a useful prognostic marker in breast cancer.

Introduction

Breast cancer (BC) is a complex heterogeneous disease due to a combination of genetic and epigenetic factors that ultimately alter molecular and cellular processes including proliferation, apoptosis and angiogenesis (1, 2). Breast cancer is the leading cause of cancer death in women worldwide, with an estimated 2 million new cases diagnosed in 2018 (3). Consequently, BC is a major global challenge (4).

Several studies have reported that BC is often triggered by an over-expression of biomarkers that are most commonly defined by estrogen-receptor (ER), progesterone receptor (PR), and Human Epidermal growth factor Receptor 2 (HER2) status. Estrogen-receptor and PR markers have been demonstrated to be important prognostic factors for endocrine therapy (5, 6), whereas, HER2 gene facilitates in the onset, growth, and metastasis of breast cancer (7).

There are various factors involved in cancer, including tumor suppressor oncogenes and genes. Any alteration from their normal activity may lead to unnecessary cell division and apoptotic evasion. Among these tumor suppressor genes is the BCL2-Associated X (BAX) interacting factor-1 (*BIF-1*) gene. The *BIF-1* gene can induce autophagy, a process which can protect normal cells by conserving intracellular homeostasis (8). This process of autophagy involves the interaction of *BIF-1* with the protein Beclin-1 via facilitation of Ultraviolet irradiation resistant-associated gene (UVRAG) that controls phosphatidylinositol 3-kinase complex 3 (PI3KC3) (9).

Current therapies rely on the expression of ER and HER2 receptors for the treatment of Triple Negative Breast Cancer. Unfortunately, these have proven to be ineffective for the treatment of metastatic carcinoma. Hence, there is a need for the development of new therapies relying on molecular biomarkers that will be discussed. In this study, *BIF-1* was selected as a candidate for a molecular biomarker and its expression status in breast cancer patients with hormone receptors (ER, PR, HER2) compared to patients without these hormone receptors. Patients with breast carcinoma often have elevated sensitivity to hormone based therapy if they have high PR and ER (10).

Researchers have long sought to develop early diagnostic methods for identifying cancer. Advances in the use of biomarkers has been effective in the diagnosis and treatment of breast cancer and has led to the application of some of these markers in patients. Common methods for measuring biomarkers such as immunohistochemistry, immunocytochemistry and ELISA are widely accepted and repeated methods in different laboratories (11). However, the non-quantitative results and the numerous and lengthy workflows are limitations of these techniques. Consequently, this has prompted researchers to search for alternative molecular methods such as Quantitative Real Time PCR (Q-RT-PCR) that detects DNA amplification (12–16). The Q-RT-PCR technique is a fast, cost-effective, and high-specificity assay for the evaluation of gene expression (12). Q-RT-PCR technique has considerable potential in biomarker detection relating to mammaglobin (MGB) and metastasis of the lymph nodes (17). For example, it has been recently shown that RT-PCR had considerable diagnostic prediction for detecting mammaglobin biomarker for lymph node metastasis in breast cancer patients (17).

Final endorsement of this method for use in diagnostic laboratories requires multiple validations including housekeeping genes. Several large-scale gene expression studies have been performed in which hundreds of housekeeping genes have been identified (18–20).

In the present study, the *BIF-1*gene was selected as a candidate for a molecular biomarker and its expression in normal individuals and breast cancer patients was investigated. The *BIF-1*gene was

examined according to its histopathological characteristics (stage and grade status) in patients' tumors, as well as the expression status of hormonal receptors (ER, RR, HER2) which have prognostic value.

The aim of this study was to confirm the role of *BIF-1* gene as a predictor of breast cancer for better and proper management of training.

Moreover, our study of molecular biomarkers, including the *BIF-1* gene, could pave the way for biomarkers with therapeutic value in the near future; second, it could lead to the production of effective drugs for the treatment of breast cancer patients and especially those patients with Triple Negative Breast Cancer.

Materials And Methods

Sampling:

Fifty breast tumor tissues (during early diagnosis and no chemotherapy or radiation therapy at the time of sampling) along with **50 adjacent normal tumor** specimens were obtained by following ethical rules in Helsinki Experimental Medical Studies (Declaration of Helsinki (DoH). All the patients signed informed consent. The ethics code number is IR. NIGEB.EC.1395.11.10. I. Samples were received from Milad and Khatam al-Anbia Hospitals and transferred (Transfer to tank containing liquid nitrogen) to National Institute of Genetic Engineering and Biotechnology, Tehran, Iran, in accordance with the principles of transfer and maintenance. All samples were stored in a freezer at -70 ° C for <2 hours.

RNA extraction and cDNA synthesis

50-100 mg of each tissue was cut on dry ice and powdered with Chinese mortar by liquid nitrogen and homogenized in 1 ml of TriPure Isolation Reagent (Roche) solution according to RNA manufacturer's instructions. The resulting RNA was extracted from the tissues, resulting in good quality RNA for the cDNA fabrication, and two gel 18S and 28s ribosomal RNA (rRNA) bands were identified, confirming the non-degradation of RNA, using quantitative absorption spectroscopy. Ultra-violet (UV) concentration and purity of the extracted RNA sample were obtained and the concentration of extracted RNA was in the range of 1000-4000 ng / µl. One Microliter of this RNA was transformed into cDNA using the Revert AID First Strand cDNA synthesis kit.

Internal control and target genes

The housekeeping gene *β-ACTIN*, commonly used in gene expression studies in breast cancer, was selected as an internal control. Its expression stability was assessed to normalize *BIF-1* gene expression. All primers were verified by Generunner v.3.05 design software and Primer Express 3.0 software (Table 1). Duplication efficiency of each primer was determined using standard curve of one-tenth of the cDNA serial dilutions using SPSS (LinReg, Statistical Package for Social Science IL, USA, V.16, SPSS Inc., Chicago) software. The cDNA used for serial dilution of a mixture of 15 tumor samples was equal in proportion.

Table 1: Characteristics of Primers Used in Real Time RT-PCR Reaction

Primer	Sequences	Length(bp)	GC Content	Tm	PCR Product Size(bp)
BIF-1	**F:5'**CTAGAGGGAATCAGCAGTACACATG**3'**	25	48%	60.74	**174 bp**
	R:5'AGGTGTCACAGAAGTCTGATTGTTG**3'**	25	44%	61.20	
b-actin	**F:5'** GAGACCTTCAACACCCCAGCC **3'**	21	61%	62.93	**161bp**
	R:5' AGACGCAGGATGGCATGGG **3'**	19	63%	62.41	

Tumor tissue samples and the tissue adjacent to the tumor lesion from patients were tested using Real Time PCR. The *β-ACTIN* gene was also used for normalization. First, the expression of the *β-ACTIN* gene in the normal tissue around the tumor was investigated. This was conducted in order to obtain a control for distinguishing normal expression from overexpression of this gene.

Real Time PCR Quantitative

Q-RT-PCR reaction was performed using Roche Applied Science SYBR Green I Master Mix Kit (480) in 10 µl reactions. The final concentration of each of the sweep primers was 0.3 µM BIF-1 and beta-actin genes. Table 2 shows the real-time RT-PCR reaction conditions.

Table 2: Thermal program used in Real Time RT-PCR reactions for *BIF-1* and *β-ACTIN* gen

Step	Cycle Number	Temperature (C°)	Duration
Primitive denatured	**1**	**95**	**10'**
denatured		**95**	**20"**
Connecting primers	**40**	**62**	**15"**
Expansion of primers		**72**	**15"**
Melting stage or temperature gradient of 72 to 95 (C°)	**1**	**95**	**5"**

Fluorescence levels were measured using a rotor-gene 6000 corbet manufacturer and data were analyzed by SPSS (LinReg, REST SPSS software, USA, V.16, SPSS Inc., Chicago).

Data analysis

Raw time data from Real Time RT-PCR were analyzed by Lininger software and reproduction efficiency and CT numbers were obtained for each reaction. Next, the expression changes of the studied genes were evaluated by Ringer software using Linger output. To obtain the difference in expression of the target genes and the reference gene, the Levak $2^{-\Delta\Delta}$CT method was used (21). Finally, SPSS software version 16 was used for data analysis and statistical analysis. Kruskal Wallis analysis (due to data normalization) was used to compare the expression of genes with response to treatment and clinical symptoms. The confidence interval was 95% in all experiments and $P < 0.05$ was considered significant.

Results

The afore mentioned institute adhered to all ethical guidelines relating to biological banks for the preservation and use of human specimens. ER, PR and HER2 / neu[1] biomarkers were performed in 50 patients, each with positive and negative biomarkers, as listed in (Table 3).

Table 3- Percentage of positive and negative cases of breast cancer biomarkers in the studied patients

Specification	
Age(years)	
Mean	**50±10.57691**
Range	**30-70**
Cancer Type	**Number and frequency of cases**
Ductal carcinoma	**32(64%)**
Lobular carcinoma	**10(20%)**
Ductal & Lobular carcinoma	**5(10%)**
Unknown	**3(6%)**
Stage at diagnosis	
Stage I	**9(18%)**
Stage II	**36(72%)**
Stage III	**5(10%)**
Lymph node status	
N0	**3(6%)**
N+	**27(54%)**
Nx	**20(40%)**
Hormone-Receptor status(IHC)	
ER and/or PR positive	**35(70%)**
ER and/or PR negative	**9(18%)**
Unknown	**6(12%)**
HER-2 status(IHC)	
HER-2 Positive	**23(46%)**
HER-2 Negative	**21(42%)**
Unknown	**6(12%)**
Triple Negative Breast Cancer ($HER\text{-}2^-$, PR^-, ER^-)	**5(10%)**

The expression levels of *BIF-1* gene in different groups were evaluated for hormone receptor status. The average expression of the *BIF-1* gene in different tumor groups is plotted in Figure 1.

In relation to estrogen hormone receptor status, 34 patients (68%) had estrogen hormone receptor (ER +) while 10 patients (20%) did not have the estrogen receptor (ER). In 6 patients the status of this hormone receptor was unclear.

Thirty-five patients (70%) had progesterone hormone receptor (PR+), while 9 patients (18%) did not have the progesterone receptor (PR-). Also, for human epidermal growth factor receptor type 2 (HER2), twenty-three patients (46%) had type 2 human epidermal growth factor receptor (HER2 +), whereas 21 patients (42%) had no type 2 epidermal human growth factor (HER2). In 6 patients the status of this hormone receptor was unclear.

Alternatively, 5 patients (10%) were missing all three hormone receptors while 39 patients (78%) possessed at least one hormone receptor. In 6 patients the status of hormone receptors was unclear.

Footnote:

1.Human Epidermal growth factor Receptor 2

Discussion And Conclusion

To our knowledge, this study is the first to investigate the relationship between hormone receptor status (ER, PR HER2) and different levels of *BIF-1* gene expression in patients with breast cancer. There was no statistically significant relationship between *BIF-1* gene expression and estrogen hormone receptor (P> 0.05). Additionally, the relationship between *BIF-1* gene expression and progesterone hormone receptor was not statistically significant (P> 0.05). Our findings indicate that the expression in *BIF-1* was increased in patients who have at least one hormone receptor. However, in patients with triple-negative hormone receptors, expression was reduced. There was no statistically significant relationship between *BIF-1* gene expression and all three hormone receptors (ER, PR, HER2) (P> 0.05).

Alternatively, there was no statistically significant relationship between *BIF-1* gene expression in patients with breast cancer (ductal, lobular and ductal & lobular carcinoma) (p> 0.05).

Additionally, there was no statistically significant relationship between *BIF-1* gene expression and disease stage (p> 0.05).

Regarding tumor size, 32 patients (64%) had a tumor equal to or more than two centimeters, and 14 patients (28%) had a tumor size less than two centimeters.

According to the results of linear regression, there was no significant relationship between gene expression and tumor size in breast cancer patients (P> 0.05).

Furthermore, *BIF-1* can also act as a tumor suppressor because of its role in regulating the BAX gene. *BIF-1* accelerates BAX degradation directly by binding to BAX and enhancing apoptosis induction kinetics in

response to innate apoptotic signals, thereby increasing the permeability of the mitochondrial outer membrane.

Previous studies correspond with our findings, For example, Cuddeback et al., reported that the *BIF-1* protein was silent in 17% (192.33 patients) of all prostate cancer patients. These findings indicate the activity of tumor suppression and pro-apoptotic *BIF-1* (22).

Impaired expression of *BIF-1* in cancer cells, compared to adjacent healthy tissue, has been observed in various types of cancer, including colorectal cancer (23), prostate cancer (24), pancreatic cancer (25), invasive bladder cancer (26), and gastric cancer (27).

In another study, Coppola et al. found that *BIF-1* lacked expression in approximately 45% of patients with malignant pancreatic cancer, but had a high level of expression in patients with benign pancreatic cancer (25).

Similarly, Fan et al., observed that patients with high *BIF-1* expression compared to patients with low *BIF-1* gene expression, had a shorter survival time, Their study correlates *BIF-1* expression to survival time (28).

Takahashi et al. showed that suppression of the *BIF-1* gene in mice promoted tumour progression which corresponds with our study's findings. In this study, it was observed that BIF-1 decreased in expression in patients with tumor size equal to or more than two centimeters and increased in expression in patients with tumor size less than two centimeters. This result suggests the induction function of *BIF-1* apoptosis (29).

Unfortunately, few studies have been conducted to quantitatively evaluate the expression of *BIF-1*. Some studies of *BIF-1* gene activity, have been inconsistent. In addition, several studies have compared the expression of *BIF-1* in patients' tumor cell lines with that of the control group (30). We contend that this is not a tenable measure for evaluating both tumor tissue of patients and healthy tissue samples. It should be noted that normal tissue (adjacent to the tested tumor) was confirmed by our results.

Current information in molecular markers is also inadequate when evaluating treatment response in breast cancer patients.

Consequently, it is time to develop a reference method to evaluate *BIF-1* gene expression. Second, investigation of the role of *BIF-1* gene expression in the development of various cancers is necessary. Next, differences in research results may be due to dissimilar methods used to study gene expression. Since various researchers have pointed to this aspect, a reference method for the expression of *BIF-1* and its role in the growth of various cancers needs to be developed.

Lastly, some studies examining BIF-*1* gene expression in breast cancer have been limited to breast cancer cell lines and normal cell lines. Therefore, in the present study, the expression of *BIF-1* gene in tumor and

normal human tissue was investigated. It is well known that gene expression in clinical specimens are lower than cell lines.

The lack of a suitable quantitative method to determine the exact amount of gene expression is an important issue that may lead to difficulties with data interpretation. The major methods of gene expression analysis are: Western blotting, immunohistochemistry, flow cytometry, RT PCR, and Real Time RT-PCR. Therefore, given the high sensitivity of the Real Time RT-PCR method, as well as, the ability of the RNA and cDNA of cells to be stored and reused, it also appears to be faster and more efficient than flow cytometry. Real time RT-PCR is an optimal way in evaluating *BIF-1* gene at the mRNA level.

Other limitations of our study include the high cost of consumables for molecular analysis, the small number of laboratories equipped with the necessary devices and the unwillingness of some hospitals to cooperate in providing the necessary samples, which led to a limit on the number of patients to perform the necessary tests.

Conclusion

Information obtained so far from molecular markers has been inadequate, thus prompting the identification of suitable biomarkers in evaluating response treatment in breast cancer patients.

To study the expression of genes in patients' clinical samples, it is necessary to use a sensitive method, since the expression of genes in clinical samples is less than cell lines. Real Time RT-PCR provides the highest sensitivity and accuracy for determining the amount of expression.

Due to the consistency of the obtained information with Real Time RT-PCR and due to the speed and accuracy of Real Time RT-PCR, this method is recommended for a quantitative study of such markers.

Our findings note that *BIF-1* expression changes can be introduced as a strategic marker in breast cancer patients. In general, the expression level of *BIF-1* increases at the protein level in line with the mRNA level.

Therefore, due to the high sensitivity of the Real Time RT-PCR method and in addition that the RNA and cDNA obtained from the cells can be stored and reused, it also seems to be faster and cheaper than the flow cytometry method. Consequently, an optimal way for evaluating *BIF-1* gene activity at the mRNA level is to use Real Time RT-PCR.

Declarations

Acknowledgments

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Ethics approval and consent to participate

All the specimens were obtained by following ethical rules in Helsinki Experimental Medical Studies (Declaration of Helsinki (DoH). This study was approved by the ethics committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), IRAN (is IR. NIGEB.EC.1395.11.10. I). All the patients signed informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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None.

Author Contributions Statement

"Mahdiyeh Salimi conceived of the presented idea. She developed the theory and performed the computations. S. Abdolhamid Angaji and Kazhaleh Mohammadi verified the analytical methods. Mahdiyeh Salimi encouraged Kazhaleh Mohammadi to investigate [investigation the relationship between hormone receptors (ER, PR, HER2) status and different levels of BIF-1 gene expression] and supervised the findings of this work. Foroozandeh Mahjoobi contributed to sample preparation. Mahdiyeh Salimi, S. Abdolhamid Angaji and Foroozandeh Mahjoobi conceived and planned the experiments. Kazhaleh Mohammadi carried out the experiment. Kazhaleh Mohammadi developed the theoretical formalism, performed the analytic calculations and performed the numerical simulations. Mahdiyeh Salimi, S. Abdolhamid Angaji and Kazhaleh Mohammadi contributed to the interpretation of the results. All authors discussed the results and contributed to the final manuscript. Arthur Saniotis took the lead in writing the manuscript. Kazhaleh Mohammadi and Arthur Saniotis wrote the manuscript with support from Mahdiyeh Salimi, S. Abdolhamid Angaji and Foroozandeh Mahjoobi."

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Ethical Statement

Hereby, I (Kazhaleh Mohammadi) consciously assure that for the manuscript (Association study of BIF-1 gene expression with histopathological characteristics and hormone receptors in breast cancer) the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

I agree with All the specimens were obtained by following ethical rules in Helsinki Experimental Medical Studies (Declaration of Helsinki (DoH). This study was approved by the ethics committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), IRAN (is IR. NIGEB.EC.1395.11.10. I). All the patients signed informed consent and also I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Date: 10-1202021

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Figures

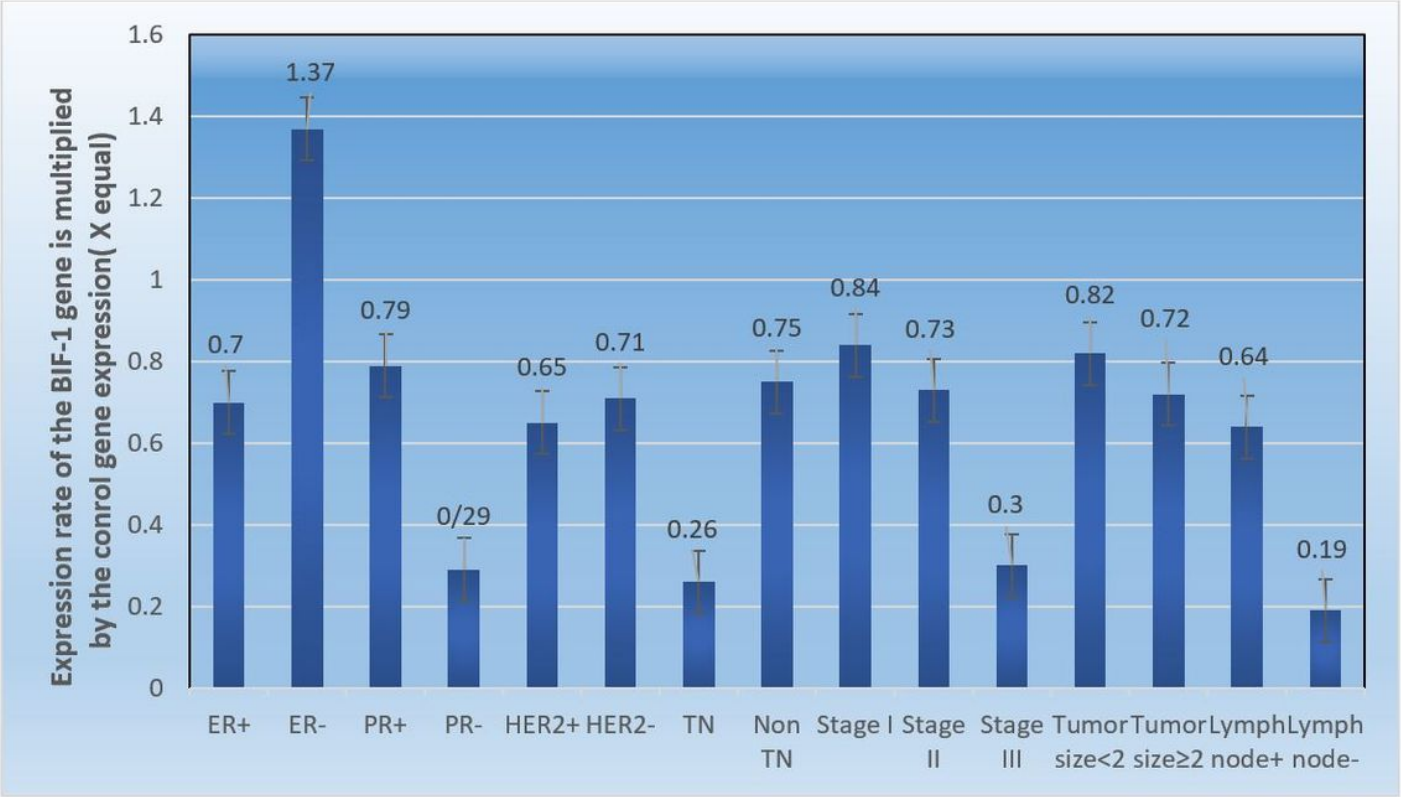

Figure 1

Average expression of *BIF-1* gene in different tumor groups (ER estrogen receptor, PR progesterone receptor, HER_2 Human epidermal growth factor 2, TN= Triple Negative, non-TN= non Triple Negative).