Biotechnology Master Program

Bethlehem University
Faculty of Science

Palestine Polytechnic University
Deanship of Graduate Studies and Scientific Research

Biotechnology Joint Master Program

Promoter Methylation Status of *PTEN* Tumor Suppressor Gene among Palestinian Breast Cancer Patients

By

Mariam Ishaq Khamis Al-Ajrami

This Thesis is submitted in Partial Fulfillment of the Requirements for the Degree of Master of Biotechnology

September, 2014

The undersigned hereby certify that they have read and recommend 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:

"**Promoter Methylation Status of *PTEN* Tumor Suppressor Gene among Palestinian Breast Cancer Patients**"

By

Mariam Ishaq Khamis Al-Ajrami

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

Graduate Advisory Committee:

Dr. Areej Al-Khatib, Bethlehem University

Committee Chair Name, University Date

Dr. Areej Al-Khatib , Bethlehem University

Committee Member Name, University Date

Dr. Rami Arafeh, Palestine Polytechnic University

Committee Member Name, University Date

Dr. Yousef Hamamreh, Augusta Victoria Hospital

External Committee Member Name, University Date

Approved for the Faculties

Dean of Faculty of Science
Research and Higher studies
Palestine Polytechnic University

Date 20/9/2014

Dean of Faculty of Science
Bethlehem University

Date 20/9/2014

"**Promoter Methylation Status of *PTEN* Tumor Suppressor Gene among Palestinian Breast Cancer Patients**"

By Mariam Ishaq Khamis Al-Ajrami

ABSTRACT

Background: The phosphatase and tensin homolog (*PTEN*) gene, is a tumor suppressor gene located on chromosome 10q23. Several studies showed that 30-40% of sporadic breast cancer cases show a loss of *PTEN* protein levels due to mutations, loss of heterozygosity, or promoter hypermethylation. The *PTEN* promoter methylation is a major epigenetic silencing mechanism leading to the development of tumors. ***Purpose***: The purpose of this study was to explore *PTEN* gene promoter methylation status in Palestinian breast cancer patients and to investigate if the methylation status in *PTEN* gene related to breast cancers has been correlated with a triple-negative phenotype. ***Methods***: In this study, DNA samples extracted from formalin fixed paraffin embedded (FFPE) blocks after characterizing the *PTEN* expression by immunohistochemical staining, for 38 patients diagnosed with breast cancer were analyzed, and DNA methylation in the 5' CPG island spanning the *PTEN* transcription start site was determined using methylation specific polymerase chain reaction (MSP) assay. ***Results***: Promoter methylation of *PTEN* was detected in three (8%) out of 38 specimens, all the methylated tumors had loss of *PTEN* expression. The Promoter methylation of *PTEN* gene was not correlated with the clinicopathological parameters including the loss of *PTEN* expression and the triple negative breast cancer. ***Conclusion***: We reported here for the first time the status of *PTEN* methylation among Palestinian breast cancer patients. Our data show that methylation of the *PTEN* promoter in 38 breast cancer women is not associated with the loss of *PTEN* protein expression, and triple negative breast cancer subtype (TNBC) in these samples. Although, the methylated cases are rare among the Palestinian patients, but further studies using large sample size and high throughput analysis is highly recommended.

" حالة مثيلة البروموتر للجين القامع للورم ***PTEN*** لدى مرضى سرطان الثدي الفلسطينيين"

مريم اسحق خميس العجرمي

ملخص

الخلفية: **الفوسفاتيز والتينسن المماثل لجين *PTEN* هو الجين القامع للورم ويقع على الصبغي (الكروموسوم) 10q23. وأظهرت العديد من الدراسات أن 30-40٪ من حالات سرطان الثدي متفرقة تظهر خسارة مستويات البروتين *PTEN* بسبب الطفرات، وفقدان تغاير الزيجوت، أو مثيلة البروموتر. مثيلة البروموتر ل *PTEN* هو أكبرآلية إسكات جينية مما يؤدي إلى نمو الاورام.**

الغرض: وكان الغرض من هذه الدراسة هو استكشاف وضع مثيلة البروموتور لجين *PTEN* لدى مرضى سرطان الثدي الفلسطينيين وللتحقق إذا كان وضع المثيلة لجين *PTEN* مرتبطة بالنمط الظاهري لسرطان الثدي سالب المستقبلات الثلاثية .

الطرق: **في هذه الدراسة عينات الحمض النووي المستخرج من القوالب الشمعية المثبته في الفورمالين والمضمنة في البارافين FFPE بعد تميز تعبير *PTEN* بواسطة الصبغ الكيميائي النسيجي المناعى immunohistochemical ، على 38 مريضا شخصت اصابتهن بسرطان الثدي وتحليلها، والحامض النووي في الجزيرة 5' CPG التي تغطي موقع بداية النسخ ل*PTEN* تم تحديدها باستخدام فحص مثيلة محدد تفاعل البلمرة المتسلسل (MSP).**

النتائج: **تم الكشف مثيلة البروموتر ل *PTEN* في ثلاثة (8٪) من أصل 38 عينة، كانت جميع الأورام المميثلة عندهاخسارة لتعبير *PTEN*. مثيلة البروموترلجين *PTEN* لم تكن مرتبطة مع المعلمات الإكلينيكية بما في ذلك خسارة تعبير *PTEN* وسرطان الثدي سالب المستقبلات الثلاثية.**

الاستنتاج: **أبلغنا هنا لأول مرة وضع مثيلة *PTEN* بين مرضى سرطان الثدي الفلسطينيين. تظهر بياناتنا أن مثيلة البروموترلجين *PTEN* في 38حالة سرطان الثدي لدى النساء لا يرتبط مع فقدان التعبيرللبروتين *PTEN* ، و النوع الفرعي سالب المستقبلات الثلاثية من سرطان الثدي (TNBC) في هذه العينات. وعلى الرغم من أن الحالات المميثلة نادرة بين المرضى الفلسطينيين، ولكن ينصح بشدة إجراء المزيد من الدراسات باستخدام عينة كبيرة الحجم وتحليل إنتاجية عالية.**

DECLARATION

I declare that this Master Thesis entitled" *PTEN* Gene Mutations and their Correlation with Triple Negative Breast Cancer among Palestinian Patients " 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: Mariam Ishaq Khamis Al-Ajrami

Date: 20th of September 2014.

mariam_ishaq@outlook.com

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation form, reproduction, or publication of this thesis may be granted by my main supervisor, or in[his/her] absence, by the Dean of Higher Studies when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature: Mariam Ishaq Khamis Al-Ajrami

Date: 20th of September 2014.

Dedication

"Praise be to Allah who created the heavens and the earth, and made the darkness and light"(The Qur'an, Al-An'am 1:165).

In the name of Allah, I am dedicating this thesis

To my great teacher and messenger, Mohammed,

To my homeland Palestine,

To my great parents, who never stop guiding me through the challenges of my study to the light of hope and support,

To my soul,

And my beloved brothers, sisters, and friends .

MAY ALLAH BLESS YOU ALL AND GRANT YOU SUCCESS AND JANNAH AMEEN

Acknowledgments

In the name of Allah the Most Compassionate and the Most Merciful .

Firstly, I want to express and offer all thanks to the one above all of us, to our creator, Allah, who gave me everything. I would like to heartily thankful to my supervisor Dr. Areej Al-Khatib for giving me great support and facilities to work in the molecular department at Augusta Victoria Hospital, providing many suggestions, enabled me to develop an understanding of my thesis.

Additionally, my sincere gratitude goes to my co-supervisor Dr.Monther Abu-Rmaileh , for helping me to solve so many problems. His guidance and patience will always be remembered and appreciated, and I am very thankful for Dr.Rami Arafeh our joint master program assistant professor and coordinator of (Biotechnology Research Center BRC) in Palestine Polytechnic University for providing me the statistical tutorial, supporting me with valuable feedback to build up my thesis, and for agreeing to be my internal examiner. Additional gratitude is offered to Dr.Yousef Hamamra, the head of oncologists in Augusta Victoria Hospital for agreeing to serve as my external examiner.

I would also like to offer my special thanks to Dr.Moein Kanaan, Dr. Rami Aqeilan, Dr. Yacoub Al –Ashhab, and Dr. Imad Maatouk.

I feel fortunate to have had the opportunity of working in the molecular department at the Augusta Victoria Hospital with such comfortable environment, and I am really thankful for all members in the laboratory for helping me in doing my master thesis.

I am just very lucky because I am working as a pathology technician in the Center of Advanced Pathology Laboratories (CAP labs), which ease from my thesis work process.

Last, but not least, special thanks to my parents, for never ending love, encouragement, and support. I really cannot find words to describe what I feel to thank you enough for everything. I am glad also to acknowledge my family: Ala', Wafa', Mohammad, Hamza, Shrouk, Hayat, Sami, Bilal, and Wael, you were always with me. Thank you.

Abbreviations

TNBC	Triple Negative Breast Cancer
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome Ten
ER	Estrogen Receptor
PR	Progesterone Receptor
Her-2	Human Epidermal Growth Factor Receptor
IHC	Immnonohistochemistry
MMAC1	mutated in multiple advanced cancers
TEP1	TGF-b regulated and epithelial cell enriched phosphatase 1
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PIP2	Phosphatidylinositol-4,5 bisphosphate
LOH	Loss Of Heterozygosity
MSP	Methylation Specific Polymerase Chain Reaction
AJCC	The American Joint Committee on Cancer
PS	Proportion Score
IS	Intensity Score
ASCO	American Society of Clinical Oncology

List of Figures

List of Tables

Table of Contents

CHAPTER 1

1 Introduction

1.1 Background

Worldwide, breast cancer accounts for 15% of all cancers, being the second most common type of cancer after lung cancer [1].The incidence of annually new diagnosed breast cancer cases is about 31% in women, while about 1% in men worldwide [2] . In Palestine, breast cancer occurs more in younger patients. In the mid year 2013, the incidence of diagnosed female breast cancer patients in Palestine was 34.1% being the most prevalent cancer among Palestinian females [3].The widely mentioned gene *PTEN* is a candidate tumor suppressor with lipid phosphatase activity which plays an important role in proliferation and apoptosis [4]. In particular, the *PTEN* gene epigenetic silencing by the promoter methylation has been shown in breast cancer progression [5]. Recent studies have shown the association between the loss of *PTEN* expression and poor prognosis of breast cancer [6-7]. Also, it is observable that 10.3% of all deaths in Palestine are due to cancer, thus breast cancer being one of the top leaders of mortality in the occupied Palestinian territory, and breast cancer is the major cause of deaths among Palestinian females with 21·1% of all cancer deaths [8], and it is the commonest cancer among Arab females[9].In the present study, randomly selected breast cancer patients were studied for *PTEN* promoter methylation after immunohistochemistry staining for estrogen (ER), progesterone (PR), her-2, *PTEN*, and after reviewing different factors such as: age, stage, grade, tumor type, surgical procedure, treatment. Moreover, methylation specific polymerase chain reaction (MSP) was used to assess the *PTEN* promoter methylation status in 38 breast cancer patients. Our previous data indicated that about 30% of the cases are triple negative breast cancer (TNBC) [10], and since this type of cancer has an aggressive course due to limitation of treatment options. Extensive studies tried to discover a mechanism other than hormonal and target therapy, tumor suppressor genes and tumor repair genes are the genes that are now under investigation for their role in the TNBC pathogenesis[11].Scientists have found a significant correlation between the loss of *PTEN* expression in cases within TNBC

subtype in comparison with other non triple negative subtypes. *PTEN* loss was also documented in the Palestinian patients and was strongly correlated with this subtype; the mechanisms behind *PTEN* expression decrease or loss like the gene mutation or deletion are not playing a major role [12], proposing to investigate epigenetic mechanisms such as promoter methylation.

1.2 The role of *PTEN* tumor suppressor gene

PTEN plays an important role in the regulation of the phosphatidylinositol 3-kinase pathway (PI3K) which is involved in important physiological processes such as cellular proliferation [13]. PI3K pathway genetic changes play a major role in the formation of breast malignancies [14].For instance, *PTEN* primary targets are highly specialized membrane lipids, although it progresses dephosphorylation of the phosphate group from the inositol rings. Furthermore, *PTEN* antagonizes the phosphatidylinositol-3-kinase (PI3K) signaling pathway. *PTEN* acts on the phosphatidylinositol-3,4,5-triphosphate (PIP3) which is generated through the action of phosphoinositide-3 lipid kinase (PI3K). PIP3 is a second lipid messenger in tumorigenesis that induces cellular events, such as survival, growth, proliferation, and invasion [15] (Fig 1). Indeed, *PTEN* decreases the PIP3, inhibiting the signals of growth, survival, and tumor suppressor creation [16].

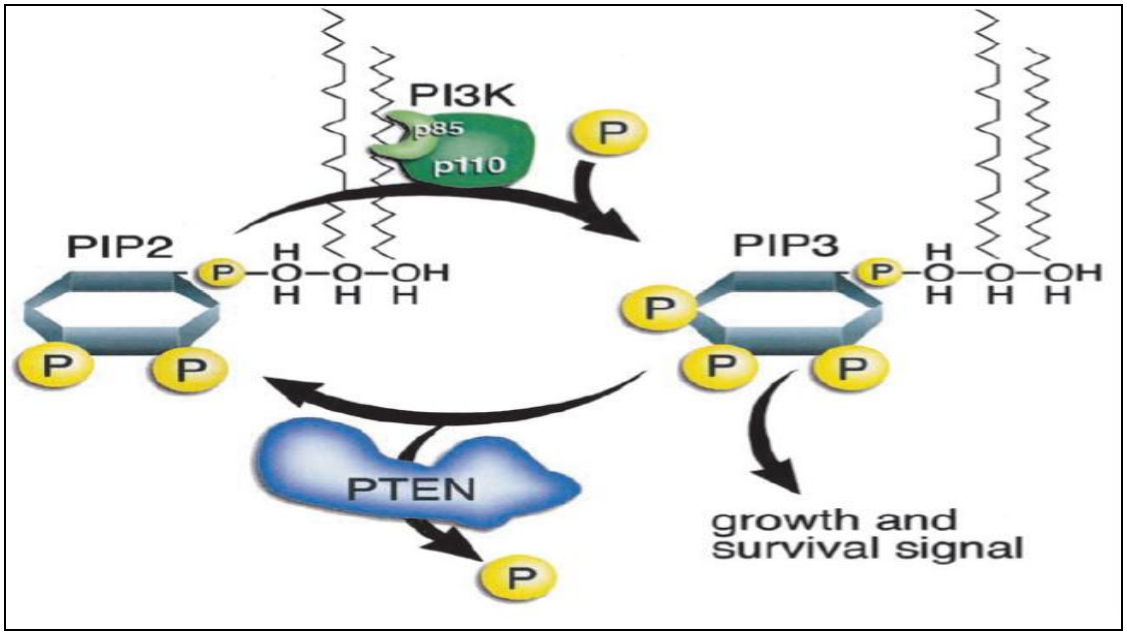

Figure 1: The role of the tumor suppressor gene *PTEN* (blue), hydrolyses phosphatidylinositol-3,4,5 trisphosphate(PIP3)to phosphatidylinositol-4,5 bisphosphate (PIP2), PIP3 is a second lipid messenger in tumorigenesis that induces cellular events, such as survival, growth, proliferation, and invasion . Indeed, *PTEN* decreases the PIP3, inhibiting the signals of growth, survival, and tumor suppressor creation, thus loss of *PTEN* therefore ease entry to the cell cycle, through generation of inositol phospholipid signals after growth factor stimulation [17].

1.3 Evidence on correlation between the loss of *PTEN* protein and breast cancer

Saal L.et al. [18] reported that *PTEN* alterations are connected with metastasis and poor prognosis. In mice, heterozygous loss of *PTEN* has been shown to cause different tumors in various organs or systems such as lymphatic system, thyroid, breast and endometrium [19].Gordon V.et al. [20] reported that phosphoinositide 3-kinase (PI3K) was approved to play a main role in TNBC formation. Mevlude I.et al. [21] reported also the evidence that loss of *PTEN* is associated with metastasis and poor clinical outcome of the triple negative breast cancer patients, though it is an important negative regulator of breast carcinogenesis. Ma W.et al.[22] reported that loss of *PTEN* is associated with poor disease outcome through the increase in the

activation of the Akt signaling that have been known as important mechanism contributing to breast cell proliferation.

1.4 Importance of *PTEN* promoter methylation status assessment

Recently, there has been wide progress in studying the mechanisms that are associated with the inactivation of *PTEN*. *PTEN* has been associated with progression of breast tumerogenesis through a number of mechanisms, such as: loss of heterozygosity (LOH), germline and somatic mutations, *PTEN* promoter methylation, *PTEN* protein degradation and post-translational modifications [23]. Jovanovic J. et al. [24] reported that DNA methylation occurs on cytosine residues of CpG dinucleotides in gene promoters or first exons. In multiple tumor-suppressor genes the methylation has been detected with correlation to poor prognoses cancers [25].

The epigenetic mechanisms has been reported as regulatory mechanisms in the differentiation of normal tissues dictating there function and structure [26]. Studies have shown frequent alterations in *PTEN* gene in many types of solid tumors [27,28,29,30].The methylation alteration in breast cancer has been reported with high level than that of normal breast tissue [31]. Also, methylation as a prognosis factor has been reported in ovarian malignant tissue [32], acute lymphocytic leukemia [33], bladder malignancies [34] and head and neck malignancies [35]. Overall, studies suggest that epigenetic silencing by promoter methylation of *PTEN* has been proposed as a process by which *PTEN* expression can be suppressed in breast cancer.

1.5 Objectives

The overall objectives of this thesis are to contribute to the understanding of the correlation between *PTEN* promoter region DNA methylation with the loss of *PTEN* expression and with triple negative breast cancer subtype. Also, to help to explore the methylation status of *PTEN* gene in a sample from Palestinian patients.

CHAPTER 2

2 Materials and Methodology

2.1 Patients and tumor characteristics

A total of 38 female patients, diagnosed with breast cancer in Augusta Victoria hospital, and Center of Advanced Pathology Laboratory were included into the study from 2009-2014. All the patients were interviewed and a full explanation of the study was offered, and the patients were consented for participation in the study (annex 1). The pathological features of the patients including breast cancer subtype, grade, their immunohistochemistry for ER, PR, Her-2, *PTEN* protein expression were performed using IHC staining method , patients clinical parameters such as: surgical procedure, age, stage, treatment (neo- adjuvant, adjuvant) were also known. The breast cancer stages were performed according AJCC/UICC TNM classification and stage grouping and were taken from the medical reports [36]. The promoter methylation status was detected in all patients.

2.2 Immunohistochemistry (IHC)

Immunohistochemical staining procedure was accomplished according to standard methods using the automated immunostainer named as Benchmarck -GX (from Ventana), according to manufacturer's protocol. Formalin-fixed, paraffin-embedded human breast tissues were sectioned (3 μm thick) and mounted on super frosted plus slides. Sections of breast tumor tissue were incubated on oven at 65°C overnight, sections were then submitted into the immunostainer. Sections were incubated with 100μL of primary antibodies: *PTEN*(human/mouse/rat *PTEN* antibody, antigen affinity-purified polyclonal rabbit IgG from R&D systems) diluted 1:50 , 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) diluted 1:100 for a total of 32 minutes, then slides

were taken from the stainer dehydrated, and mounted with mounting media (Sigma)and cover slipped (Table 1).

Table 1: Biomarkers used for IHC

Biomarker	**Pretreatment**	**Dilution**
Estrogen	H	1:100
Progesterone	H	1:100
Her-2	H	1:100
PTEN	H	1:50

H: Heating

Control immunostaining slides were performed. Normal breast tissue was used as positive control for the ER, PR, and breast cancer tissue known to stain positive for Her 2. Normal breast tissue also was used as positive control for *PTEN*.

2.3 Biomarker Evaluation: ER, PR , Her-2, and *PTEN* by Immunohistochemistry (Scoring System)

The PR, ER expression in primary breast cancer patients were tested according to the pathologist's interpretation of the proportion score (PS) and intensity score (IS) of IHC stained positive tumor cell nuclei under light microscope with X200 magnification. An Allred score scaled between 0 and 8 was used which is represented by the pathologist who did not know the results of the biomarkers or the patient outcome as a total score (TS) that composed of the following scores:

- A proportion score (PS) was assigned representing the estimated proportion of positive staining tumor cells (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) was scaled from 0 to 3 (0 = one; 1 = weak; 2 = intermediate; 3 = strong) [37] (Table 2).

Table 2: Allred Score for ER and PR Status (0-8)*

Percentage staining score or PS	**Proportion of positive staining cells**	**Intensity score (IS)**	**Average of intensity of positively stained cells**
0	None	0	None
1	<1/100	1	Weak
2	1/100 to 1/10	2	Intermediate
3	1/10 to 1/3	3	Strong
4	1/3 to 2/3		
5	>2/3		
*Allred Score= PS+IS Total Score Sum of PS and IS Interpretation 0-2 Negative 3-8 Positive			

According to ASCO-CAP guidelines the IHC of Her-2 is considered :negative if the qualitative score of the IHC staining is 0 or +1 depending on the staining intensity, +2 classified as equivocal (borderline) , and +3 as positive. When Her-2 is considered +2 then fluorescence in situ hybridization (FISH) is used to evaluate the number of the Her-2 gene DNA copies and is scored negative with < 2 copies[38] (Table 3).

Table 3: ASCO-CAP guidelines for the evaluation of the IHC of Her-2

Score	**Her-2 Status Assessment**	**Definition**
0	Negative	No immunostaining
+1	Negative	Weak immunostaining, incomplete membrane staining of >10 % of the tumor cells.
+2	Equivocal	Complete membranous staining, either uniform or weak in at least 10% of the tumor cells
+3	Positive	Uniform, intense membranous staining in at least30 % of the tumor cells.

The normal breast cells showed strong expression of *PTEN* protein and were considered as positive control. The evaluation of *PTEN* expression included staining distribution and intensity. The evaluation performed with a scoring system.

"Intensity was scored as strong, moderate, or weak. Distribution was scored as diffuse if 50% of tumor show staining, regional if 15 to 50% of tumor show staining, and focal if 15% of tumor show 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"[39] (Table 4, Fig 8).

Table 4: *PTEN* expression scoring system .

***PTEN* Status Assessment**	**Definition**
Negative	Weak staining with any Distribution
Negative	Moderate to regional, moderate to focal
Positive	Intense to diffuse, Intense to Regional, intense to focal, and moderate to diffuse staining

2.4 DNA extraction from the paraffin tissue

We selected paraffin-embedded blocks, and we cut 40 μm-thick tissue sections from each. A total of 38 blocks were selected. All of the sections were deparaffinized by using heat in oven for 2 minutes, then tissues were collected (by gentle scratching for tissues that were submitted after cutting on the slides using a sterile blade) in 1.5 ml micro tube, followed by series of xylene treatment to remove paraffin wax, and then using a graded ethanol series of washing. Dissected tissues were purified by DNA extraction kit (QIAamp DNA FFPE Tissue Kit from Qiagen) according to manufacturer's instructions, RNase was used to remove contaminating RNA. Sodium acetate and isopropanol were added to precipitate the DNA, followed by centrifugation pellet the DNA. Washing with 70% ethanol was held to remove excess salts, with repeating for centrifugation step to re-pellet the DNA. The DNA that was used was extracted from HCT cell line with defective DNA methylation machinery to ensure unmethylated state so it was used as negative control. Positive control is same DNA after the in vitro methylation treatment.

2.5 Bisulfate treatment and methylation specific PCR assay (MSP)

Bisulfate conversion of the extracted DNA from FFPE breast cancer tissues were done to prepare the samples for methylation analysis following the instructions of manufacturer EpiTect Bisulfate Kit (Qiagen). Treated DNA was amplified in a total

volume of 50 µL solution containing 2X PCR buffer II, 2.5 mM $MgCl_2$, 200 nM of each primer mix, 200 µM of dNTPs and 0.6 U Amplitaq Polymerase (Life Technologies, Carlsbad, CA). The following PCR primers were designed using MethPrimer tool to assay the methylation status of cytosine and guanine separated by only one phosphate; phosphate links any two nucleosides together in DNA (CpGs) from the *PTEN* promoter region **:**one specific for the methylated sequence (M,sense:5'-TATTTCGAGTAAAGGAAGAAGACGA,temperature=59.82; antisense: 5'-ACTACTTTCCGAAAAAAATCACGTA -3' temperature = 59.54; PCR product, 109bp) ,and the other for the unmethylated sequence (U,sense:5'-GTTATTATTTTTAGGGTTGGGAATGT-3'temperature=59.02; antisense: 5'-CACACAAAAAATTTAAAACCAAC-3' temperature= 55.64 ; PCR product, 122bp).

PCR was performed as follows: one cycle of 94°C for 5 minutes and 3 cycles of 94° C for 30 seconds, 58° C for 30 seconds, and 72° C for 30seconds, and then 39 cycles of 94° C for 15 seconds, 56° C for 15 seconds, and 72° C for 15 seconds and one cycle of 72° C for 10 minutes. Electrophoresis through an 2.5% agarose gel, and the gel will be stained with ethidium bromide to visualize the DNA under UV illumination. If the product presents in the methylation reaction this would be an indication of the presence of methylated *PTEN* gene.

2.6 Study population

The methylation specific polymerase chain reaction was carried out for thirty eight breast cancer patients. All the patients were participated in the study. The tumor was assessed for being satisfactory for study, slides having tumor cells more than 50% were chosen for each case. The general characteristics of the study population (as presented in table 5).

Table5: Characterization of the study population

All		Triple Negative Breast Cancer	Non-Triple Negative Breast Cancer
Age(years)	(26-67)		
Total number of Patients	38	18	20
Grade	Total=38		
1 - 2	17	6	11
3	21	12	9
Stage	Total=38		
I	2	1	1
II	18	11	7
III	18	6	12
Surgical Procedure	Total=38		
Mastectomy	21	9	12
Lumpectomy	14	7	7
Core biopsy	3	2	1
Type of breast cancer	Total=38		
NOS (not otherwise specified)	36	18	18
Colloid	1	0	1
Invasive lobular carcinoma	1	0	1
Neoadjuvant therapy	Total =38		
Neoadjuvant (Yes)	8	3	5
Neoadjuvant (No)	30	15	15
Adjuvant therapy	Total =38		
Adjuvant (Yes)	37	18	19
Adjuvant (No)	1	0	1

All		Triple Negative Breast Cancer	Non-Triple Negative Breast Cancer
Estrogen receptor (ER)	Total=38		
Positive	14	0	14
Negative	24	24	0
Progesterone receptor (PR)	Total=38		
Positive	11	0	11
Negative	27	27	0
Her-2	Total=38		
Positive	8	0	8
Negative	30	30	0
PTEN	Total=38		
Positive	16	0	16
Negative	22	22	0

All the patients included in the study were females; they represented 100% of the study specimens, without male patients. According to the present study, the age range of the 38 patients was between 26 and 67 years, with a mean of 49 years. 23 (61%) cases were below 50 years old, while 15(39%) cases were more than 50 years old (Fig. 2).

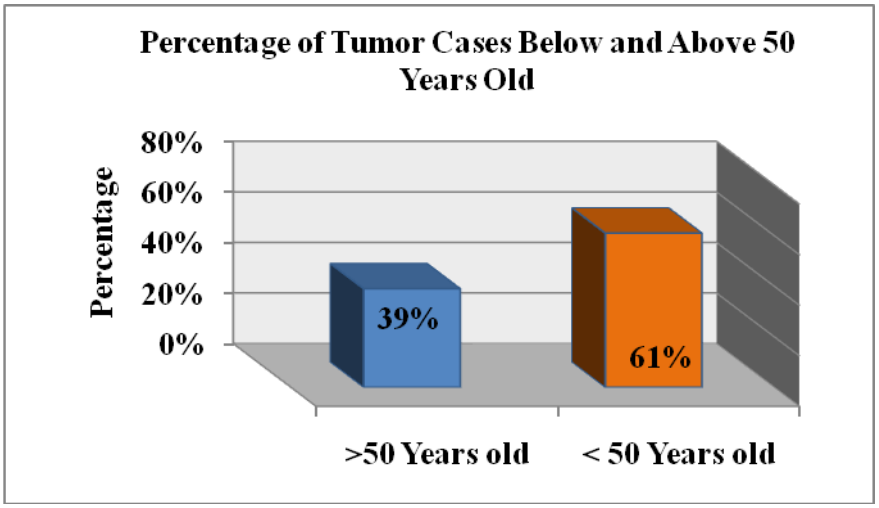

Figure 2: The percentage of tumor cases below and above 50 years old histogram.

Forty seven percent (18/38 cases) were TNBC and 53% (20/38cases) of the cases were Non-TNBC. The most common subtype of breast cancer within the study population was NOS (not otherwise specified) affecting (95%) patients. Representative results showing the incidences of the main variables are illustrated in Fig 3.

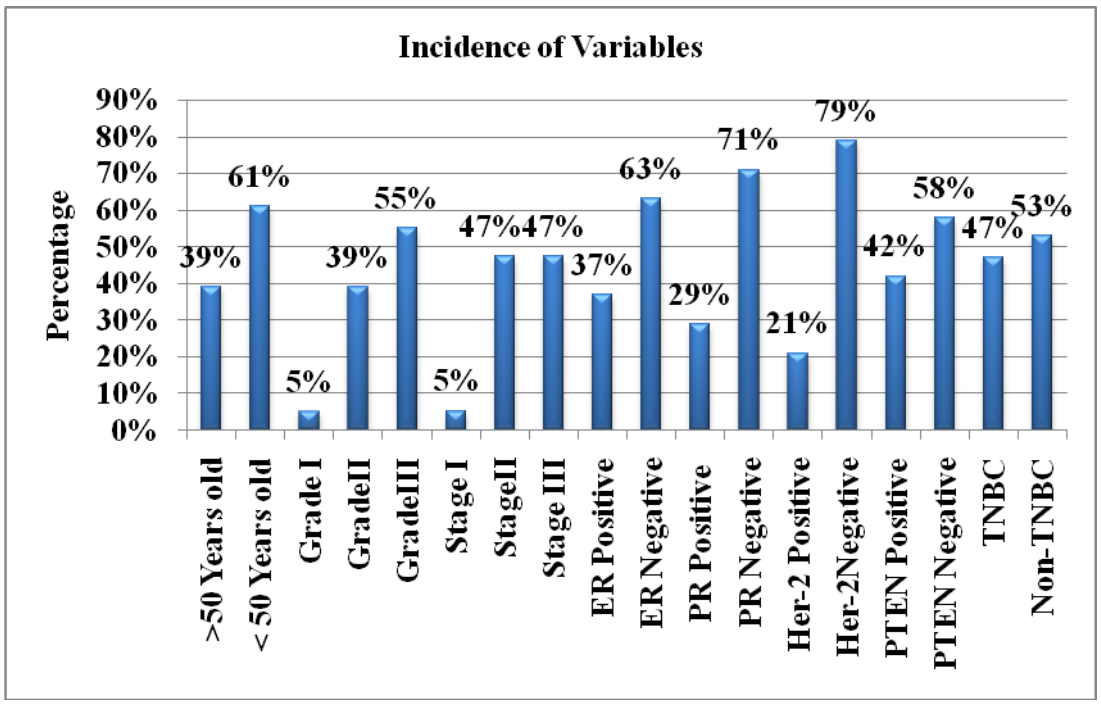

Figure 3: The resulting histogram for the incidences of variables present in the study.

CHAPTER 3

3 Statistical evaluations

3.1 Sampling of the study

The study population composed of breast cancer patients. Thirty eight cases were chosen randomly according simple random sampling process. The variables in the present study were age, grade, stage, ER, PR, Her-2, TNBC, neo-adjuvant therapy, adjuvant therapy, type of breast cancer and *PTEN* protein expression.

3.2 Statistical tests

The statistical methods that used to evaluate the statistically significance of association between *PTEN* methylation and the parameters: age, grade, stage, ER, PR, Her-2 , TNBC, neo-adjuvant therapy , adjuvant therapy, type of breast cancer and *PTEN* protein expression include:

Chi square test for independence: evaluates statistically significant differences between proportions for two or more groups such as the difference in the proportion between *PTEN* methylation group and different clinicopathological characteristics including TNBCs.

All of the parameters were analyzed with contingency tables and Pearson's χ^2 test. For all tests *p*-value of 0.05 was taken as threshold for statistical significance. The statistical comparisons were carried out with SPSS "Statistical package (Chicago, IL) Software" for Windows Version 21.

CHAPTER 4

4 Results

Table 6 presents the results of *PTEN* expression by IHC for both TNBC and Non-TNBC. Twenty two percent of the breast cancers showed loss of *PTEN* expression, while sixteen percent showed positive *PTEN* expression.

Table 6: Results of *PTEN* expression by IHC

***PTEN* IHC**	**TNBC Count**	**Non-TNBC Count**	**Count percentage within TNBC**
***PTEN* Negative**	12 67%	10 50%	22 22%
***PTEN* Positive**	6 33%	10 50%	16 16%
Total	18 100%	20 100%	38 100%

Of the 18 TNBC, the loss of *PTEN* was seen in 12 (67%) cases, while the loss of *PTEN* was seen in 10(50%) cases of the 20 Non-TNBC Fig. 4.

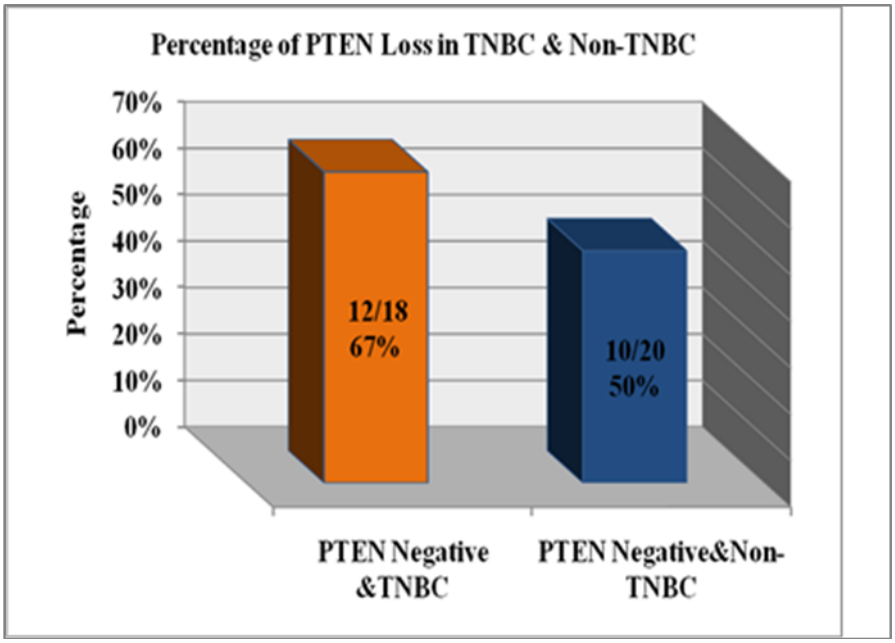

Figure 4: The resulting histogram of the percentage of *PTEN* loss in TNBC and Non-TNBC.

4.1 *PTEN* promoter methylation by MSP

The DNA methylation status was assessed for patients using methylation specific polymerase chain reaction method. The resulted gel electrophoresis for the methylation specific polymerase chain reaction is showed in Fig.5.

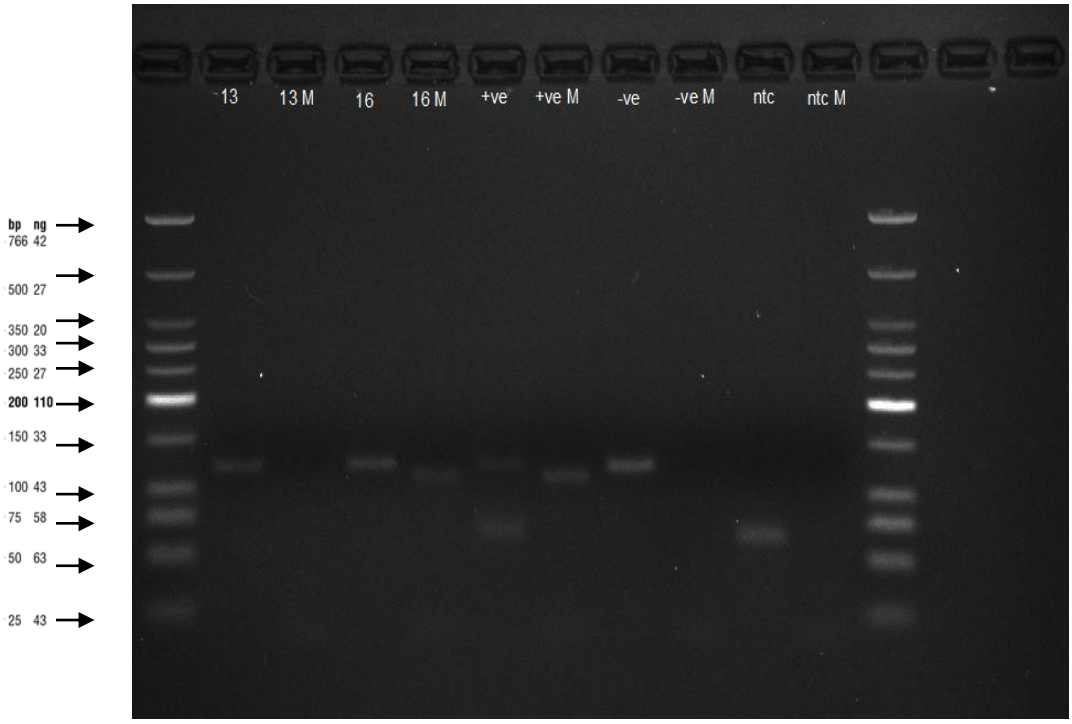

Figure5: Methylation specific polymerase chain reaction gel electrophoresis. The methylated *PTEN* promoter (M) with 109 bp MSP product ,while 122 bp product is indicative of an unmethylated *PTEN* promoter(U), the positive control for the methylated primer (+ve M) and for the unmethylated primer positive control (+ve UM), the negative control (-ve UM, and –ve M for unmethylated and methylated primer respectively) .

The results show that the promoter methylation of *PTEN* gene was detected in 3 out of 38 specimens (8%), while un-methylation was detected in 35 out of 38 specimens (92%) Fig. 6.

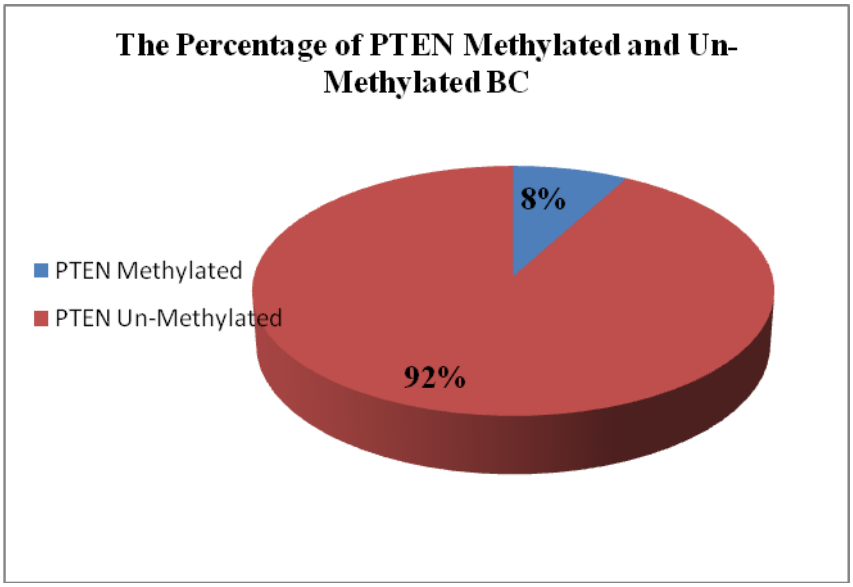

Figure6: Pie chart shows the percentage of methylated and un-methylated breast tumors.

The association between *PTEN* gene methylation status with clinicopathological characteristics are presented in Table 7. There was no correlation between the *PTEN* methylation and loss of *PTEN* expression (p=0.124). There was no correlation between the methylation of *PTEN* and TNBC (p =0.485), tumor type (p =0.913), surgical procedure (p =0.227), neo-adjuvant treatment (p =0.587), adjuvant therapy (p =0.767), age (P =0.145), grade (p =2.637), Stage (p =0.755), ER status (p =0.896), PR status (p =0.861), and Her-2 status (p =0.351) representative results showing the correlation between *PTEN* methylation and variables in table7 .

Table 7: Association between *PTEN* promoter methylation and clinicopathological characteristics in breast cancers

Clinicopathological Factors		**Number of Cases**	***PTEN* Methylation Positive (%)**	***PTEN* Normal**	***p*-value**
Age (years)	<50	23	3(13%)	20(87%)	0.145,NS
	>50	15	0(0)	15(100%)	
Grade	I	2	0(0)	2(100%)	2.637,NS
	II	15	0(0)	15(100%)	
	III	21	3(14%)	18(86%)	
Stage	I	2	0(0)	2(100%)	0.755,NS
	II	18	2(11%)	16(89%)	
	III	18	1(6%)	17(94%)	
Type of breast cancer	NOS (not otherwise specified)	36	3(8%)	33(92%)	0.913,NS
	Colloid	1	0(0)	1(100%)	
	Invasive lobular carcinoma	1	0(0)	1(100%)	
Surgical Procedure	Mastectomy	21	1(5%)	20(95%)	0.227,NS
	Lumpectomy	14	1(7%)	13(93%)	
	Core biopsy	3	1(33%)	2(67%)	

Clinicopathological Factors		**Number of Cases**	***PTEN* Methylation Positive (%)**	***PTEN* Normal**	***p*-value**
Neoadjuvant therapy	Neoadjuvant (Yes)	8	1(12.5%)	7(87.5%)	0.587,NS
	Neoadjuvant (No)	30	2(7%)	28(93%)	
Adjuvant therapy	Adjuvant (Yes)	37	3(8%)	34(92%)	0.767,NS
	Adjuvant (No)	1	0(0)	1(100%)	
ER	Positive	14	1(7%)	13(93%)	0.896,NS
	Negative	24	2(8%)	22(92%)	
PR	Positive	11	1(9%)	10(91%)	0.861,NS
	Negative	27	2(7%)	25(93%)	
Her-2	Positive	8	0(0)	8(100%)	0.351,NS
	Negative	30	3(10%)	27(90%)	
PTEN	Positive	16	0(0)	16(100%)	0.124,NS
	Negative	22	3(14%)	19(86%)	
TNBC	Present	18	2(11%)	16(89%)	0.485,NS
	Absent	20	1(5%)	19(95%)	

NS: Not Significant

4.1.1 The association between *PTEN* gene methylation status with loss of *PTEN* expression

Of the 22 cases with loss of *PTEN* expression, 14 %(3/22 cases) had methylated *PTEN*, while 86 %(19/22cases) of the negative *PTEN* expression tumors without methylation, the percentage of *PTEN* methylation in relative to *PTEN* loss showed in Fig.7. Of the 38 cases, 42% (16 /38 cases) express *PTEN*, without *PTEN* promoter methylation.

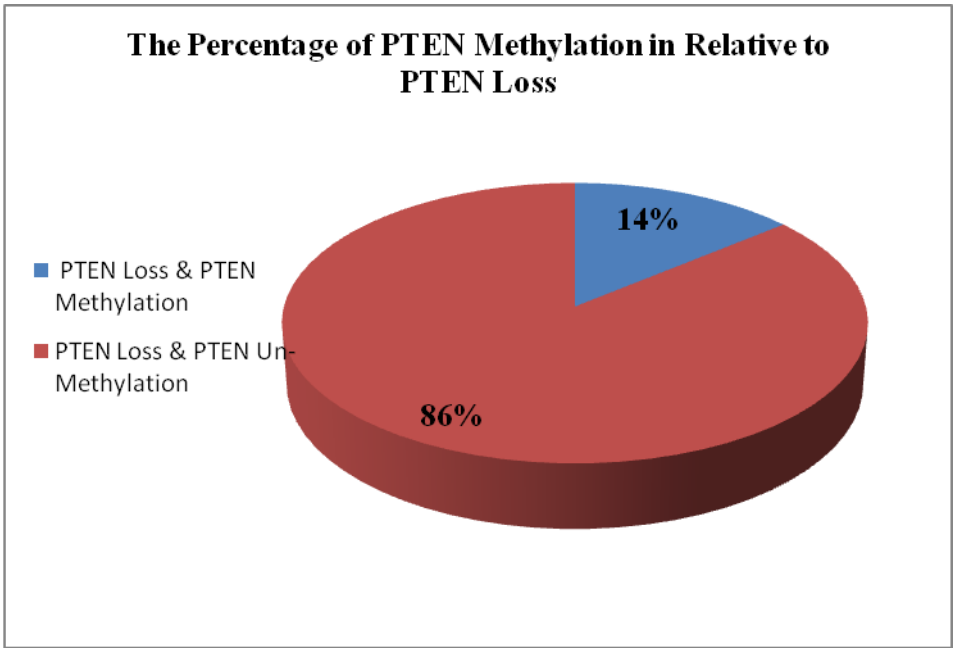

Figure 7: Pie chart illustrates the percentage of methylated and un-methylated breast tumors in relative to *PTEN* loss.

4.1.2 The association between *PTEN* gene methylation status with TNBC

The association between *PTEN* gene methylation status with TNBC is presented in Fig 8. The results show 11 % (2/18 cases) of breast tumor methylated cases with TNBC, and 89 %(16/18cases) of the TNBC cases without methylation.

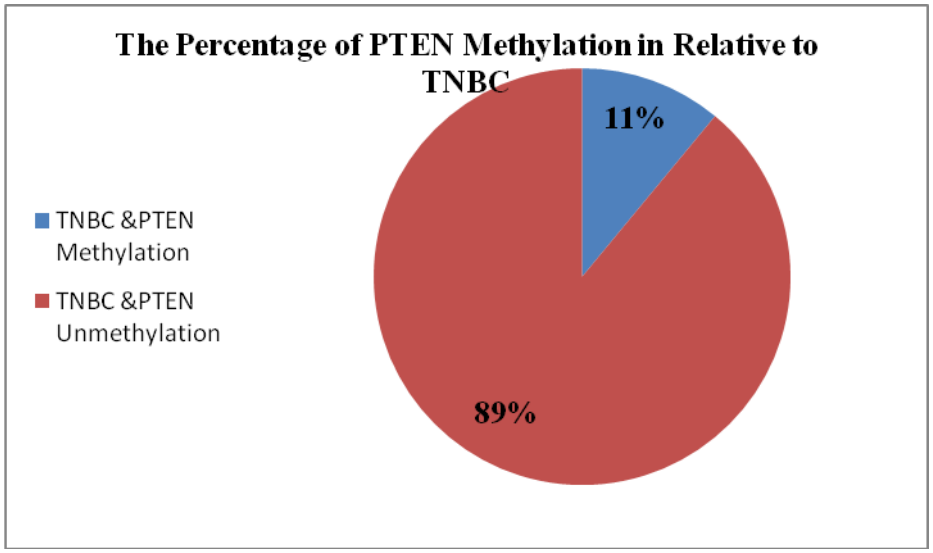

Figure 8: The resulting pie chart illustrates the percentage of methylated and un-methylated breast tumors in relative to TNBC.

CHAPTER 5

5.1 Discussion

In the present study, we found that 22% of the Palestinian women breast cancers in the study sample exhibited loss of *PTEN* expression. In breast cancers, results showing that *PTEN* protein loss are associated with poor prognosis and might contribute to the development of breast cancer [40]. Forty seven percent of the cases were TNBC and loss of *PTEN* gene expression was seen in 67% of TNBC cases. These results are in agreement with Marty B. et al. [41] who reported that triple negative breast cancers often exhibit alterations in *PTEN* gene .In addition, the study of Al-Tamimi DM et al.[42] showed similar incidence of the TNBC, which was 39% in Saudi Arabia population. A recent study by Yanyuan Wu. et al. [43] also come with our results with some discrepancies which may be due to small sample size since the prevalence of the TNBC was 28 % in African-American, and 19% in Hispanics women, with significant association between *PTEN* loss and TNBC in African-American women. In this study, we analyzed the PTEN gene promoter methylation using methylation specific polymerase chain reaction (MSP) in breast cancer patients. We performed MSP because it clears any bias comes from the existence of DNA methylation in the normal epithelial breast tissue. The methylation analysis has been reported that it offers number of advantages when compared with other DNA alterations in cancer [44]. To avoid contamination with normal epithelial cells we performed methylation specific polymerase chain reaction on the paraffin tissues that consist of breast tumor tissue only. In the present study, PTEN promoter methylation was investigated to understand its correlation with the clinicopathological characteristics, especially the poor prognosis subtype of breast cancer (TNBC), and loss of PTEN expression, but the results showed no correlation. In breast carcinomas results regarding methylation suggesting that gene methylation may be associated to different clinicopathological factors[45].We did not detect significant association between PTEN promoter methylation and age, grade, stage, surgical procedure, tumor type, ER, PR, and Her-2. Furthermore, there was no association between PTEN promoter methylation and treatment with adjuvant and neo adjuvant therapy. In the

present study, the analyzed 38 specimens of breast cancer showed that PTEN promoter methylation was detected in 8%. Our findings are in concordance with previous study, reporting that the PTEN promoter methylation has been detected in 14.3% of ductal carcinoma in situ Sweden patients [46]. In addition, low methylation of PTEN (6%) were detected by Tserga A. et al. [47] without clinicopathological correlation in agreement with our results . PTEN promoter methylation was detected in 18% of Korean patients [48].The low percentage of PTEN promoter methylation detected in this study suggests that the methylation may be not the mechanism of inactivation among Palestinian breast cancer patients. In contrast with our results, the findings that were detected in Taiwanese patients by Hou M. etal. [49] who studied PTEN methylation in breast cancer, and 70% of breast malignancies found to be methylated. In addition, Vallian Sadeq et al. [50] reported methylation in 70% of the Iranian breast cancer patients. The different methylation percentages can be explained by the methodology used detect methylation results, the primers used in each study, and ethnic discrepancy. Fourteen percent of the cases (3/22 cases) were methylated with loss of PTEN protein expression. Of the 38 cases, 42% (16 /38 cases) express PTEN, without PTEN promoter methylation. Therefore, no significant correlation between methylation and loss of PTEN expression (p-value=0.124). In agreement of several studies which showed the presence of PTEN methylation in cases with loss of PTEN expression, such as Khan S. et al. [51] who found methylation in the PTEN promoter region in 60% of breast cancer patients with loss of PTEN expression . The divergence between the incidence of the loss of PTEN expression that was 58%, with the incidence of PTEN promoter methylation in our thesis, and the detection of 86 %(19/22cases) unmethylated breast tumors that had loss of PTEN expression; suggest other inactivation mechanisms that affect the loss of PTEN expression. These include loss of heterozygosity (LOH), though this has been reported in only a minority of breast cancers [52].The results show that the majority of the TNBC cases were without methylation. We showed that the PTEN promoter methylation was not correlated with TNBC (p-value=0.485).These findings are consistent with the findings showed by Holm K. etal. [53] who stated that the TNBC subtype have low methylation frequencies of several CPG islands in comparison with Luminal B tumors which showed a high methylation frequencies. The lack of significant correlations in the present study may be due to the small sample size. Nevertheless

our data was the first to present the methylation status of the promoter region of PTEN gene in Palestinian patient's samples.

In conclusion, from our present observations, we detect *PTEN* promoter methylation in 8% of the breast tumors. Our data show that methylation of the *PTEN* promoter in 38 breast cancer women is not significantly associated with the loss of *PTEN* protein expression, and triple negative breast cancer subtype (TNBC) in these samples. Although, the methylated cases are rare among the Palestinian patients, but further studies using large sample size and high throughput analysis is highly recommended.

Annexes

Patient's consent format

نموذج موافقة معلم

انا الموقع ادناه

الاسم:
رقم البطاقة الشخصية :
العنوان : المنطقة :

أ) اصرح بموجب هذه الوثيقة بأني أوافق على المشاركة في هذه الدراسة الوارد وصفها في هذا النموذج
ب) اصرح بموجب هذه الوثيقة انني لا اشارك اثناء توقيع هذه الوثيقة في اية دراسة اخرى , وألتزم بأن لا افعل ذلك خلال سير الدراسة الحاليه
ت) اصرح بموجب هذه الوثيقة انني قد أعلمت من قبل :

الاسم : دكتورةأريج الخطيب او ما ينوب عنها بالدراسة

1- ان الدكتورة اريج الخطيب تلقت من مستشفى اوغستا فكتوريا تفويضا لإجراء دراسة وراثية على الانسجة كما هو منصوص عليه في قانون الصحة العام لعام 1993.
2- ان الباحث الرئيسي ليس له منفعة ذاتيه بأي من المشتركين في هذه الدراسة.
3- ان موضع هذه الدراسة هو :الدراسة الوراثية لأنسجة سرطان الثدي للنساء في فلسطين.
4- أني حر في ان اقرر عدم المشاركة في هذه الدراسة , وأني حر في ان اتوقف عن المشاركة في هذه الدراسة في اي وقت.
5- ان جميع الافراد القائمين بهذه الدراسة او المشاركين فيها سيحافظون على السرية المطلقه فيما يتعلق بهويتي التي لن تنشر حتى في النشرات العلمية.
6- اني مخول بأن اتلقى إجابات على جميع ما لدي من أسئلة , وأني مخول بأن اتبادل الرأي مــع شهود اخرين (مثل طبيب اسرتي او أفراد أسرتي) فيها يتعلق بمشاركتي أو استمرار مشاركتي في هذه الدراسة
7- أني مخول – عند اية مشكلة تتعلق بالدراسة أن أتصل بالدكتورة أريج الخطيب على رقم الهاتف :0598366871

ث) أصرح بموجب هذه الوثيقة أني قد اعطيت معلومات تفصيلية عن هذه الدراسة خصوصاً فيما يتعلق بالمواضيع التالية (المفصله في النموذج المرافق الملحق بهذا النموذج)

1- الاهداف
2- الأساليب
3- التوقيت والمتابعة
4- الفوائد (للمشارك)
5- المخاطر المرافقة
6- المضايقات المرافقة

ج) اصرح بموجب هذه الوثيقة أنني أعرف أنني غير أهل لأية تعويضات مالية عن مشاركتي في هذه الدراسة . بما في ذلك العوائد المالية التي قد تنتج عن براءات الاختراع العلمي الخاصة بنتائج الدراسة الحاليه.
ح) أصرح بموجب هذه الوثيقة بأنني قد وافقت طوعاً على المشاركة في هذه الدراسة وأنني ادركت كل ما قيل انفا . كما وأعطيت نسخة عن نموذج الموافقة والنموذج المرافق . وبتوقيع هذا النموذج فأنني أخول الشخص المسؤول عن هذه الدراسه –الدكتورة أريج الخطيب- الوصول الى ملفاتي الطبية بغرض التحقق من الأساليب المستخدمة في هذه الدراسة والبيانات السريريه , وان الوصول الى المعلومات الطبية سيتم بمنتهى السرية طبقا لقوانين وإجراءات السرية الطبية.

اسم المشارك	التوقيع	التاريخ

اسم المشاهد	التوقيع	رقم البطاقة الشخصية	التاريخ

تصريح الباحث :
تم احراز موافقتي بعد أن شرحت للمشارك كل التفاصيل الواردة انفا وبعد أن تأكدت من ان المشارك قد ادرك كل ما شرحته له

اسم الباحث المفسر	التوقيع	رقم البطاقة الشخصية	التاريخ

ملحق لنموذج الموافقة المعلمة

القسم : مختبر الوراثة الجزيئية التشخيصي
الباحث الرئيسي : دكتورة أريج الخطيب
مشروع البحث : الدراسات الوراثية لسرطان الثدي للنساء في فلسطين

أنت على وشك أن تشارك في مشروع الدراسة الأنفة الذكر بعد ان تتلقى شرحا شاملا من قبل أحد الباحثين المسؤولين حول موضوع الدراسة. والطريقة التي سنجري بها , وكذلك حول الفوائد والمخاطر المتضمنة لمشاركتك , وتوقيعك على نموذج الموافقة بوجود شاهد هو شرط إلزامي لمشاركتك في هذه الدراسة , وفي القسم التالي سنشرح لك محتويات هذه الدراسة بالتفصيل .
" أن هذا النموذج المرافق هو جزء مكمل لنموذج الموافقة المعلمة , وتوقيعك على هذا النموذج المرافق شرط ضروري لمشاركتك في الدراسة الحاليه "

1- أهداف الدراسة :

تهدف هذه الدراسة إلى الحصول على معلومات بيانية عن طريق تعبئة نموذج . وكذلك لجمع عينات نسيج الثدي لأناس يعانون من سرطان الثدي . وهدفنا هو دراسة الأمراض لتحديد العيوب الوراثية او الجينية المسببة لها .

2- بروتوكول الدراسة :
تبدأ هذه الدراسة بمقابلة لمدة نصف ساعة يطلب منك خلالها ان تعطي تفاصيل تتعلق بتاريخك الطبي وذلك بتعبئة نموذج بيانات . كما يطلب منك تقديم تفاصيل مشابه تتعلق بأسرتك . ومن المحتمل ان يطلب منك تمنح أذنا لمراجعةتقاريرك الطبية المتعلقة بمرضك أو أية وثائق طبية أخرى . سيتم أخذ عينات الانسجة لاستخلاص الحامض النووي الغير أكسجيني (DNA).وسيتم اخذ جزء بسيط جدا (شريحة) من عينة النسيج بعد التاكد من الإنتهاء من اي فحص طبي كان مقرر لها . وجميع المواد البيولوجية التي جمعت سيتم تخزينها في قسمالمختبر التشخيصي الوراثي/مستشفى أوغستا فكتوريا ,

وسيرمز لكل مادة بشيفرة معروفة للباحثين الرئيسين فقط . وفي نهاية الدراسة وبعد تحديد المتغاير /المتغايرات الاحيائية (methylation) التي تسببت في المرض سيتم اتلاف الحامض النووي الغير أكسجيني ((DNA) . وحتى هذه المرحلة , فان عينات الحامض النّنوي الغير أكسجيني ستستخدم فقط لأغراض الدراسة الحالية .

3- المخاطر :

ستتم الفحوصات في هذة الدراسة على الانسجة الماخوذة مسبقا لغرض الفحص والتشخيص الطبي بعد التاكد من انتهاء الفحص ولن يتم اجراء اي فحوصات عليك مباشرة

جميع المعلومات التي ستعطينا إياها او أية بيانات سيتم الحصول عليها نتيجة لهذه الدراسة لن تنقل لأي طرف ثالث دون موافقتك الخطية المسبقة . والمعلومات المشتقة من هذه الدراسة ستنقل إليك ولا سيما في حالة احتمال أن تؤثر هذه المعلومات على صحتك , أو على خيارك في الرعاية الصحية أو على ما تخطط له أسرتك – وكل هذا فقط اذا تم التعبير رسميا عن رغبتك في تلقي المعلومات . وسيكون الباحث الرئيسي مسؤولا بأن ينقل اليك جميع المعلومات الوثيقة الصلة بالموضوع والمتعلقة بالمخاطر المرافقة للدراسة الحالية

4- المتابعة :

في حالة الكشف عن تغيرات وراثية ذات قيمة تشخيصية فانه سيتم استدعاءك لغرض الارشاد الوراثي من ناحية والمتابعة من ناحية اخرى .

5- الخيارات :

انت حر في ان تقرر عدم المشاركة في هذه الدراسة . وإذا ما تم الحصول على اية معلومات قد تؤثر على مشاركتك في هذه الدراسة فيستم نقلها اليك دون أي تأخير . كما وان لك الحق في ان تطلب اتلاف عينات الحامض النووي الغير أكسيجيني (DNA) الخاصة بك في اي مرحلة من مراحل هذه الدراسة.

6- السرية :

اي معولمات ستنقلها الينا ستحفظ في سرية مطلقة ولن يتم نقل أو نشر أية معلومة مماثلة . كما وأن جميع المواد والصور والمعلومات الطبية ستنظم في سجل مدوّن , وسيكون السّجل المدوّن تحت تصرف الباحثين الرئيسين فقط . والعينات التي اخذت منك لن تستخدم لاي غرض اخر غير الاغراض المفصلة في هذه الدراسة . نتصل بهم من تلقاء أنفسنا وذلك حفاظا على السرية الطبية .

7- الفوائد التي يحققها المشارك :

ان فهماً افضل لسبب مرضك قد يؤدي الي تحسين الطريقة التي يشخص بها والطريقة التي سيعالجبها وفي ذلك فائدة شخصية لك ولأسرتك ولجميع المرضى المصابين .بمثل هذا المرض. وأيضا من المحتمل ان تفيد هذه الدراسة المجتمع ككل وذلك من المعرفة التي تتجمع نتيجة لمشاركتك .
وكنتيجة لمشاركتك في هذه الدراسة سيتم التأكد على تشخيص حالتك وسوف تتلقى معلومات حول طريقة التعامل مع هذه النتائج .

8- التعويض المالي :

لا يوجد تعويض مالي نتيجة لمشاركتك في هذه الدراسة . قد تؤدي نتائج هذه الدراسة الوراثية الي تطبيق تجاري , أو قد تؤدي إلي براءة الاختراعات العلمية وتطوير عقار , والمشاركون في هذه الدراسة ليس لهم الحق فيما يتعلق ببراءة الاختراعات العلمية والعقاقير والتطبيقات الطبية التي قد تنتج عن الدراسة الحالية

9- معلومات اضافية :

اذا ما كان لديك أية أسئلة فيما يتعلق بهذه الدراسة يمكنك أن تسأل هذه الاسئلة وتحصل على معلومات اضافية من الباحثين الرئيسين على رقم هاتف (026279946) أما المعلومات التي تتعلق بالجوانب الاخلاقية لهذه الدراسة يمكنكالحصول عليها من لجنة حقوق المريض على رقم الهاتف ( ) وأيضا فأن المشاركة في هذه الدراسة تتضمن انك غير مسجل في الوقت الحاضر في دراسة اخرى.

10- الموافقة المعلمة والانسحاب من الدراسة :

توقيعك على نموذج الموافقة المعلمة يعبر عن رغبتك في أن تشارك في هذه الدراسة . وأنت مخول للانسحاب من الدراسة الحالية في اي مرحلة .

اسم المشارك :
توقيع المشارك/الوصي :
توقيع الطبيب :
توقيع المشاهد :

Table of methylation status results

Lab#	P10 Meth	P10IHC	ER	PR	Her-2	Age Range	Age	Tumor Size	Stage	Lymph Node Metastatis	Rejonal Lymph Node	Distant Metastasis	Grade	NeoadjuvantChrmotherapy	Surgical Procedur	Adjuvant Chemotherapy	Tumor Type
2000-10	M	N	N	N	N	40-49	40	T2	IIIA	Present	N2	M0	III	Yes	Lumpectomy	Yes	NOS
2193-10	U	N	N	N	N	30-39	39	T1	IIA	Present	N1	MX	III	No	Mastectomy	Yes	NOS
2018 H 12	U	N	N	N	N	50-59	54	T3	IIIA	Present	N2	M0	III	Yes	Mastectomy	Yes	NOS
754-12	U	N	N	N	N	30-39	33	T3	IIIA	Present	N1	M0	III	No	Mastectomy	Yes	NOS
52-14	U	N	N	N	N	50-59	55	T3	IIB	Absent	N0	M0	III	No	Mastectomy	Yes	NOS
1011	U	N	N	N	N	40-49	41	T3	IIB	Absent	N0	ND	III	No	Core biopsy.	Yes	NOS
1050-13	U	N	N	N	N	50-59	52	T2	IIB	Present	N1	M0	II	No	Mastectomy	Yes	NOS
446-13	U	N	N	N	N	50-59	56	T3	IIIA	Present	N1	MX	I	No	Lumpectomy	Yes	NOS
2402	U	N	N	N	N	50-59	55	T2	IIA	Absent	N0	Mn/a	II	No	Lumpectomy	Yes	NOS
1140	U	N	N	N	N	40-49	47	T1	IIA	Present	N1	Mn/a	II	Yes	Mastectomy	Yes	NOS
2280	M	N	N	N	N	20-29	26	T2	IIA	Absent	N0	ND	III	No	Core biopsy	Yes	NOS
506-11	U	N	N	N	N	50-59	59	T2	IIA	Present	N0	Mn/a	III	No	Mastectomy	Yes	NOS
1596-1a	U	N	P	N	0	60-69	67	T1	IIA	Present	N1	MX	II	Yes	Lumpectomy	Yes	NOS
965-1B	U	N	P	P	0	30-39	39	T2	IIB	Present	N2	MX	III	No	Mastectomy	Yes	NOS
281	U	N	N	N	1	40-49	48	T2	IIIA	Present	N2	MX	II	No	Core biopsy	Yes	NOS
5432-12	U	N	P	N	1	40-49	46	T2	IIIA	Present	N2	M0	III	No	Mastectomy	Yes	NOS
978M12	M	N	P	P	0	40-49	40	T2	IIB	Present	N1	M0	III	No	Mastectomy	Yes	NOS
1277-13	U	N	P	P	0	40-49	44	T2	IIA	Absent	N0	Mn/a	II	No	Lumpectomy	Yes	NOS
388-13	U	N	P	P	0	40-49	46	T2	IIIA	Present	N2	M0	II	No	Mastectomy	Yes	NOS
50	U	N	P	P	0	50-59	52	T2	IIA	Absent	N0	M0	I	No	Lumpectomy	Yes	Colloid Carcinoma
659-11	U	N	N	P	0	40-49	47	T1	I	Absent	N0	Mn/a	III	No	Mastectomy	Yes	NOS
324-13	U	N	P	N	0	40-49	48	T1	IIA	Present	N1	Mn/a	III	Yes	Lumpectomy	Yes	NOS
3411-13	U	P	N	N	N	60-69	60	T2	IIB	Present	N1	MX	III	No	Lumpectomy	Yes	NOS
73-1B-14	U	P	N	N	N	40-49	48	T1	I	Absent	N0	M0	III	No	Lumpectomy	Yes	NOS
1054	U	P	N	N	N	40-49	47	T2	IIIA	Present	N2	Mn/a	II	No	Mastectomy	Yes	NOS
229	U	P	N	N	N	40-49	44	T2	IIA	Absent	N0	Mn/a	III	No	Lumpectomy	Yes	NOS
826	U	P	N	N	N	50-59	54	T2	IIIB	Present	N3	MX	III	No	Mastectomy	Yes	NOS
2264-10	U	P	N	N	N	60-69	64	T2	IIA	Absent	N0	Mn/a	II	No	Lumpectomy	Yes	NOS
4607	U	P	P	N	N	50-59	55	T2	IIIA	Present	N2	M0	II	Yes	Lumpectomy	No	NOS
658-c	U	P	N	N	P	40-49	48	T4	IIIB	Absent	N0	MX	II	No	Mastectomy	Yes	NOS
441-13	U	P	N	N	P	60-69	64	T2	IIA	Absent	N0	MX	II	No	Mastectomy	Yes	NOS
959-12-IG	U	P	P	P	P	40-49	48	T3	IIIB	Present	N3	MX	III	No	Mastectomy	Yes	NOS
1623-13	U	P	P	P	N	30-39	38	T1	IIIA	Present	N2	MX	II	Yes	Mastectomy	Yes	NOS
1542-1B-12	U	P	N	N	P	30-39	36	T3	IIIA	Present	N2	MX	II	No	Lumpectomy	Yes	NOS
22-13	U	P	P	P	P	60-69	62	T2	IIIA	Present	N2	M0	III	No	Mastectomy	Yes	NOS
297-13	U	P	P	P	N	40-49	45	T3	IIIB	Present	N3	M0	III	No	Mastectomy	Yes	NOS
1033-1B	U	P	P	P	N	40-49	47	T3	IIIA	Present	N1	M0	II	No	Mastectomy	Yes	Invasive Lobular Carcinoma
224-13	U	P	N	N	P	50-59	57	T1	IIIB	Present	N3	Mn/a	III	Yes	Lumpectomy	Yes	NOS

References

1World Health Organization. 2009. World Health Statistics. [Online] (Updated 3 May 2013) .http://www.who.int/whosis/whostat/2009/en/index.html.

2Giordano SH, Buzdar AU, Hortobagyi GN., 2002. Breast cancer in men. Ann Intern Med.;137(8),PP.678–687.

3Ministry of Health, Midyear 2013 . The annual health report. Palestinian Health Information Center [Online] (Updated October 2013) Available at: http://www.moh.ps/attach/536.pdf.

4Benjamin D. Hopkins, Cindy Hodakoski, Douglas Barrows, Sarah M. Mense, Ramon E. Parsons , 2014. *PTEN* function: the long and the short of it . Trends in Biochemical Sciences. 39(4), PP. 183-190.

5Song, M.S., Salmena, L. , Pandolfi, P.P., 2012. The functions and regulation of the *PTEN* tumour suppressor . Nature Reviews Molecular Cell Biology. 13(5), PP. 283–296.

6Dean SJ, Perks CM, Holly JM, Bhoo-Pathy N, Looi LM, Mohammed NA, Mun KS, Teo SH, Koobotse MO, Yip CH, Rhodes A , 2014. Loss of *PTEN* expression is associated with IGFBP2 expression, younger age, and late stage in triple-negative breast cancer. Am J Clin Pathol . 141(3), PP. 323-33.

7Katrien Berns, Hugo M. Horlings, Bryan T. Hennessy, Mandy Madiredjo, E. Marielle Hijmans,Karin Beelen,Sabine C. Linn, Ana Maria Gonzalez-Angulo,Katherine Stemke-Hale, Michael Hauptmann,Roderick L. Beijersbergen,

Gordon B. Mills, Marc J. van de Vijver, and Rene Bernards, 2007. A Functional Genetic Approach Identifies the PI3K Pathway as a Major Determinant of Trastuzumab Resistance in Breast Cancer . Cancer Cell . 14(4) , PP. 297-299.

8 Husseini A, Abu-Rmeileh NME, Mikki N.,2009.Cardiovascular diseases, diabetes mellitus, and cancer in the occupied Palestinian territory. Lancet .373, PP. 1041–9.

9Chouchane L, Boussen H, Sastry KS.,2013. Breast cancer in Arab populations: molecular characteristics and disease management implications. Lancet Oncol. 14(10),PP.417-24.

10Khatib A, AL Abed MN., 2011.*PTEN* expression in Palestinian women with breast cancer, particularly in triple-negative subtype. J Clin Oncol. 29,suppl 27; abstr.

11Foulkes, William D., Ian E. Smith, and Jorge S. Reis-Filho,2010. "Triple-negative breast cancer." New England journal of medicine .363(20), PP. 1938-1948.

12Salmena, Leonardo, Arkaitz Carracedo, and Pier Paolo Pandolfi, 2008. Tenets of *PTEN* tumor suppression. Cell , PP. 403-414.

13Besson A, Robbins SM, Yong VW., 1999. *PTEN*/MMAC1/TEP1 in signal transduction and tumorigenesis. Eur J Biochem.263,PP.605-11

14Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, Nolden LK, Horlings H, Berns K, Hung MC, van de Vijver MJ, Valero V, Gray JW, Bernards R, Mills GB, Hennessy BT., 2008. An integrative genomic and proteomic analysis of PIK3CA, *PTEN*, and AKT mutations in breast cancer. Cancer Res. 68,PP.6084-91.

15Shaw RJ and Cantley LCRas. PI(3)K and mTOR signaling controls tumour cell growth. Nature;144,PP. 424-430.

16Engelman J, Luo J and Cantley L., 2006.The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet . 7,PP. 606-619.

17Sansal I and Sellers WR., 2004. The biology and clinical relevance of the *PTEN* tumor suppressor pathway. J Clin Oncol.22, PP.2954-2963.

18Saal L, Johansson P, Holm K, 2007. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant *PTEN* tumor suppressor pathway activity. Proc Natl Acad Sci USA .104,PP.7564-9.

19Podsypanina K, Ellenson L, Nemes A., 1999.Mutation of *PTEN*/Mmac1 in mice causes neoplasia inmultiple organ systems. Proc Natl Acad Sci USA.96, PP.1563-8.

20Gordon V, Banerji S, 2013. Molecular Pathways: PI3K Pathway Targets in Triple-Negative Breast Cancers. Clin Cancer Res.19, PP. 3738-44.

21Mevlude Inanc, Metin Ozkan, Halit Karaca, Veli Berk, Oktay Bozkurt, Ayse Ocak Duran, Ersin Ozaslan, Hulya Akgun, Fatos Tekelioglu, Ferhan Elmali 2013. Cytokeratin 5/6, c-Met expressions, and *PTEN* loss prognostic indicators in triple-negative breast cancer . Medical Oncology ..31, P. 801.

22Ma WW, Adjei AA, (2009) Novel agents on the horizon for cancer therapy. CA Cancer J Clin .59.PP. 111-137.

23Nadia C. Correia, Ana Gırio, Ines Antunes ,Leila R. Martins, Joao T. Barata,2014. The multiple layers of non-genetic regulation of *PTEN* tumour suppressor activity . European Journal of Cancer .50,PP.216-225.

24Jovanovic J, Ronneberg JA, Tost J and Kristensen , 2010.The epigenetics of breast cancer. Mol Oncol. 4, PP.242-254 .

25 Issa JP, 2003. Methylation and prognosis: of molecular clocks and hypermethylator phenotypes. Clin Cancer Res.9, PP.2879-2881.

26 Hawkins RD, Hon GC, Lee LK, Ngo Q, Lister R, 2010. Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell . 6, PP. 479–491.

27Duerr E. M., Rollbrocker B., Hayashi Y., Peters N., Meyer-Puttlitz B., Louis D. N., Schramm J., Wiestler O. D., Parsons R., Eng C., von Deimling A., 1998.*PTEN* mutations in gliomas and glioneuronal tumors. Oncogene. 16 , PP.2259-2264.

28Perren A., Weng L-P., Boag A. H., Ziebold U., Thakore K., Dahia P. L., Komminoth P., Lees J. A., Mulligan L. M., Mutter G. L., Eng C.,1999. Immunohistochemical evidence of loss of *PTEN* expression in primary ductal adenocarcinomas of the breast. Am. J. Pathol. . 155 , PP. 1254-1260.

29Mutter G. L., Lin M-C., Fitzgerald J. T., Kum J. B., Baak J. P., Lees J. A., Weng L. P., Eng C., 2000. Altered *PTEN* expression as a diagnostic marker for the earliest endometrial precancers. J. Natl. Cancer Inst. (Bethesda). 92, PP. 924-930.

30Cairns P., Okami K., Halachmi S., Halachmi N., Esteller M., Herman J. G., Jen J., Isaacs W. B., Bova G. S., Sidransky D. , 1997. Frequent inactivation of *PTEN*/MMAC1 in primary prostate cancer. Cancer Res. . 57, PP. 4997-5000.

31 Avraham A, Cho SS, Uhlmann R, Polak ML, Sandbank J, 2014. Tissue Specific DNA Methylation in Normal Human Breast Epithelium and in Breast Cancer. PLoS ONE . 9(3), P.91805.

32 Wei SH, Chen CM, Strathdee G, Harnsomburana J, Shyu CR,Rahmatpanah F, Shi H, Ng SW, Yan PS, Nephew KP, 2002. Methylation microarray analysis of late-stage ovarian carcinomasdistinguishes progression-free survival in patients and identifiescandidate epigenetic markers. Clin Cancer Res .8, PP.2246-2252.

33 Shen L, Toyota M, Kondo Y, Obata T, Daniel S, Pierce S, Imai K,Kantarjian HM, Issa JP, Garcia-Manero G , 2003.Aberrant DNA methylation of p57KIP2 identifies a cell cycle regulatory pathway with prognostic impact in adult acute lymphocytic leukemia. Blood.101, PP.4131-4136.

34 Maruyama R, Toyooka S, Toyooka KO, Harada K, Virmani AK,Zochbauer-Muller S, Farinas AJ, Vakar-Lopez F, Minna JD, Sagalowsky A, 2001. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features.Cancer Res . 61, PP.8659-8663.

35 Ogi K, Toyota M, Ohe-Toyota M, Tanaka N, Noguchi M, Sonoda T,Kohama G, Tokino T, 2002. Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin Cancer Res., 8, PP.3164-3171.

36The American Joint Committee on Cancer (AJCC),2014. [Online] (Updated November2014).Availableat:http://www.cancer.gov/cancertopics/pdq/treatment/breast/healthprofessional/page3.
[Accessed November 2014] .

37Vamesu S. 2007. Angiogenesis and progesterone receptor status in primary breast cancer patients: an analysis of 158 needle core biopsies. Rom J Morphol Embryol. 48,PP.267-74.

38Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A. 2007. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. J Clin Oncol. 25, PP.118-145.

40Park SB, Choi TY, Cha SJ, 2001. Loss of expression of the *PTEN* gene product in the infiltrating ductal carcinoma of the breast and its relationship with clinicopathologic factors. J Korean Surg Soc.60,PP: 600-5.

41Marty B, Maire V, Gravier E, et al.,2008. Frequent *PTEN* genomic alterations and activated phosphatidylinositol 3-kinase pathway in basal-like breast cancer cells. Breast Cancer Res.10, P.101.

42Al-Tamimi DM, Bernard PS, Shawarby MA, Al-Amri AM, Hadi MA. 2009. Distribution of molecular breast cancer subtypes in middle eastern-saudi arabian women: a pilot study. Ultrastruct Pathol. 33, PP.141-50.

43Yanyuan Wu equal contributor,Marianna Sarkissyan equal contributor, Yahya Elshimali,Jaydutt V. Vadgama, 2013. Triple Negative Breast Tumors in African-American and Hispanic/Latina Women Are High in CD44+, Low in CD24+, and Have Loss of *PTEN*. PLoS ONE [online](Updated 22 October 2013) Availableat:http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0078259 [Accessed 22 October 2013]. 8(10) .

44 Widschwendter M, Jones PA,2002. The potential prognostic, predictive, and therapeutic values of DNA methylation in cancer. Commentary re: J. Kwong et al., Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin. Cancer Res., 8: 131-137, 2002, and H-Z. Zou et al., Detection of aberrant p16 methylation in the serum of colorectal cancer patients. Clin. Cancer Res., 8: 188-191, 2002. Clin Cancer Res. 8(1),PP.17-21.

45 Li ShaoYing, Rong M., Iacopetta B., 2005. DNA hypermethylation in breast cancer and its association with clinicopathological features.Cancer Letters .237 (2), PP.272 – 280.

46Aslaug Aa Muggerud, Jo Anders Ronneberg, Fredrik Warnberg, Johan Botling, Florence Busato, Jovana Jovanovic, Hiroko Solvang, Ida Bukholm,Anne-Lise Borresen-Dale, Vessela N Kristensen, Therese Sorlie and Jorg Tost, 2010. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and *PTEN* in ductal carcinoma in situ and early invasive breast cancer. Breast Cancer Research .12(1), R3 .

47 Tserga A., Michalopoulos, N., Levidou, G., Korkolopoulou, P., Zografos, G., Patsouris, E., & Saetta, A. 2012. Association of aberrant DNA methylation with clinicopathological features in breast cancer. Oncology Reports.27(5), PP.1630-1638.

48 Sun Hee Chang·Shi Nae Lee,Min Sun Cho, Heasoo Koo, Woon Sup Han, Seock-Ah Im, Byung-In Moon, Hyun Suk Suh, Hye-Young Choi, Sun Hee Sung, 2005, Loss of PTEN Expression in Breast Cancers. The Korean Journal of Pathology. 14(7), PP.672-6.

49Hou M., Chen P. and Yeh Y., 2009. Promoter Methylation and Phosphorylation Status of *PTEN* in Taiwanese Breast Cancer . Cancer Research [Online] (Updated 15 December2009)Availableat:http://cancerres.aacrjournals.org/cgi/content/abstract/69/24_MeetingAbstracts/4060 [Accessed 13 December 2009]. 69(24) .

50Vallian Sadeq, Nassiri Isar, Tavassoli Manoochehr, 2011. Association of sporadic breast cancer with *PTEN*/MMAC1/TEP1 promoter hypermethylation. Medical Oncology [Online] (Updated June 2011)
Available at: http://link.springer.com/article/10.1007/s12032-010-9473-8
[Accessed June 2011], 28(2) , PP. 420-423 .

51Khan S, Kumagai T, Vora J, Bose N, Sehgal I, Koeffler PH, Bose S., 2004. *PTEN* promoter is methylated in a proportion of invasive breast cancers. Int J Cancer. 112(3), PP. 407-10

52 Bose S, Wang SI, Terry MB, Hibshoosh H, Parsons R., 1998. Allelic loss of chromosome 10q23 is associated with invasion in breast carcinomas.
Oncogene .17,PP.123–7.

53 Holm K, Hegardt C, Staaf J, Vallon-Christersson J, Jonsson G, Olsson H, Borg A, Ringner M, 2010. Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. Breast Cancer Res.12(3),R36.