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Master Program in Biotechnology

Breast Cancer*: PTEN* Expression in Palestinian Women

Triple Negative Subtype

BY

May Naji Fadel Al-Abed

In Partial Fulfillment of the Requirement for the Degree Master of Biotechnology.

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The undersigned hereby certify that they have read and recommended to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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Breast Cancer*: PTEN* Expression in Palestinian Women Triple Negative Subtype

By (May Naji Fadel Al-Abed)

ABSTRACT

***Introduction*:** According to data from the Palestinian Ministry of Health, breast cancer is the most common malignancy affecting Palestinian women, being 30% of all cancers affecting female patients. Sixty percent of women diagnosed with breast cancer had the disease already spread to other parts of the body. Very few studies were conducted to explore clinical and pathological characteristics of this cancer in this population. Triple-negative breast cancer (TNBC) has distinct clinical and pathological features; it is a clinical problem because of its relatively poor prognosis and aggressive behavior. The fact that this type is more aggressive despite lacking the expression of growth promotion receptors, suggests other players; such as defects in tumor suppressors such as the *PTEN* gene.

***Aim* :** The aim of this study is to establish data base about the clinical and pathological characteristics of breast cancer in Palestinian women including; age, grade, stage and status of estrogen receptor (ER), progesterone receptor (PR), and HER-2 receptor, then to study the status of *PTEN* protein expression by immunhistochimestry in breast cancer samples specifically in TNBC.

***Materials and methods*:** 100 confirmed cases of breast cancer were collected from different pathology centers, clinical and pathological data, age, grade, stage for each case were specified. Each case was evaluated for the expression of hormone receptors status and for *PTEN* protein expression by Immunhistochimestry (IHC) to see if a certain pattern is noted.

***Results*:** The mean age of the cases at presentation was 53 years. Fifty three (53%) of 100 breast tumors were high grade and 70% presented with advanced stage. Fifty eight percent of cases were ER positive and 46.0% were PR positive. HER-2 positive breast cancer cases counted for 26.0% of all cases. Thirty percent of all breast cancer cases satisfied the definition of TNBC subtype. The majority of TNBC cases (63.0%)

were below 50 years of age. Loss of *PTEN* expression was seen in 44.0 % of breast cancer cases evaluated. Sixty percent of TNBC cases lost *PTEN* expression, which was statistically significant *(P*=0.035). No significant correlation between *PTEN* loss and ER, PR, HER-2, grade or stage was noted.

Conclusion: Palestinian women have high incidence of TNBC in comparison to Caucasian population, and are presented with high grade, and advanced stage tumors. There is a significant correlation between *PTEN* loss and TNBC that needs more research and study.

سرطان الثدي : تقييم وجود جين *PTEN* في سرطان سالب المستقبلات الثلاثية عند المرأة الفلسطينية

مي ناجي العبد

ملخص

المقدمة: وفقا لبيانات سرطان الثدي تبين انه الأكثر شيوعا عند المرأة الفلسطينية ، وأنه واحد من أنواع السرطان الأكثر عدوانية ، مما تسبب في ارتفاع عدد الوفيات ، وعدد قليل جدا من الدراسات تم القيام بها لاستكشاف خصائص هذا السرطان في هذه الفئة من السكان

أن سرطان الثدي سالب المستقبلات الثلاثية يتميز بالعديد من المظاهر السريرية و المرضية , حيث انه يفتقر إلى مستقبلات هرمون البروجستيرون , مستقبلات ه رمون الاستروجين و مستقبلات عامل النمو . ومشكلة التشخيص السريري سببها السلوك العدواني و الافتقار إلى العلاج . حقيقة أن هذا النوع هو أكثر عدوانية على الرغم من غياب التعبير عن مستقبلات تعزيز النمو ، تشير إلى وجود عوامل أخرى مثل خلل في الجينات التي تحافظ على الخلايا و تمنع حدوث الأورام مثل جين *PTEN* . يوجد هذا الجين *PTEN* على الكر وموسوم رقم 10 و يعتبر من أهم الجينات المرشحة التي تسبب حدوث الأورام . أظهرت الدراسات وجود العديد من الطفرات في هذا الحين.

والهدف من هذه الدراسة هو وضع بيانات عن الخصائص المرضية لسرطان الثدي في النساء الفلسطينيات ، بما في ذلك: السن,المرحلة, درجة السرطان وحالة مستقبلات هرمون الاستروجين, مستقبلات هرمون البروجسترون ولها مستقبلات عوامل النمو - 2 ، ودراسة حالة *PTEN* باستخدام immunhistochimestry في سرطان الثدي ، وتحديدا في TNBC.

الطريقة: لقد تم جمع 100 حالة من سرطان الثدي بالإضافة إلى المعلومات الطبية لكل حالة و قد تم جمعها من العديد من المراكز الطبية . لقد تم تقييم هذه الحالات من حيث وجود أو عدم وجود المستقبلات الثلاثية , ثم تم تقييمها من حيث وجود أو عدم وجود جين *PTEN* باستخدام Immunohistochemistry

النتائج: من 100 حالة كان متوسط العمر 53.3 عام years . الدرجة العالية (53.0 ٪) ومرحلة متقدمة (70 ٪) ، الاستروجين الموجب (58.0٪) ، البروجستيرون الموجب (46.0 ٪) ، و مستقبل عامل النمو- 2- الموجب 26.0 ٪. ثلاثون بالمئة من حالات سرطان الثدي كانت سالب المستقبلات الثلاثية وكانت معظم حالاته (63.0 ٪) أقل من 50عام . ستون بالمئة من حالا سرطان الثدي سالب المستقبلات الثلاثية فقدت الجين *PTEN*. وقد وجد خسارة التعبير *PTEN* في 44 ٪ من أورام الثدي. وجد ارتباط كبير بين خسارة و *PTEN* سرطان الثدي سالب المستقبلات الثلاثية *معامل الارتباط* = (*0.035*) ، ولكن لم نجد ارتباط بين وفقدان *PTEN* و الاستروجين والبروجستيرون و مستقبل عامل النمو ، المرحلة المتقدمة و الدرجة .

الاستنتاج: نسبة سرطان سالب المستقبلات الثلاثية عند المرأة الفلسطينية نسبة عالية جدا كما انه يصيب النساء اللواتي اعمارهن اقل من 50 عام.درجة و مرحلة سرطان الثدي عند المرأة الفلسطينية متقدمة. توجد علاقة بين جين *PTEN* و سرطان سالب المستقبلات الثلاثية و هذه العلاقة تحتاج إلى بحث و دراسة.

DECLARATION

I declare that this Master Thesis entitled" Breast Cancer: *PTEN* Expression in Palestinian Women Triple Negative subtype" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: May Naji Fadel AL - Abed

Date: 18/07/2011

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Date: 18/07/2011

Dedication

I dedicate my thesis to my family specially to my parents and my sister Muna, who was patient and took me in the toughest conditions and gave me support and advice. Also I dedicate my thesis to my advisor Dr. Areej AL Khatib who was always by my side, and gave me help and advice throughout that period.

Acknowledgment

I would like to thank my advisor Dr Areej AL Khatib for her support and help on the research contributing to this thesis. I would like to thank Mr. Adel Al Baba the lab technician in Center of Advanced Pathology Labs (CAP) where I conducted my thesis, for providing me with information and helping me when I needed .

List of Abbreviation

TNBC	**Triple Negative Breast Cancer**
ER	**Estrogen Receptor**
PR	**Progesterone Receptor**
HER-2	**Human Epidermal Growth Factor Receptor**
PTEN	**Phosphatase and Tensine homolog Deleted on chromosome Ten**
CS	**Cowden's disease**
Rb1	**Retinoblastoma gene**
IHC	**Immnonohistochemistry**
MMAC1	**Mutated in Multiple Advanced Cancer**
PIP3	**Phosphatidylinositol-3,4,5- Triphosphate**
PIP2	**Phosphatidylinositol-3,4- Bisphosphate**
PI3K	**Lipid Kinase Phosphoinositide 3-Kinase**
GRB2	**Growth factor receptor-bound protein 2**
SOS	**Son-of-sevenless protein**
PKB	**Serine/Threonine Protein Kinase or Akt**
MAPK	**Mitogen-Activated Protein Kinase**
PDK1	**phosphoinositide-dependent kinase 1**
PKB OR Akt kinases	**serine/threonine protein kinase B**
FAK	**Focal Adhesion Kinase**
IS	**Intensity Score**
CAP	**Center of Advanced Pathology Labs**
TBS	**Tris Buffered Saline**

DAB	**3,3 diaminobenzidine tetra hydrochloride**
TS	**Total Score**
PS	**Proportion Score**
SPSS	**Statistical Package Service Solution Software**
H&E	**Hematoxylin and Eosin**
LOH	**Loss of Heterzygozity**
MSP	**Methylation specific PCR**
IDC	**Invasive ductal carcinoma**
CN	**Copy Number**
FISH	**fluorescence *in situ* hybridization**
RTKs	**Tyrosin Kinase Receptor**
DF	**Degree of freedom**
NLS	**Nuclear localizing signal**

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CHAPTER 1

1 - Introduction:

Breast cancer is considered as a heterogeneous disease. It includes a number of biological entities which are associated with specific morphological features and clinical behavior [1]. This is in addition to the variation in incidence and mortality in relation to different ethnicity or race [2].

Breast cancer was the most common cause of cancer deaths among Palestinian women from 1999 through 2003 [3]. The Palestinian Ministry of Health reported that 60% of women in the Gaza Strip were diagnosed with breast cancer after the disease had already spread to other parts of the body [4]. The cancer registry center (CRS) reported that breast cancer occupied the most prevalent type in Palestinian women (31%) ranking it as the first of all cancers in women [5]. According to their data breast cancer is the most common malignancy affecting Palestinian women and it is one of the most aggressive cancers. Up to date this is the first study in this population taking clinical and pathological features into consideration, and after discovering the unique characteristics of this cancer in Palestine, further evaluation of the triple negative subtype and relation to *PTEN* gene expression was done.

DNA Microarrays and hierarchical clustering analysis have classified breast cancer based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor -2 (HER-2) into five groups;

luminal A (ER/PR+, HER-2–), luminal B (ER/PR+, HER-2+), HER-2-enriched tumors (ER/PR–, HER-2+), basal-like (ER/PR–, HER-2–) and normal-like [6,7]. Basal-like subtype is composed almost entirely of triple negative breast cancers

(TNBC) that lack the expression of hormone receptors ER, PR, and HER-2 [8]. TNBC accounts for 10–17% of all breast carcinomas depending on thresholds used to define estrogen receptor (ER), and progesterone receptor (PR) positivity, as well as methods and criteria for HER-2 assessment [8-12].

Recently, there has been great attention towards studying TNBC. This is because it is the most aggressive type of breast cancer; the majority of cases are of histological grade 3 and are invasive ductal carcinoma [12]. Furthermore, TNBC tumors are associated with high rate of recurrence, distant metastases and poorer outcome in terms of overall survival and disease-free interval [13]. These tumors are more prevalent in young women, less than 50 years old [14], and in women of African and Hispanic descent [15,17]. It was found that the frequency of TNBC among Caucasian women is 16%, while it is 26 %among African American women and it constitutes 82% of African Ghanaian women [16]. Furthermore, the prevalence of this type was found to be 23.1% among Hispanic patients [17]. As TNBC is lacking the expression of hormone receptor, it remains the biggest challenge moving forward. In a hormone receptor–positive breast cancer, endocrine therapy is added to chemotherapy. For those that are HER-2+, anti–HER-2 therapy is added, and there is a substantial improvement in outcome because of the addition of a targeted therapy to chemotherapy. Meanwhile, in TNBC, clinicians are entirely reliant on chemotherapy [18].

Currently, there are potential approaches towards finding targeted therapy for TNBC [18]**.** The aggressive behavior of the disease has stimulated large public interest and research initiatives to help in the early detection and to develop new therapies. All efforts are directed at the cellular targets involved in regulating tumor growth and metastasis [19]. Breast cancer results from genetic alterations of normal cells, and

possibly from epigenetic changes [20]. A growing understanding that these alterations are associated with cellular pathway involved in growth and development, In particular, they are associated with tumor suppressor genes, that act as a negative regulator for growth of cell, and their loss of function results in the promotion of malignancy. A number of tumor suppressor genes are known to contribute to breast malignancy including; *P53* [21], *P27* [22], *Skp2* [23], *BRCA-1* [24], *BRCA-2* [25], *PTEN* [26], *p16* [27] and *Rb1* [28].

PTEN is phosphatase and tensine homolog on chromosome ten [29]. *PTEN* encodes a 403 amino acid protein that belongs to the family of protein tyrosine phosphatase [30] and is known to play major roles in suppressing cancer, apoptosis, cell migration and embryonic development [31,32]. *PTEN* protein is a unique phosphatase that has the ability to dephosphorylate proteins and lipids [33].

The main targets of *PTEN* are plasma membrane lipids, phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3,4- bisphosphate (PIP3), that are produced during cellular signaling events by the action of the lipid kinase phosphoinositide 3-kinase (PI3K) [34]. Genetic alteration in *PTEN* had been described in wide variety of tumors including endometrial, breast, prostate, lung, brain and ovarian cancers [35-40].

It was found that germ line deletion of *PTEN* is associated with several autosomal dominant tumor predisposition syndromes such as Cowden's disease (CS)[41,42], with a tendency toward malignant transformation of developing breast cancer [43]. Cowden's disease (multiple hamartoma syndrome) is characterized by mucocutaneous lesions, especially facial trichilemmomas and other follicular malformations, oral papillomas [44] benign hamartomas, macrocephaly, gangliocytomas of the cerebellum and increased predisposition to breast, thyroid, and endometrial carcinoma [45].

Many studies have investigated the role of *PTEN* in the tumorgenesis of breast cancer. Investigation by Ghosh's group suggested that *PTEN* acts as a transcriptional repressor, inhibits the AKT-mediated cell survival signaling pathway, and negatively regulates human breast cancer cell growth through modulating c-Myc gene [46,47]. Research carried out by Yang etal. using Immunohistochemical analysis showed that 48% of breast cancers demonstrated loss of PTEN protein expression [48].

1.1 The Phosphoinositide 3-kinase/Akt Pathway:

The lipid phosphatase function of *PTEN* acts as a negative regulator of the AKT pathway. *PTEN* dephoshprelates (PIP3) at the D3 position generating (PIP2), thus decreasing the cellular PIP3 levels (Figure1.1) [49,50,31].

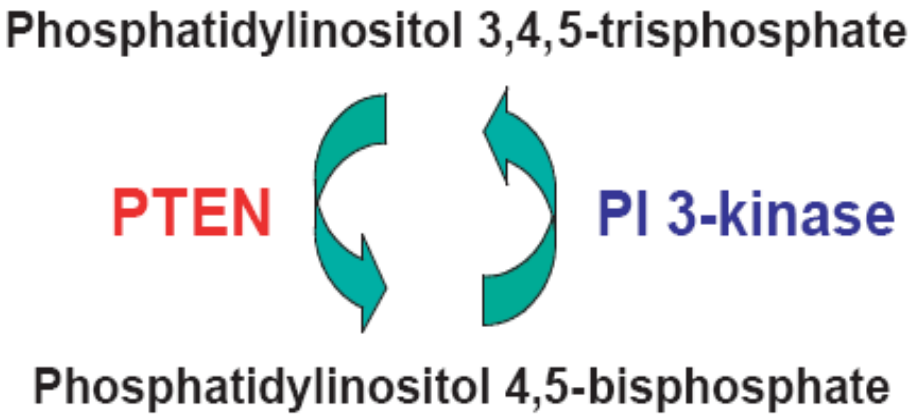

Figure 1.1: *PTEN* a lipid phosphatase. PI3K lipid kinase catalyzes the transfer of phosphate group to PIP2, thus generating PIP3. PTEN removes the phosphate group, and regenerates PIP2.

Signaling through the PI3K pathway is initiated by receiving cell growth and survival signals, that are sensed and transmitted by receptor tyrosine kinases (RTKs) spanning the plasma membrane to the internal cellular environment [51]. Upon ligand activation, RTKs activate the PI3K resulting in the recruitment of PI3K to the

membrane and the generation of PIP3 [52,53]. Once generated, the phospholipids PIP3 recruits the serine/threonine protein kinase B (PKB) to the plasma membrane, also known as Akt kinases and phosphoinositide-dependent kinase 1 (PDK1) [54]. Upon membrane localization, Akt is phosphorelated by PIP3 and PDK1 and becomes active [55], and capable of phosphorylating a number of downstream targets, that are important for cells growth, proliferation, apoptosis, metabolism and survival (Figure 1.2) [46,56]. The dephosphorelation of PIP3 by *PTEN* will inactivate this signaling pathway [57].

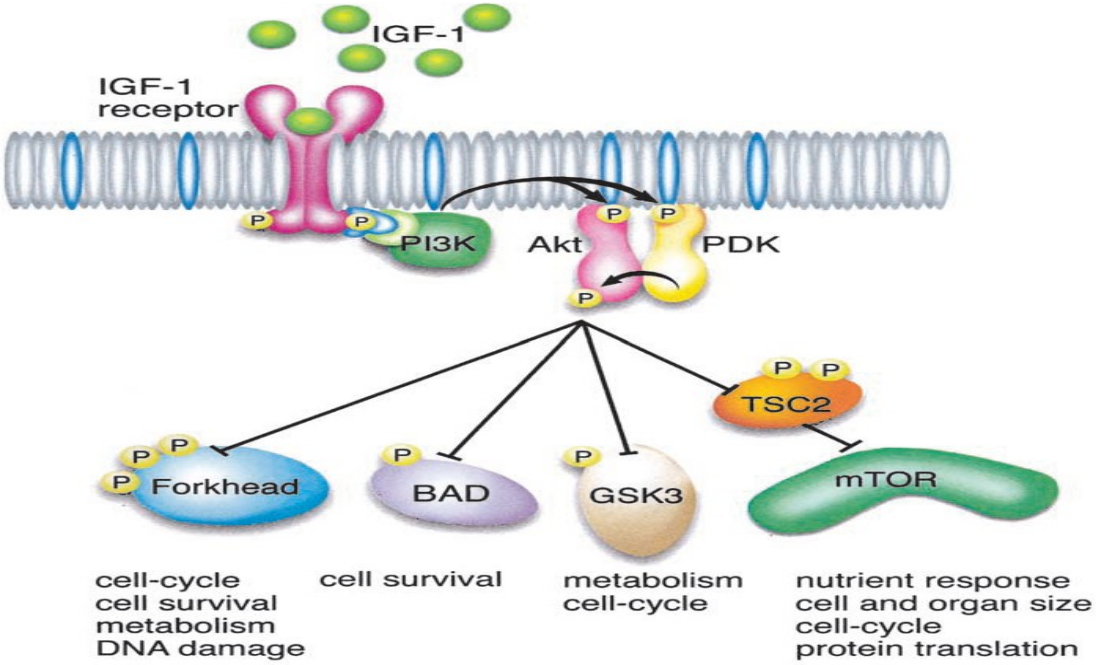

Figure 1.2: The PI3K Pathway. Upon binding of the legend (green) to the receptor(pink) , PI3K is activated thus it phosphrelate PIP2 to produce PIP3 . PIP3 in turn recruits PDK1(green) to the plasma membrane which will activate AKT pathway that controls cellular processes . The lipid phospatase activty of *PTEN* dephosphoralates PIP3 and produces PIP2, resulting in reducing AKT activity.

PTEN has protein phosphates activity has been shown to inhibit the SHC/SOS/GRB2 and mitogen-activated protein kinase (MAPK) pathway. The dephosphorelation process of SHC by *PTEN* decreases the phosphorelation of (MAPK). As a result, *p27* is up regulated and levels of cyclin D are reduced leading to G1 arrest (Figure1.3)

[58-60]. Additionally, *PTEN* has been shown to dehposphorelate focal adhesion kinase (FAK), which inhibits cell spreading and migration [61].

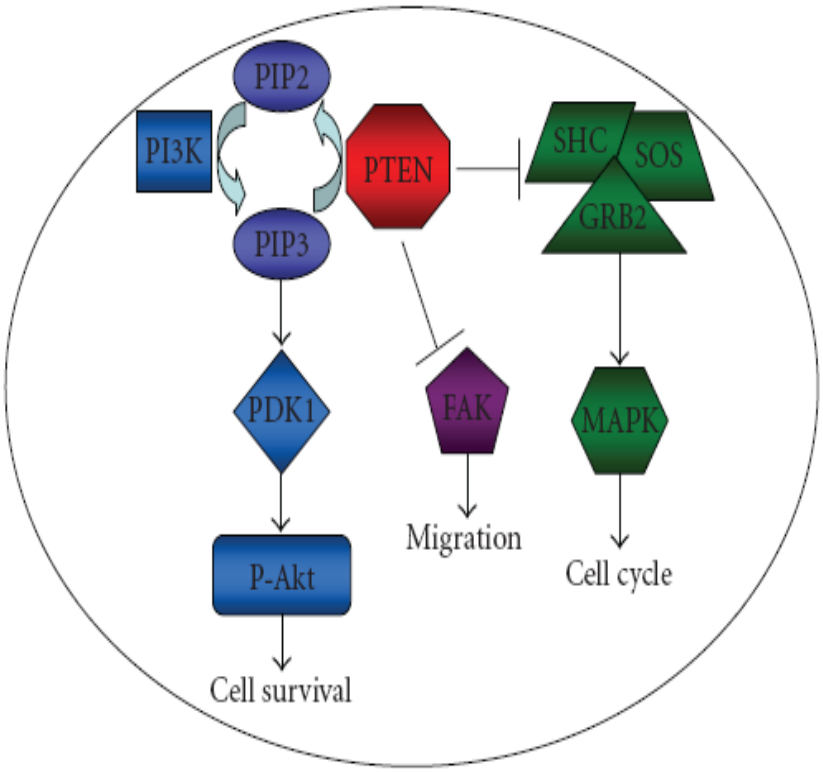

Figure 1.3: *PTEN* protein signaling pathways.

1.2 Location of *PTEN*: *PTEN* nucleo- cytoplasmic shuttling in breast cancer:

It is well established that *PTEN* regulates cell growth and cell cycle arrest [62]. To prove that *PTEN* localizes in both cytoplasm and nucleolus and shuttles between them, Chung and Eng, analyzed downstream *PTEN* readouts using MCF-7 Tet-Off breast cancer cell lines stably transfected with two different NLS mutant *PTEN* constructs, which do not localize to the nucleus, and compared these with cells transfected with wild-type *PTEN* and empty vector control cells [63]. They found that cytoplasmic *PTEN* downregulates phosphorylation of Akt and up-regulates *p27kip1*, whereas nuclear *PTEN* down-regulates cyclin D1 and prevents the phosphorylation of

MAPK. Additionally, they observed that nuclear *PTEN* is required for cell cycle arrest, and cytoplasmic *PTEN* is required for apoptosis [63].

The overall goal of this study is to establish database for the clinical and pathological characteristics of breast cancer among the Palestinian women including; age, grade, stage, status of estrogen receptor (ER), progesterone receptor (PR) and HER-2 receptor. In addition, we studied the status of *PTEN* protein expression by immunhistochimestry in breast cancer and specifically in TNBC.

CHAPTER 2

2-Materials and Method:

One hundred Cases were selected randomly between 2008 and 2010, from different pathology centers including; Center of Advanced Pathology Labs (CAP), al Makassed Islamic charitable Hospital and Beit Jala Governmental Hospital. The clinical and pathological data were obtained. Clinical data included site of biopsy, procedure name, and age. Pathological data included type of cancer, grade, ER, PR, and HER-2. The study included the pathological TNM stage (tumor nodal metastasis) where T specifies exact tumor size, N specifies the number of metastatic lymph nodes over the total number removed and M specifies metastatic site [64].

TNM stage was available for 63 of the total number of cases, the age was not available for only one case, procedure name was unknown for 13 of cases, site of biopsy was unknown for 18 of cases and type of cancer was unknown for 20 of cases (Table 2.1).

The study was conducted in Center of Advanced Pathology Labs (CAP). The original diagnosis for each case was re-evaluated to confirm the presence of tumor using Hematoxylin-Eosin stain and to re-evaluate the grade of the tumor. All cases were evaluated for ER, PR, HER-2, and for *PTEN* by Immunohistochemistry.

Clinical and pathological data	Number of cases
Age	available for 99 case (15-87year)
Grade	
High grade	53
Low grade	47
Total	100
Stage	
Early stage	32
Advanced stage	31
Total	63
Site of Biopsy	
Left breast	47
Right breast	35
Total	82
Procedure Name	
Modified Radical Mastectomy:	42
Lumpectomy	13
Quardectomy	1
True cut biopsy	31
Total	87
Type of cancer	
Invasive ductal carcinoma(IDC)	75
Colloid carcinoma	1
Invasive lobular carcinoma	3
Papillary carcinoma	1
Total	80

Table 2.1: Distribution of the clinical and pathological data

2.1 Immunohistochemistry:

Immunhistochimestry steps were run as previously described [65, 66].

Sections from formalin – fixed, paraffin – embedded tissues, were cut at 4 μ thicknesses, mounted onto Super frost Plus slide, and left to dry overnight at 65°C. Sections were then deparaffinized in xylene, 2 stations 3 minutes for each and rehydrated in ethanol series: 100% ethanol 2 stations for 1 minute each, 95% ethanol, 2 stations 1 minute each and 70% ethanol 2 stations for 1 minute each. Then they were washed with running water for 2 minutes.

Endogenous peroxidase activity was blocked by incubating the slides for 5 minutes in 3% hydrogen peroxide (Biolab). Antigen retrieval was achieved by heat retrieval using pressure cooker. Slides were placed in Coplin jars containing enough citrate buffer pH 6.0 (Diagnostic Biosystem) to cover the sections, then slides were cooked for 30 minutes at 100°C. Slides were removed from pressure cooker and cooled in a water bath for 15 minutes, and then they were placed into 3% hydrogen peroxide for 5 minutes. Slides were removed, tissue sections were dried around and circled with a pap pen. The sections were incubated with 100- 200μL of diluted primary antibodies: *PTEN*, ER, PR and HER-2 for 20 minutes. The dilution of the primary antibodies against ER (mouse monoclonal antibody from Biocare Medical, clone 1D5) and PR (rabbit monoclonal antibody, Biocare Medical, clone SP2) and for HER-2 (mouse monoclonal antibody, Biocare Medical, clone CB11) was 1:100. The dilution for *PTEN* primary antibody (mouse monoclonal anti *PTEN* antibody from Diagnostic Biosystem, clone 28H6) was 1:50.

After the incubation period, primary antibody was washed using Tris Buffered Saline (TBS) (pH 7.6, sodium azaid and thimerosal free) for 5 minutes. After washing, binding of antibody was detected by incubation for 10 minutes with Peroxidase – labeled polymer conjugated to universal detection (Zytomed Systems /HRP).

Then slides were washed by TBS buffer, then the chromogenic reaction was carried out by adding 3,3 diaminobenzidine tetra hydrochloride (DAB, Biocare) as chromogen to produce the characteristic brown stain for 5-15 minutes. Finally, after rinsing with tap water, the slides were counterstained with hematoxylin, dehydrated and mounted with mounting media (Sigma)and cover slipped.

For each run of staining, positive control slides were prepared. The positive control slides were prepared from normal beast tissue which is known to be positive for ER, PR, and from breast carcinoma known to over express HER- 2. Positive control for *PTEN* was prepared from tonsil, besides the internal control of normal beast tissue that exists in some slides for ER, PR and *PTEN*, which are positive in normal breast tissue .

2.2 Staining Interpretation:

Evaluation of ER, PR staining takes into consideration both the proportion and intensity of stained cells [67]. The proportion score (PS) estimates the proportion of positive tumor cells and ranges from 0 to 5 (0 = none; 1 <1/100; 2 = 1/100 to <1/10; 3 = 1/10 to<1/3; 4 = 1/3–2/3; 5 => 2/3). The intensity score (IS) ranges from 0 to 3 (0 = one; 1 = weak; 2 = intermediate; 3 = strong)

The PS and IS are added to obtain a total score (TS) that ranges from 0 to 8. (Table 2.2).

Table2. 2 : Guide lines for the interpretation of ER and PR staining

Proportion Score(PS)	Observation	Intensity Score(IS)	Observation
0	NONE	NONE	0
1	Weak	<1/100	1
2	Intermediate	1/100 to <1/10	2
3	Strong	1/10 to< 1/3	3
4		1/3–2/3	
5	> 2/3		

HER-2 neu was scored on a 0 to 3 scale according to the criteria set by ASCO-CAP guidelines [68].

The staining was scored as: negative (0) when no membrane staining was observed, or when membranous staining was observed in less than 10% of the tumor cells; weak positive (1+) if weak focal membrane staining was seen in more than 10% of the tumor cells; intermediate (2+) if weak to moderate, complete membrane staining was seen in more than 10% of the tumor cells; and strongly positive (3+) if intense membrane staining with weak to moderate cytoplasmic reactivity was seen in more than 10% of the tumor cells (Table 2.3).

Table 2.3: ASCO-CAP guidelines for HER2 scoring

Score to Report	HER-2Protein Over expression Assessment	Staining Pattern
0	**Negative**	**No staining or faint membrane staining is observed in < 10%of the tumor cells**
+1	**Negative**	**A faint /barely perceptible membrane staining is detected in >10%of tumor cells. The cells exhibits incomplete membrane staining**
+2	**Equivocal**	**Non uniform or weak complete membrane staining is observed in 10%of tumor cells**
+3	**Positive**	**A strong complete membrane Staining is observed in >30% of tumor cells**

Evaluation of *PTEN* expression was based on staining intensity and distribution. Intensity was scored as strong, moderate, or weak. Distribution was scored as diffuse if 50% of tumor shows staining, regional if 15 to 50% of tumor shows staining, and focal if 15% of tumor shows staining. Tumors with intense to diffuse, intense to regional, intense to focal, and moderate to diffuse staining were considered positive for *PTEN* expression, while tumors with moderate to regional, moderate to focal, or weak staining with any distribution were considered negative [69] (Table 2.4).

Table 2.4: *PTEN* Scoring System

Observation	*PTEN* Protein expression Assessment
Intense to diffuse, Intense to Regional, Intense to Focal, and moderate to diffuse staining	Positive
Moderate to regional, moderate to focal,	Negative
Weak staining with any Distribution	Negative

CHAPTER 3

3-Statistical analysis:

3.1 Sampling of the Study:

The study population consisted of one section, which covers the patients who are suffering from breast cancer. One hundred cases of paraffin – embedded tissues were selected by Simple Random Sampling. The study includes the variables ER, PR, HER-2, *PTEN,* age, stage, grade and TNBC.

3.2 Statistical treatment:

Findings were analyzed using statistical software SPSS "Statistical Package Service Solution software" for Windows Version 13. The significance of association between *PTEN* and the parameters: grade, stage, ER, PR, HER-2, and TNBC subtype was tested by using Chi square (X^2). The level of significance was set at 0.05. SPSS program was used to find percentage and frequency for each variable.

CHAPTER 4

4- Results:

4.1 Frequency and distribution of the variables: age, grade, stage, ER, PR and HER-2.

This study includes 100 cases; the age range was between 15 years to 87 years old. The mean age was calculated for 99, and was found to be 53 years old. 49 (49 %) of the cases were below age of 50 years old (Figure 4.1).

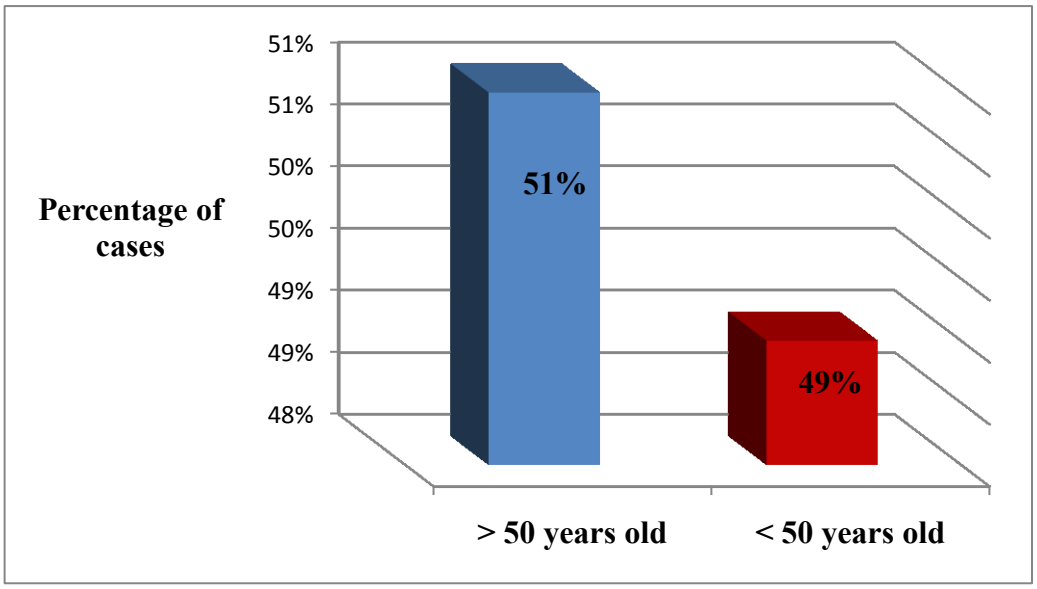

Figure 4.1: The Percentage of cases below and above age of 50 years.

In this study, 53 (53%) of the cases were high grade tumors and 47 (47%) were low grade tumors (Figure 4.2).

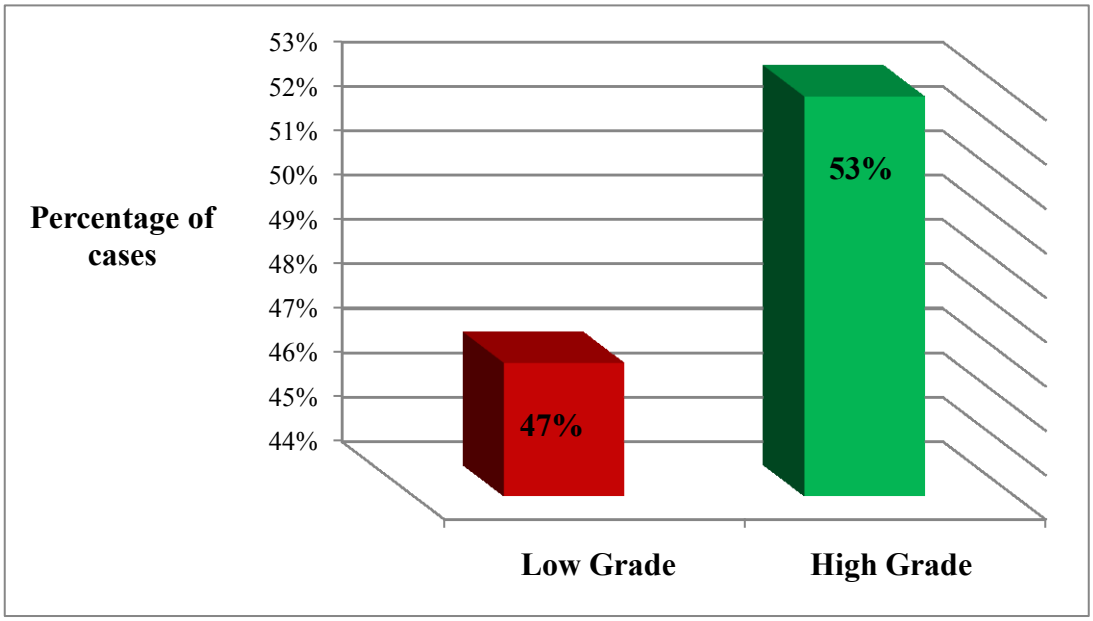

Figure 4.2: The Percentage of High Grade and Low Grade cases.

In this study, the stage was available for 63 of cases. Forty four out of 63 (70%) were tumors with advanced stage, while there were 19 out of 63 (30%) tumors with early stage (Figure 4.3).

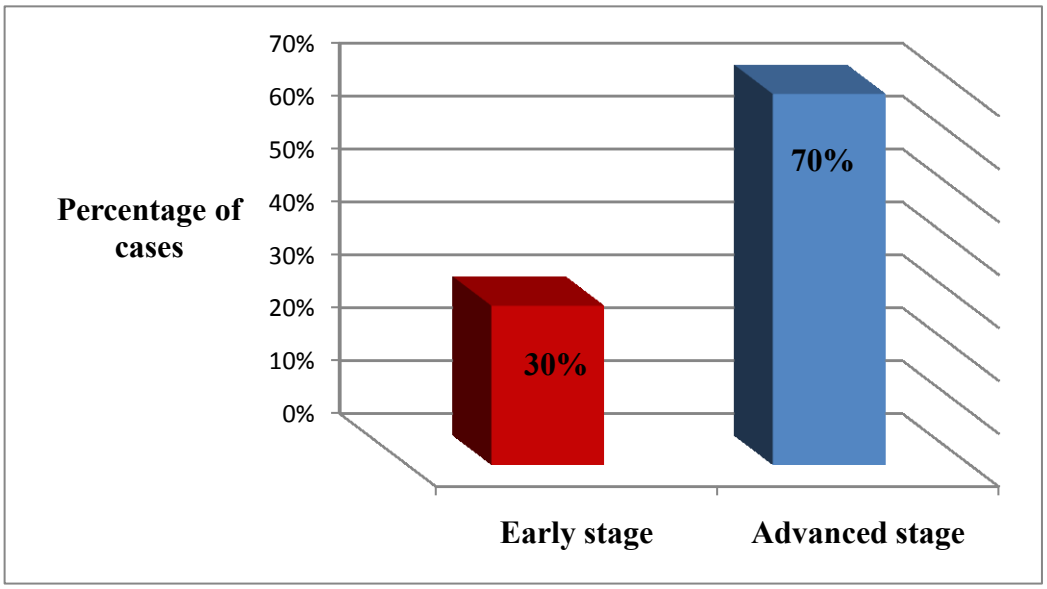

Figure 4.3: The Percentage of Early and Advanced stage

4.2 Incidence of the Variables: ER, PR, HER-2.

Of 100 cases there were 58 (58%) ER positive tumors and 42 (42%) ER negative tumors (Figure 4.4).

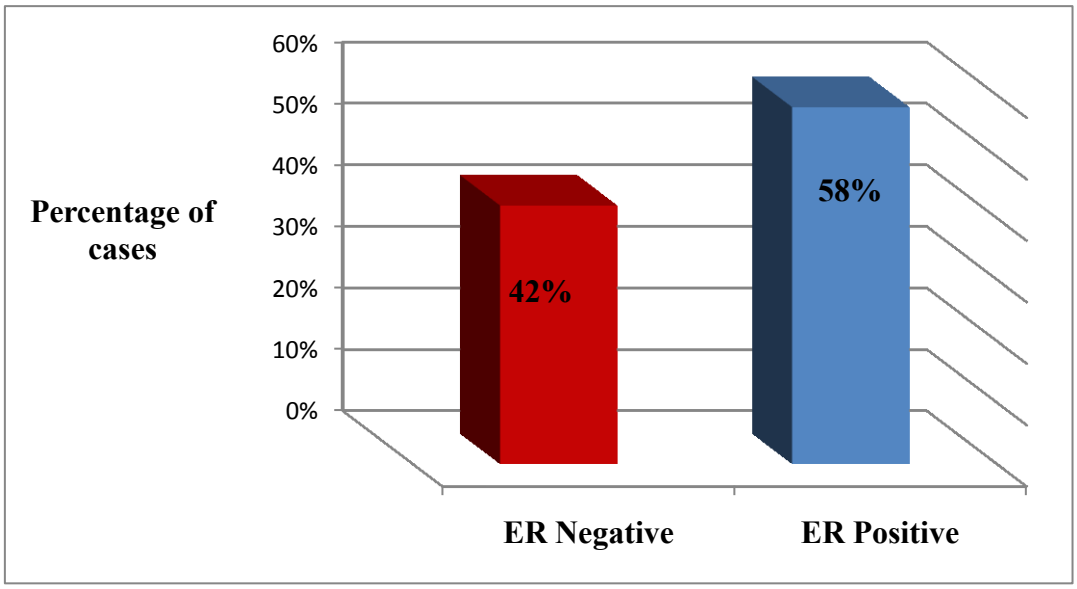

Figure 4.4: Expression profile of ER.

There were 46 (46%) PR positive tumor and 54 (54%) PR negative tumors (Figure 4.5).

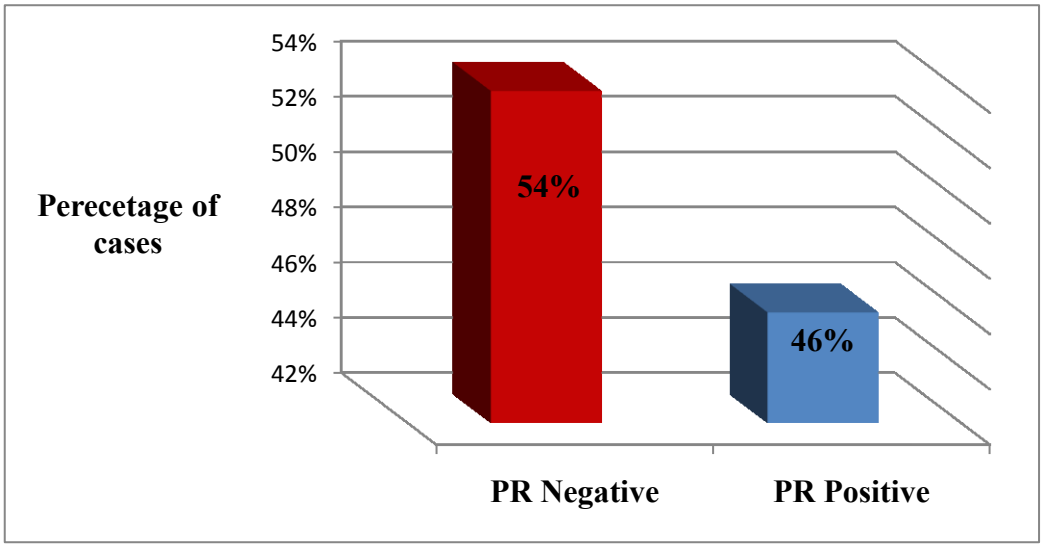

Figure 4.5: Expression profile of PR.

Twenty six of (26%) the cases were positive for HER-2 receptor while 74 (74%) were HER-2 negative (Figure 4.6).

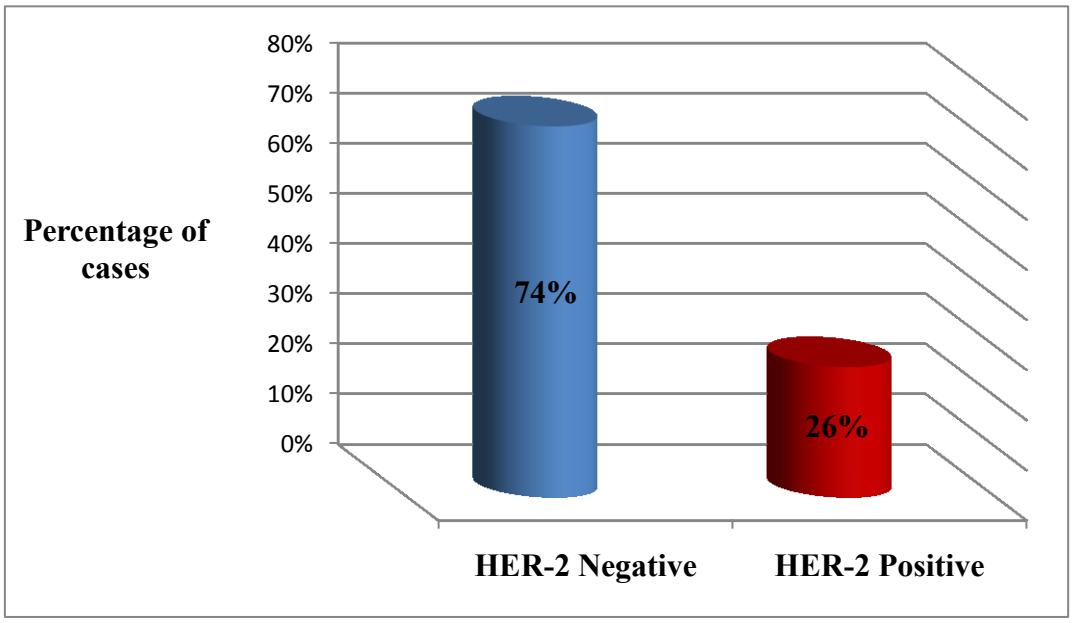

Figure 4.6: Expression profile of HER-2.

There were 30 (30%) cases of TNBC subtype (Figure 4.7). The majority of TNBC cases (19/30)(63%) were below 50 years with mean age of 43 years old, while mean age for non TNBC cases is 54 years old .

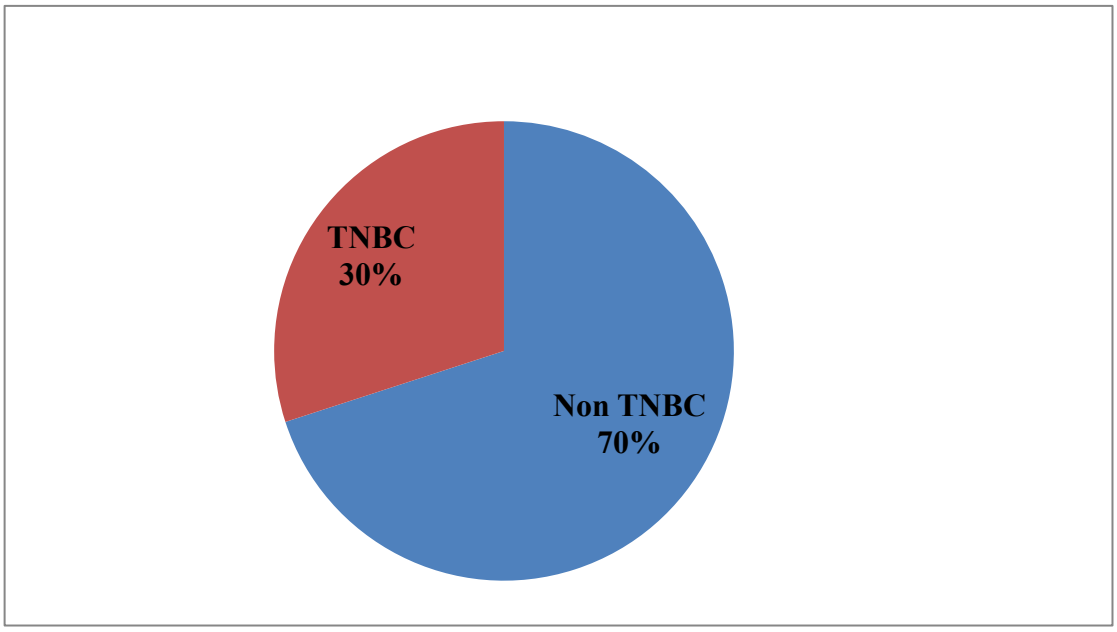

Figure 4.7: The percentage of Triple Negative Breast Cancer cases.

4.3 *PTEN* expression by Immunohistochemistry:

The *PTEN* expression was observed in nuclear and cytoplasmic compartments of the tumor cells as well as normal ductal epithelial cells. Endothelial cells and nerves showed strong *PTEN* expression and were useful as internal positive controls (Figure 4.8).

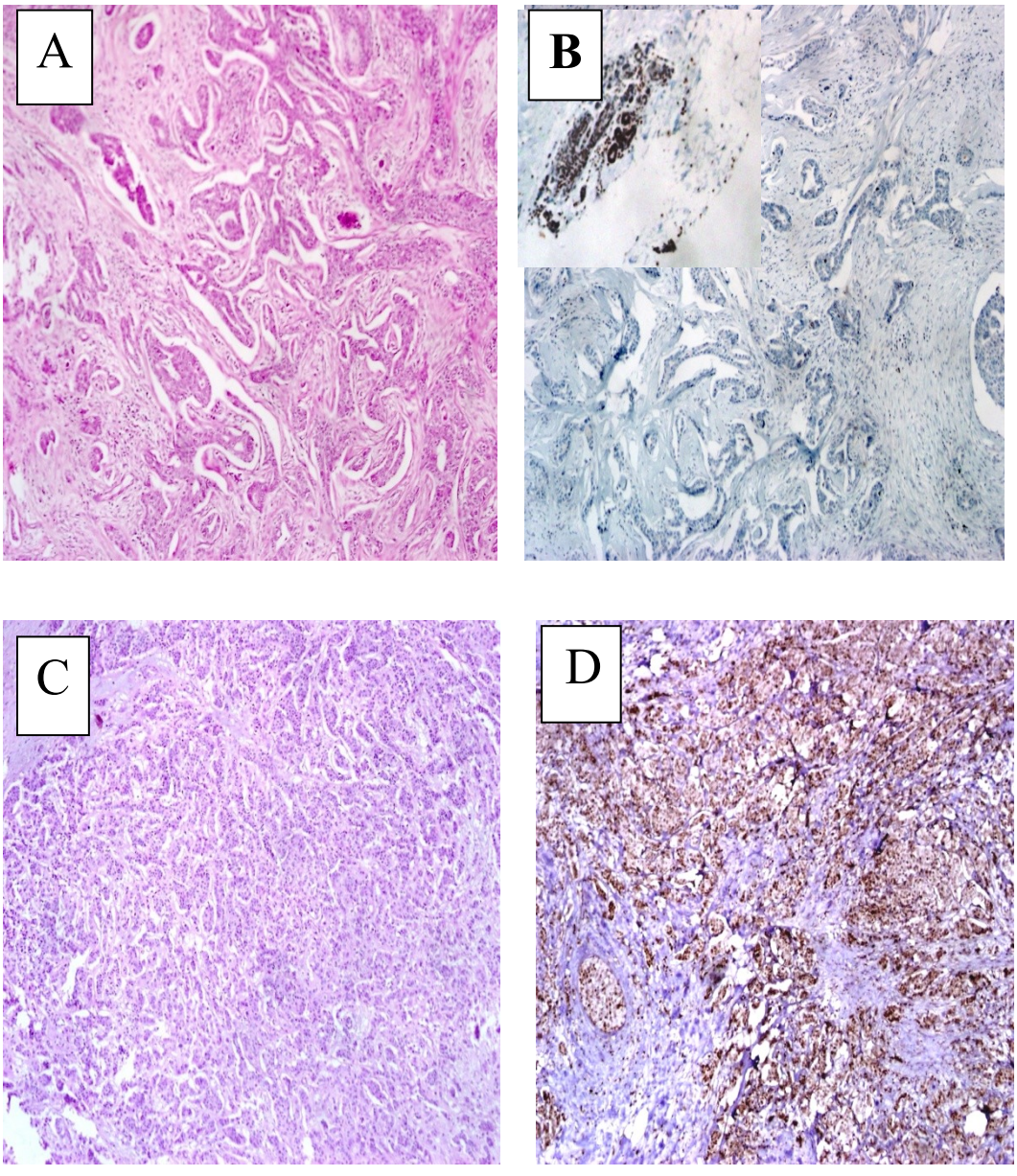

Figure 4.8: A-Hematoxylin and Eosin staining for breast cancer. B-Breast cancer with Negative *PTEN* immunohistochemistry stain -(islet)- Positive internal control for *PTEN* stain. C- Hematoxylin and Eosin stain for breast cancer. D- Positive *PTEN* immunohistochemistry stain.

Loss of *PTEN* expression was seen in 44 % of cases evaluated and was retained in 56% of them (Figure 4.9).

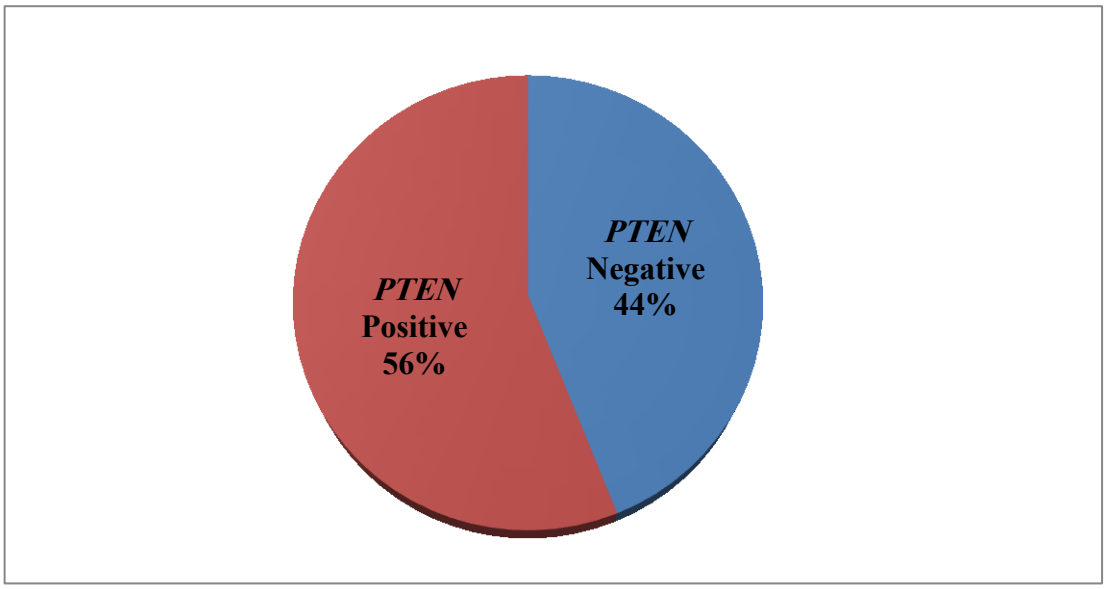

Figure 4.9: Expression profile of *PTEN*.

PTEN expression and tumor grade:

Of the 53 high grade tumors 27 (51%) were negative for *PTEN*, while 26 (49%) were *PTEN* positive. Of the 47 low grade tumors 17 (36%) were negative for *PTEN* expression while 30 (64%) were *PTEN* positive (Figure 4.10) (Table 4.1).

Table 4.1: The frequency and percentages of *PTEN* expression with respect to high and low grade tumors

	PTEN		**Total**
	Negative	**Positive**	
Grade High count	**27**	**26**	**53**
% within Grade	**51%**	**49%**	**100%**
Grade Low count	**17**	**30**	**47**
% within Grade	**36%**	**64%**	**100%**
Total Count	**44**	**56**	**100**
% within Grade	**44%**	**56%**	**100%**

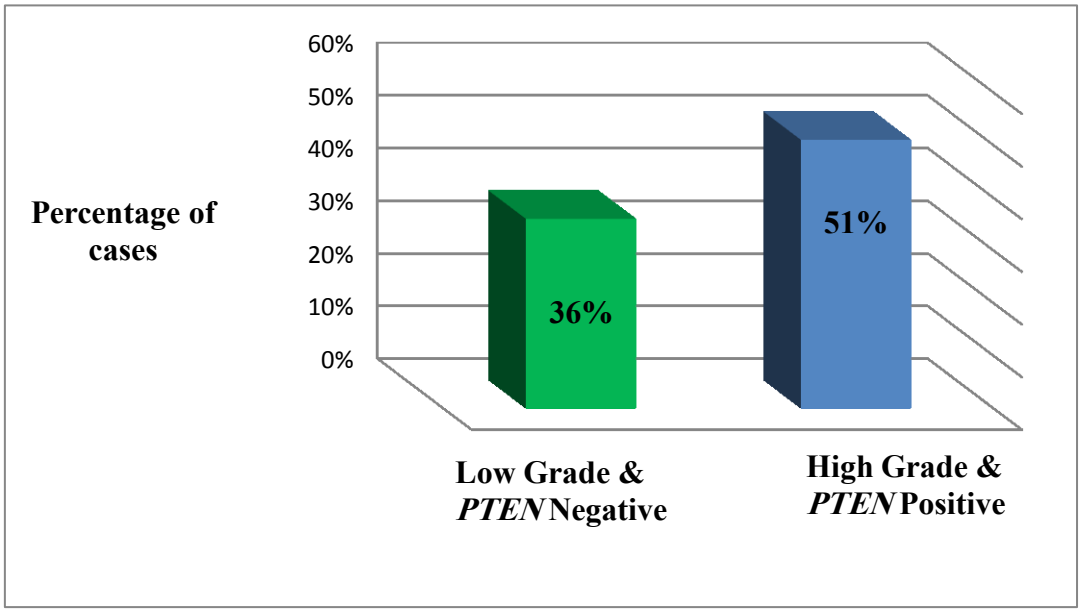

Figure 4.10: The Percentages of *PTEN* loss with respect to high and low grade tumors

PTEN expression and tumor stage:

Of the 63 advanced stage tumors 18 (41%) exhibited absent *PTEN* expression and 26 (59%) exhibited presence *PTEN* expression. Of the 19 early stage tumors 9 (47%) lost *PTEN* expression and 10 (53 %) showed positive *PTEN* expression (Figure 4.11) (Table 4.2). This result shows that *PTEN* loss is nearly the same in early and late stages, this is limited to the fact that only 63 out of 100 case had a known stage, but also it could represent an early event of *PTEN* loss; since early stage is defined as tumors that did not metastasize yet, so may be the tumor cells loose *PTEN* before additional genetic hits that cause them to metastasize**.**

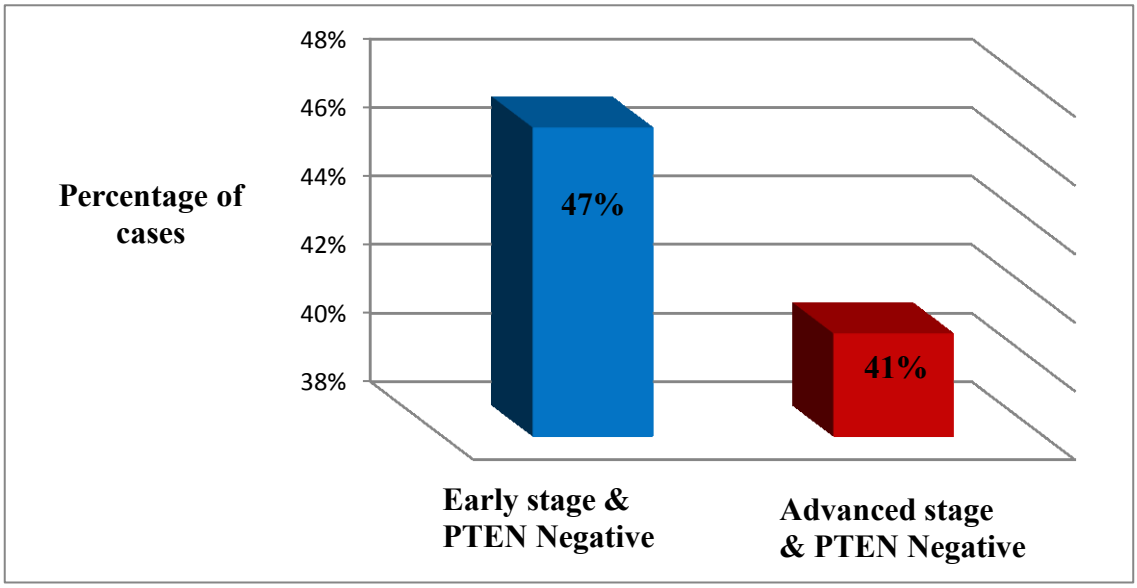

Figure 4.11: The percentages of *PTEN* loss with respect to advanced and early stage

Table4.2: The frequency and percentages of *PTEN* expression with respect to advanced and early stage

		PTEN		Total
		Negative	Positive	
Stage	Advanced count	18	26	44
	% within stage	41%	59%	100%
	Early count	9	10	19
	% within Grade	47%	53%	100%
Total	Count	27	36	63
	% within Grade	42.9%	57.1%	100%

PTEN expression and Estrogen receptor status:

Out of 42 ER negative cases, 21 (50%) lost *PTEN* expression and 21 (50%) retained *PTEN* expression. Of 58 ER positive tumors 23 (40%) were negative for *PTEN* expression (Figure 4.12), while 35 (60%) cases retained *PTEN* expression (Table 4.3).

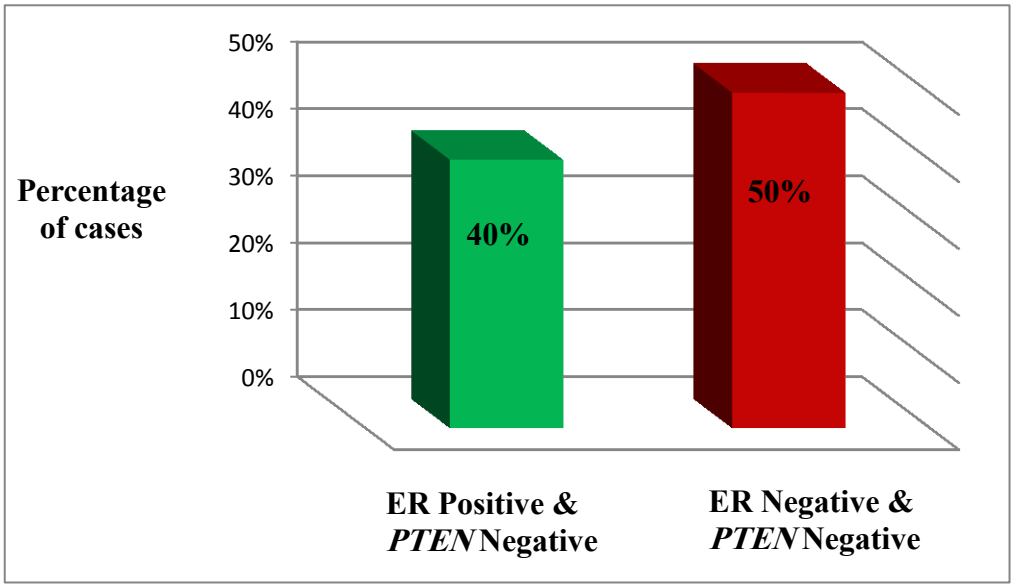

Figure 4.12: The percentages *PTEN* loss with respect to and ER receptor status

Table 4.3: The frequency and percentages of *PTEN* expression with respect to and ER receptor status

		PTEN		Total
		Negative	Positive	
ER	Neg.	21	21	42
% within ER		50.0%	50%	100%
ER	Pos.	23	35	58
% within ER		40%	60%	100%
Total	Count	44	56	100
% within ER		44	56%	100%

PTEN expression and Progesterone receptor status:

From 46 PR positive cases, there were 18 cases (39%) lost *PTEN* expression and there were 28 cases (61%) retained *PTEN* expression. Out of 54 PR negative cases, there were 26 cases (48%) that lost *PTEN* expression and 28 (52%) retained *PTEN* expression (Figure 4.13,Table 4 .4).

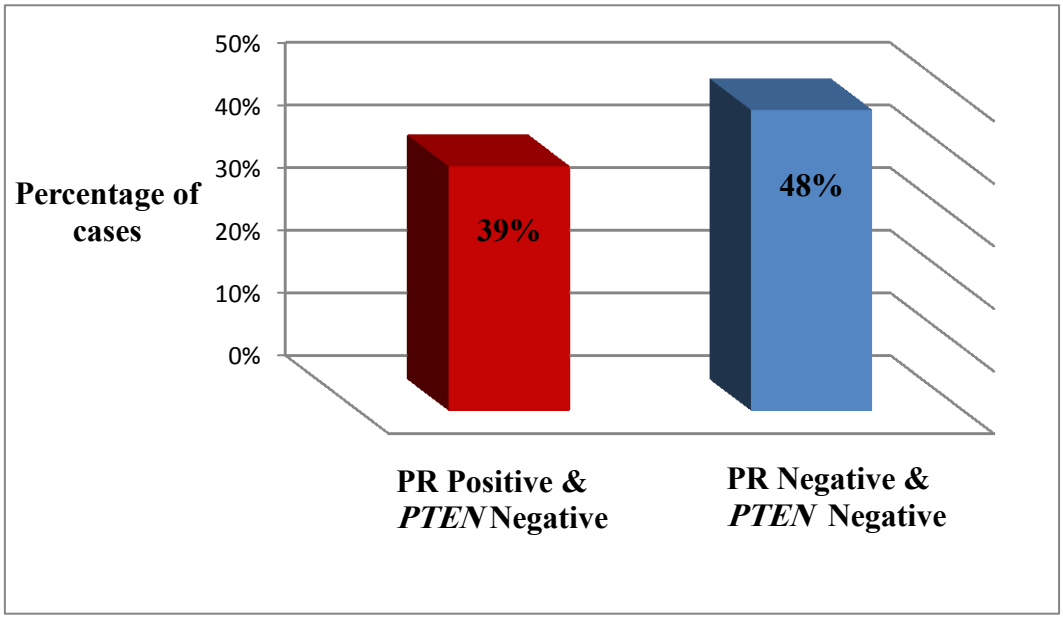

Figure 4.13: The Percentages of *PTEN* loss relative to PR receptor status

Table 4.4: The frequency and percentages of *PTEN* expression relative to PR receptor status.

		PTEN		Total
		Negative	Positive	
PR	Neg.	26	28	54
	% within PR	48%	52%	100%
PR	Pos.	18	28	46
	% within PR	39%	61%	100%
Total	Count	44	56	100
	% within PR	44%	56%	100%

PTEN expression and HER-2 receptor status:

There were 74 HER-2 negative tumors, 34 (46%) were negative for *PTEN*, and 40 (54%) retained their *PTEN* expression. There were 26 HER-2 positive tumor, 10 (39%) lost *PTEN* expression, while 16 (61%) were *PTEN* positive (Figure 4.14, Table 4.5).

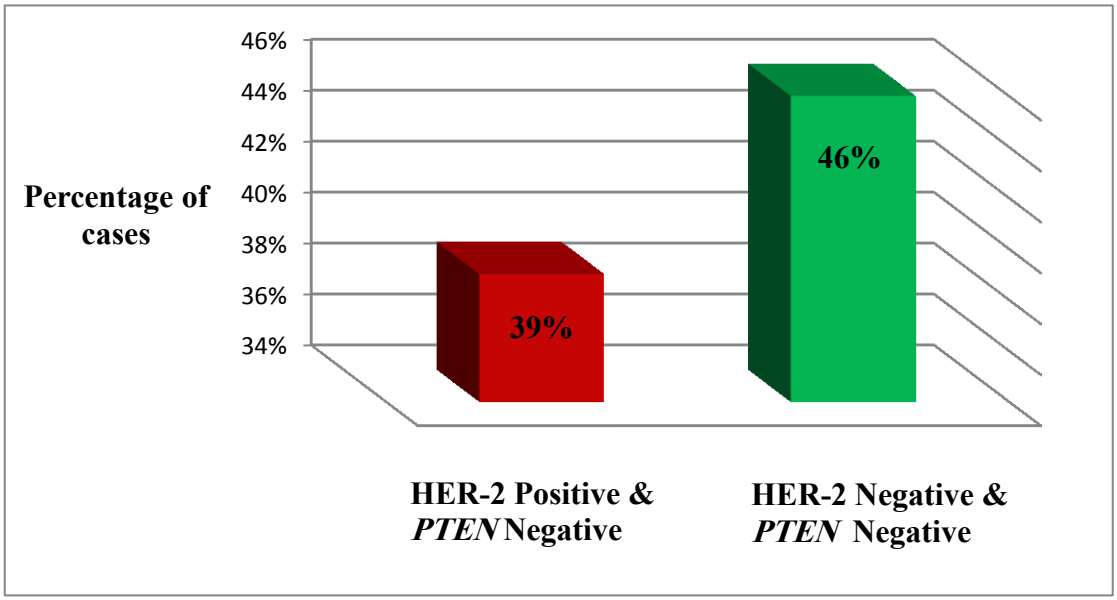

Figure 4.14: The Percentages of *PTEN* loss relative to HER-2 receptor status

Table 4.5: The frequency and percentages of *PTEN* expression relative to HER-2 receptor status.

		PTEN		Total
		Negative	Positive	
HER-2	Neg.	34	40	74
	% within HER-2	46 %	54%	100%
HER-2	Pos.	10	16	26
	% within HER-2	39%	61%	100%
Total	Count	44	56	100
	% within HER-2	44%	56%	100%

PTEN expression and TNBC:

In this study 30 case (30%) were diagnosed as triple negative breast cancer (Table 4.6), 18 (60%) case from TNBC cases lost *PTEN*, while 26 (37%) of non TNBC cases lost their *PTEN* expression (Figure 4.15).

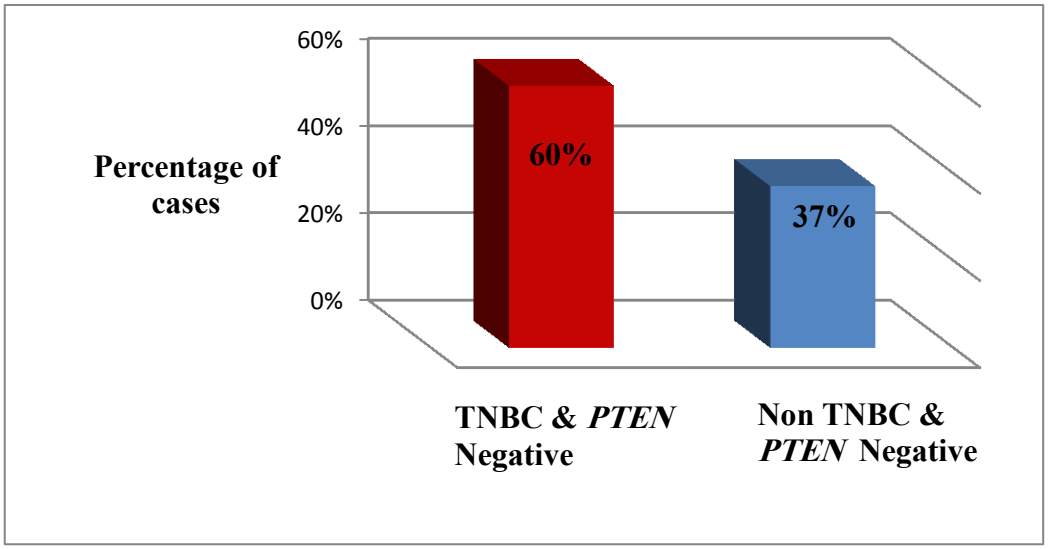

Figure 4.15: The Percentage of *PTEN* loss with respect to Triple and non Triple negative breast cancer.

From 30 cases of TNBC 12 (40%) retained their *PTEN* expression, mean while from 70 non TNBC tumors 44 (63%) were *PTEN* positive (Table 4.6) .

Table 4.6: The frequency and percentages of the *PTEN* expression relative to TNBC and non TNBC

		PTEN		**Total**
		Negative	**Positive**	
NON TNBC	**count**	**26**	**44**	**70**
	% within non TNBC	**37%**	**63%**	**100%**
TNBC	**Count**	**18**	**12**	**30**
	% within TNBC	**60%**	**40%**	**100%**
	count	**44**	**56**	**100**
	%within TNBC	**44%**	**56%**	**100%**

4.4 Correlations between *PTEN* loss and clinicopathologic parameters

We performed statistical analysis using SPSS software (version 13). We used chi square (χ^2) contingency test to examine the association between *PTEN* loss and various clinicopathological characteristics including; grade, stage, ER, PR, HER-2 and TNBC. The cut off significance was 0.05. The outputs of the chi square (χ^2) contingency test include; continuity correction factor that is used to eliminate the discrepancies that arise when approximating the distribution in χ^2 test, the likelihood ratio and Fisher exact test (Table 4.7). The likelihood ratio is another contingency test, it is almost the same as the χ^2 test [70]. Fisher exact test is improvement over the χ^2 test in cases where the expected cell frequencies are less than 5 [70,71]. In this study all of the expected numbers are greater than 5, so it's acceptable to use the chi-square test.

There was no correlation between *PTEN* loss and grade, stage, ER, PR, and HER-2. Nevertheless, a significant correlation was found between *PTEN* loss and TNBC *(P*=0.035). Table (4.8) summarizes the significance of correlations between *PTEN* loss and the variables grade, stage, ER, PR, HER-2, and TNBC.

Table 4.7: Chi Square test between the PTEN loss and grade stage, ER, PR, HER-2 and TNBC.

Test	GRADE	STAGE	ER	PR	HER2	TNBC
Chi-Square (value)	2.206(b)	0.226(b)	1.058(b)	0.820(b)	0.437(b)	4.453(b)
Asymp. Sig. (2-sided)	0.137	0.634	.304	.365	0.508	0.035
DF	1	1	1	1	1	1
Fishers exact test (2- sided)	0.161	0.782	0.317	0.422	0.647	0.048
Continuity Correction(a) (Value)	1.648	0.039	0.680	0.495	0.186	3.573
Asymp. Sig. (2-sided)	0.199	0.843	0.410	0.482	0.666	0.059
DF	1	1	1	1	1	1
Likelihood Ratio (Value)	2.218	0.225	1.057	0.822	0.441	4.446
Asymp. Sig. (2-sided)	0.136	0.635	0.304	0.365	0.507	0.035
DF	1	1	1	1	1	1
Number of cases	100	63	100	100	100	100

DF: Degree of freedom

Table 4.8: The Correlations between *PTEN* loss and the variables

Variable	Number of cases (*P Value*)
High grade	**27/53 (*P=0.137*), NS**
Advanced stage	**18/44 (*P=0.634),* NS**
ER negative	**21/42 (*P=0.304*), NS**
PR negative	**26/54 (*P=0.365*), NS**
HER-2 negative	**34/74 (*P=0.508*), NS**
TNBC	**18/30 (*P=0.035*)**

NS : not significant

CHAPTER 5

5-Discussion

In the present study, we found that Palestinian women had an early age when diagnosed with breast cancer. The majority of the cases were high grade and advanced stage tumors. We found that the positivity rate of the receptors, ER, PR and HER-2 was 58%, 46% and 26% respectively. TNBC constituted 30% of the cases and *PTEN* gene expression was absent in 60% of TNBC cases which was statistically significant (*P=0.035*). We found that, the mean age at diagnosis with breast cancer among Palestinian women was 53 years old, and 49% were below age of 50 years with mean age of 41.4 years old. Several papers in the Arab countries have reported an early age of onset for breast cancer [72-79] (Table 4.9).

Table 4.9: Percentage of breast cancer patient < 50 years old among Arab countries

Arab Countries	Percentage of patients < 50 years old
Saudi Arabia	**78%**
Al Bahrain	**48%**
Libya	**72.3%**
Lebanon	**49%**
Egypt	**44%**
Qatar	**64%**

In comparison with other races, it was reported that the mean age at diagnosis of African-American patients, is 54.17 years old [80], compared with 58.6 years old for those who are white [81] and 46.7 years old for Hispanic women [82]. Our result is

in concordance with the result among the Arab countries, Hispanic and African American patients.

In this current study 53% of the cases were high grade and 47% were low grade tumors, these results are similar to those in African women, in which high grade occurred in 53.6%, while low grade occurred in 46.4% of their cases [83,84]. Similarly, it was observed that Hispanic women were more likely to have high grade tumors [85]. In the Arab countries this was also the case, since in Oman high grade tumors were identified in 35.2% of patients [86], and in 42.68% of the patients in Saudi Arabia [87] .

In our study, 70% of the cases presented with advanced stage. In comparison with others, it was found that the percentage of advanced stage cancer was 39.7 % for Black women and 28.6 % for white women [88]. Meanwhile, Blanchard reported that compared to white women, 35.9% of Hispanics have more advanced stage tumors at time of diagnosis [89]. Among African American women 25% were diagnosed with early stage (stage1) tumors, thus 75% were diagnosed with advanced stage tumors [90]. In the Arab countries the diagnosis at advanced disease remains very common in Egypt, Tunisia, Saudi Arabia, Syria, and others [72, 77, 91].

The status of ER, PR positivity in the Palestinian population, was 58% and 46%, respectively and they were negative in 42% and 54% of the cases respectively. Numerous studies have demonstrated differences in hormone receptor status among races. Chu found that 63.9% of white American women with breast cancer were ER/PR+, and 48.3% of African American women were ER/PR+ [92]. Hormone receptor determination of 1052 Chinese breast cancer patients revealed that ER was positive in 53%, and PR was positive in 51.5% [95]. In a study conducted in Australia the positivity of ER was 80.6%, while PR yielded 61.3% positivity [96]. In another

study among Korean women, the proportion of tumors that stained positive for ER was 47.5% and 42.4% for PR [97]. In Nigerian patients, positive stain for ER was 24.0% and 13.9% for PR [98]. Increase incidence rate of ER- and PR-negative tumors was observed among Hispanic women compared with white women (14.2% in white women compared with 17.3% in Hispanic women) [93]. In a study among Asian Indian and Pakistani women, ER/PR receptors were negative in 30.6% of the cases [94].

Regarding status of hormone receptors in the Arab countries, it was found that in Tunisia, the positivity for ER was 57% and it was 54% for PR [99]. In Saudi Arabia the prevalence of ER+,PR+ was 64.6%, 57.3%, respectively [87]. In a study done on Iraqi women, 34.2% were ER+/PR+ and 43.8% were ER-/PR- [100]. In comparison with other results, our findings are more close to those of African Americans, Asians and Arabs which could be partially explained by the age at diagnosis of breast cancer.

In our study, HER-2 was positive in 26% which is considered high when compared with other international results. In Rome, HER-2 status is positive in 18.24% of the cases [101]. Among African American women, HER-2 is positive in 32% [102]. In the Arab countries, high positivity rate of HER-2 was documented in Saudi Arabia where it constitutes 35.3% of the cases [87] and 26% among Tunisian women [103].

In our study, the incidence of triple negative breast cancer was 30%. In the published literature, the frequency of TNBC is 16% among white American women [16], 15.5% in Japan [104], 30% among black women [105], 23.1% among Hispanic women [17], and 39% in Saudi Arabia [106]. Our results showed high percentage of TNBC similar to blacks and Hispanic and this is in agreement with studies done in Saudi Arabia.

In this research, we were interested in studying the expression of *PTEN* because it has become one of the most important molecules in tumor biology [107]. Mutations, or dysregulation of *PTEN* is found in many human tumors [107] and the Loss of *PTEN* activates the Akt pathway that is known to regulate divergent cellular processes, including apoptosis, proliferation, differentiation, and metabolism [111]. It has recently been shown that Akt activation as a result of *PTEN* loss is associated with a worse outcome among endocrine treated breast cancer patients [111]. Accordingly, we studied the loss of *PTEN* expression with respect to hormone receptor status and TNBC.

In our study, loss of *PTEN* was detected in 44% of the cases. This result is in agreement with Chang who found significant *PTEN* protein loss (48%) in breast cancer cases using immunohistochemical methods [108]. In addition, Park, etal. found loss of *PTEN* expression in 35.6% of breast cancer tissues [109] and Bakarakos, etal. found loss of *PTEN* protein in 72% women with a familial history of breast cancer [110].

PTEN was not significantly correlated with stage and grade, but the percentage of loss of this protein was in agreement with many studies [69], the lack of significant statistical correlation may be due to the small sample size. We found that *PTEN* was absent in about 50% of ER / PR negative tumors. In comparison with others, Depowski, etal. found that 68% of tumors that are negative for ER/PR, exhibit loss of *PTEN* expression [69]. Nevertheless, in our study we didn't find a significant correlation between *PTEN* loss and ER/PR perhaps due to the sample size. *PTEN* loss was seen in 38.5% of our HER-2 positive cases only. Since Pérez-Tenorio, found *PTEN* to sensitize breast cancers to targeted therapy with trastuzumab and consequently down-regulate the PI3K–Akt signaling pathway [112], this could be a

factor that change the disease course and make the outcome better for our HER-2 positive patients which is significantly higher than the international percentage.

In this study *PTEN* loss was detected in 60% of TNBC cases and showed statistically significant correlation. Karseladze, etal. studied the expression of the *PTEN* gene product in TNBC by an immunohistochemical method, as well as detecting the gene by fluorescence *in situ* hybridization (FISH). The gene product was absent in 56 % of the tumor cell nuclei [113], so the percentage is similar to ours, but we did not find any study showing significant correlation between the loss of *PTEN* in the other subtypes. A significant correlation was found between LOH in *PTEN* gene and the activation of AKT pathway, when LOH at the *PTEN* gene locus occurred simultaneously, the incidence of Akt activation and reduced PR expression was significant, which suggest that *PTEN* LOH may lead to PR negative expression therefore, poor prognosis breast cancer [111]. Accordingly, the potential identification of proteins or genes associated with the aggressive form of breast cancer like TNBC could shed light on the important of molecular pathways of metastasis like AKT pathway and eventually could be translated into preventing poor survival outcome of TNBC among the Palestinian women.

The remaining question is if we can depend on immunohistochimestry as a reflection of gene status, Peron, etal. had made comparison between immunohistochemistry and structural mutation data. They found that the tumors that had either no or decreased expression of *PTEN* by immunohistochemistry staining, correlate with structural monoallelic loss (LOH) of the gene [114,115]. Detecting the loss of *PTEN* protein in breast tumors has great value. Since it reflects the presence of loss of hereozygosity of the gene and explains the aggressiveness of TNBC subtype, besides, it promotes future therapy issues through targeting the AKT- pathway.

There are growing interests in studying the factors associated with cancer-related disparities among races, but information regarding these factors are still limited. Our results revealed that in comparison with other races, the biological features of breast cancer among the Palestinian women are more close to those of African Americans and Hispanic women [116]. The similar socioeconomic characteristics of Arab, African American and Hispanic women are the most powerful predictors of having similar results regarding the biological features of breast cancer [115]. The advanced nature of breast cancer in our country could be attribute to the delays in seeking medical attention. The reasons for this are multi factorial and include; the lacking of breast screening services combined with socioeconomic status, cultural and the political factors which are major factors that underpin a propensity for Palestinian women to present with advanced stage and high grade cancer [117,118] The lacking of specialized laboratories, genetic reporting services, therefore the limited number of studies about breast cancer renders it difficult to elicit the genetic mutations which contributes to the development of poor prognosis breast cancer among Palestinian women. We need intense efforts to explore the factors behind the aggressive pattern that breast cancer shows in our country, We need to know why Palestinian women have high incidence of TNBC?

As we found that TNBC is more prevalent in younger women among the Palestinian population, we need to lower the age that allow them to use Mammography, because mammography remains the gold standard for early detection of breast cancer [119].

CHAPTER 6

Conclusion:

From this study we conclude that breast cancer in Palestinian women is present with young age (53 years) at diagnosis . Palestinian women present with high grade tumors and advanced stage. The incidence of TNBC among Palestinian women that constitutes 30%, equivalent to known ethnic groups as Hispanics and blacks . Palestinian women have high percentage of HER-2 positive tumors and low positive rate of ER receptor and PR receptor.

We didn't find significant correlation between *PTEN* status and grade, stage Estrogen receptor, Progesterone receptor, and HER-2 receptor. But, we have identified the presence of significant correlation between *PTEN* status and TNBC in Palestinian women.

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