GENETIC DETERMINANTS OF TAC CHEMOTHERAPY RESPONSE IN TRIPLE NEGATIVE BREAST CANCER PATIENTS

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by

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LIST OF ABBREVIATIONS

ºC	'Degree Celcius'
%	Percent
µL	Microlitre
µM	Micromolar
5-FU	5-Fluorouracil
A260/230	Absorbance At 260 Nm And 230 Nm
A260/280	Absorbance At 260 Nm And 280 Nm
ABC	ATP-Binding Cassette
ABCB1	ATP-Binding Cassette Subfamily B Member 1
AC	Adriamycin And Cyclophosphamide
AC-T	Adriamycin, Cyclophosphamide, Taxane
ADR	Adriamycin
AE	Elution Buffer
AhRR	Aryl Hydrocarbon Receptor Repressor
AKT	Protein Kinase B
AL	Lysis Buffer
AP-1	Activator Protein 1
ARNT	Aryl Receptor Nuclear Translocator
AS-PCR	Allele Specific-Polymerase Chain Reaction
ATP	Adenosine Triphosphate
AUC	Area Under Curve
AW1	Washing Buffer 1
AW2	Washing Buffer 2

BCl2	B-Cell Lymphoma 2
BLAST	Basic Local Alignment Search Tool
BLBC	Basal Like Breast Cancer
BMI	Body Mass Index
BmiI	Polycomb Complex Protein
bp	Base Pair
BRCA 1	Breast Cancer Gene 1
BRCA 2	Breast Cancer Gene 2
CAF	Cyclophosphamide, Adriamycin and Fluorouracil
CBLB	E3 Ubiquitin-Protein Ligase
CCND1	Cyclin-D1
CCNG1	Cyclin-G1
CDK6	Cell Division Protein Kinase 6
cDNA	Complementary Deoxyribonucleic Acid
CIs	Confidence Intervals
CMF	Cyclophosphamide, Methotrexate, and Fluorouracil
COX	Cyclooxygenase
COX-1	Cyclooxygenase-2
CREB1	Camp Responsive Element Binding Protein 1
Ct	Cycle Threshold
CT scan	Computed Tomography scan
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member 5
CYPs	Cytochrome P450

D’	Coefficient of Linkage Disequilibrium
DCIS	Ductal Carcinoma *In Situ*
DFS	Disease-Free Survival
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
DOX	Doxorubicin
DPD	Dihydropyrimidine Dehydrogenase
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-Mesenchymal Transition
ENPP1	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
ER	Oestrogen receptor
FAS-L	First Apoptosis Signal Ligand
FB-XW7	F-Box And WD Repeat Domain-Cointaning 7
FEC	Fluorouracil (5FU), Epirubicin And Cyclophosphamide
FFPE	Formalin-Fixed Paraffin Embedded
FOX1	Forkhead Box 1
FOXF2	Forkhead Box F2
FOXO1	Forkhead Box Protein O1
FOXO3	Forkhead Box O3
FRA17B	Fragile Site, Aphidicolin Type, Common, Fra(17)(Q23.1)
g	Gram
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GRB2	Growth Factor Receptor-Bound Protein 2
HER2	Human Epidermal Growth Receptor Factor-2

HOTAIR	Hox Antisense Intergenic RNA
HR	Hazard Ratio
HSF2	Heat Shock Transcription Factor 2
HVP16	Human Papillomavirus 16
IDC-NOS	Invasive Ductal Carcinoma-No Otherwise Specified
JFCR	Japanese Foundation of Cancer Research
kb	Kilobase
LD	Linkage Disequilibrium
mA	Milliampere
MDR	Multidrug Resistance
$MgCl_2$	Magnesium Chloride
MIM	Missing In Metastasis
miRNAs	MicroRNAs
MITF	Melanogenesis Associated Transcription Factor
mL	Millilitre
MLH1	MutL protein homolog 1
mM	Millimolar
MMIF	Macrophage Migration Inhibitory Factor
MREC	Medical Research and Ethics Committee
MRI	Magnetic Resonance Imaging
mRNAs	Messenger RNAs
MSH2	MutS protein homolog 2
MSI	Microsatellite Instability
MTSS1	Metastasis Suppressor 1
NC1	National Cancer Institute

NF-κB	Nuclear Factor-Kappa B
ng	Nanogram
NOTCH1	Notch Homolog 1
NR5A2	Nuclear Receptor Subfamily 5, Group A, Member 2
NW	Washing buffer
OR	Odds Ratio
OS	Overall Survival
PARP1	Poly-ADP Ribose Polymerase 1
PB	Binding buffer
pCR	Pathological Complete Response
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
PDCD4	Programmed Cell Death 4
P-gp	Permeability-Glycoprotein
PR	Progesterone receptor
PRC2	Polycomb Repressive Complex 2
PRKD1	Protein Kinase D1
PTEN	Phosphatase And Tensin Homolog
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
r^2	Coefficient of Determination
RAB14	Ras-Related Protein
RECK	Reversion Inducing Cysteine Rich Protein With Kazal Motifs
REST	Relative Expression Software Tools
RFS	Relape-Free Survival

RIN	RNA Integrity Number
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SD	Standard Deviation
SE	Standard Error
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package For The Social Sciences
TAC	Taxane, Adriamycin, Cyclophosphamide
TBE	Tris-Boric Acid EDTA
TCGA	The Cancer Genome Atlas
TE	Tris EDTA
TGF-β1	Transforming Growth Factor Beta 1
TIMP1	Timp Metallopeptidase Inhibitor 1
TMEM49	Transmembrane Protein-49
TNBC	Triple Negative Breast Cancer
TNM	Tumour-Node-Metastasis
TPM1	Tropomyosin 1
TrKB	Tropomyosin Receptor Kinase B
TSS	Transcription Start Site
TUBB3	Tubulin Beta 3 Class III
U	Unit
UTR	Untranslated Region
UV	Ultra Violet
V	Voltage
VEGF-A	Vascular Endothelial Growth Factor- A

YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta
ZEB1	Zinc Finger E-Box Binding Homeobox 1
ZEB2	Zinc Finger E-Box Binding Homeobox 2

PENENTU GENETIK DALAM TINDAK BALAS TAC KEMOTERAPI DALAM PESAKIT KANSER PAYUDARA TIGA KALI NEGATIF

ABSTRAK

Kanser payudara tiga kali negatif (TNBC), salah satu subtip dalam kanser payudara yang dikategorikan sebagai fenotip yang agresif, kadar ulangan yang tinggi dan prognosis yang buruk dan merupakan cabaran klinikal yang penting kerana kekurangan terapi yang khusus. Walaupun, pesakit TNBC dirawat dengan regimen kemoterapi taxane, adriamycin dan cyclophosphamide (TAC), kerintangan ubatan dan pertumbuhan tumor ulangan adalah halangan utama dalam rawatan TNBC. Penanda prognostik bagi TNBC masih sukar difahami. Kajian ini dijalankan untuk menyelidik kesan SNP yang dipilih *CYP1B1* 142 C> G (rs10012), 4326 C> G (rs1056836) dan 4390 A> G (rs1800440), *CYP3A4* 878 T> C (rs28371759), *CYP3A5* 6986 A > G (rs776746), *ABCB1* 1236 C> T (rs1128503), 2677 G> T / A (rs2032582) dan 3435 C> T (rs1045642) dan ekspresi gen serta tahap ekspresi miRNA yang dipilih (miR-21, miR-27b, miR-34a, miR-182, miR-200c dan miR-451) dalam memodulasi tindak balas kemoterapi TAC serta hasil rawatan pada pesakit TNBC. Tujuh puluh enam (76) sampel darah and 41 tisu blok FFPE dari pesakit yang sama disahkan secara klinikal dan histopatologi pesakit TNBC. DNA (sample darah) dan keseluruhan RNA (tisu blok FFPE) diekstrak. Pengenotipan dilakukan menggunakan kaedah PCR-RFLP and AS-PCR seterusnya penjujukan DNA. Tahap ekspresi mRNA dan miRNA diukur dengan menggunakan kaedah qRT-PCR. Kesan terhadap rawatan dan penyakit dinilai setelah selesai kemoterapi. Berdasarkan respon kemoterapi, Pesakit dibahagikan kepada kerintangan kemoterapi dan respon kemoterapi. Dalam analisis perkaitan genetik, genotip homozigot dan alel mutasi *CYP1B1* 4326 C>G dan

ABCB1 3435 C>T polimorfisme, gabungan genotip *CYP1B1* 142 GG + *CYP3A4* 878 TT dan *CYP1B1* 4326 GG + *ABCB1* 1236 CT dan haplotip *CYP1B1* 4326G / 142C, *ABCB1* 3435T / 2677G / 1236T dan *ABCB1* 3435T / 2677T / 1236T dikaitkan dengan risiko yang lebih tinggi untuk kerintangan kemoterapi. Manakala, genotip *CYP1B1* 142 CG secara signifikan dikaitkan dengan respon kemoterapi yang baik. Untuk analisis ekspresi mRNA dan miRNA, tahap ekspresi tinggi bagi *CYP1B1* dikaitkan secara signifikan dengan kerintangan kemoterapi. Lebih-lebih lagi, regulasi yang tinggi bagi miR-21 dan miR-182 didapati berkaitan dengan peningkatan risiko pengulangan penyakit menjadi lebih teruk dan menjadi faktor prognosis dalam pesakit TNBC yang menjalani kemoterapi TAC. Sebaliknya, genotip *CYP1B1* 142 CG dijumpai sebagai faktor prognostik yang baik untuk meramalkan kelangsungan hidup dalam pesakit TNBC. Oleh itu, genotip *CYP1B1* 4326 GG, *ABCB1* 3435 TT, tahap regulasi *CYP1B1* mRNA yang tinggi dan tahap regulasi miR-21 dan miR-182 yang tinggi menjadi penentu genetik terhadap tindak balas kemoterapi TAC didalam pesakit TNBC. Penentu genetik ini boleh dipertimbangkan sebagai penanda bio yang berpotensi yang mungkin akan membantu pegawai perubatan, terutamanya dalam risiko berulang dan / atau prognosis yang berulang dan seterusnya meningkatkan pengurusan pesakit TNBC.

GENETIC DETERMINANTS OF TAC CHEMOTHERAPY RESPONSE IN TRIPLE NEGATIVE BREAST CANCER PATIENTS

ABSTRACT

Triple negative breast cancer (TNBC), one of the breast cancer subtypes is characterised by aggresive phenotype, high rates of recurrence and poor prognosis and is an important clinical challenge due to lack of specific targeted therapy. Although, TNBC patients are treated with taxane, adriamycin and cyclophosphamide (TAC) chemotherapy regimen, drug resistance and tumour recurrence are major obstacles in TNBC treatment. Reliable prognostic marker of TNBC remains elusive. The present study was undertaken to investigate the impact of selected SNPs of *CYP1B1* 142 C>G (rs10012), 4326 C>G (rs1056836) and 4390 A>G (rs1800440), *CYP3A4* 878 T>C (rs28371759), *CYP3A5* 6986 A>G (rs776746), *ABCB1* 1236 C>T (rs1128503), 2677 G>T/A (rs2032582) and 3435 C>T (rs1045642) and their respective gene expressions as well as expression levels of selected miRNAs (miR-21, miR-27b, miR-34a, miR-182, miR-200c and miR-451) in modulating TAC chemotherapy response and treatment outcome in TNBC patients. Seventy six (76) blood samples and 41 match paired FFPE tissues blocks available from the same group of clinically and histopathologically confirmed TNBC patients, who had undergone surgery and completed six cycles of TAC chemotherapy regimen were included in the study. DNA (blood samples) and total RNA (FFPE tissues blocks) were extracted. Genotyping was carried out using PCR-RFLP and AS-PCR methods followed by DNA sequencing. mRNA and miRNA expression levels were determined using qRT-PCR. The treatment response and disease outcome of the patients were evaluated after completion of chemotherapy. Based on chemotherapy response, patients were

categorized into chemoresistant and chemoresponse groups. In genetic association analysis, homozygous variant genotype and variant allele of *CYP1B1* 4326 C>G and *ABCB1* 3435 C>T polymorphisms, combination of *CYP1B1* 142 GG + *CYP3A4* 878 TT and *CYP1B1* 4326 GG + *ABCB1* 1236 CT genotypes, *CYP1B1* 4326G/142C, *ABCB1* 3435T/2677G/1236T and *ABCB1* 3435T/2677T/1236T haplotypes were associated with significantly higher risk for chemoresistance. Whereas, *CYP1B1* 142 CG genotype was significantly associated with good chemoresponse. For mRNA and miRNA expression levels analysis, high expression level of *CYP1B1* was significantly associated with chemoresistance. Moreover, up regulation of miR-21 and miR-182 were found to be associated with increased risk of relapse and to be a prognosis factor in TNBC patients undergoing TAC chemotherapy. On the other hand, *CYP1B1* 142 CG genotype was found to be a good prognostic factor for predicting survival in TNBC patients. Thus, *CYP1B1* 4326 GG, *ABCB1* 3435 TT genotypes, high *CYP1B1* mRNA expression level and up regulation of miR-21 and miR-182 expression levels emerged as genetic determinants of TAC chemotherapy response in TNBC patients. These genetic determinants could be considered as potential biomarkers that might help the clinician, especially in predicitng recurrence risk and/or prognosis and thus improve management of TNBC patients.

CHAPTER 1

INTRODUCTION

1.1 Breast cancer

Breast cancer is a type of cancer that develops in the cell lining of milk ducts as well as the lobules that supply the duct with milk. Breast cancer develops from normal breast epithelial cells that evolve via atypical hyperplasia (and eventually dysplasia) to ductal carcinoma *in situ* (DCIS) and subsequently to invasive breast cancer. The process involves multiple molecular alterations involving genetic and epigenetic alterations in precursor and neoplastic cells, subsequently turning to metastatic breast cancer (Rivenbark *et al.*, 2013).

Generally, breast cancer can be divided into two (2) types; sporadic and hereditary forms. The most common form of breast cancer is the sporadic form which accounts for more than 85% of cases. Sporadic breast cancer results from genetic changes that occur only in breast cancer cells (somatic mutation) due to exposure or interaction with environmental factors. However, hereditary breast cancer which accounts for less than 15% of breast cancer cases, results from inherited germline mutations in high penetrant breast cancer susceptibility genes including breast cancer genes 1 and 2 (*BRCA1* and *BRCA2* genes) (Martin, 2001).

1.2 The incidence and prevalence of breast cancer

Breast cancer is a global health issue and is the commonest form of cancer and the leading cause of cancer-related deaths in women (Beiki *et al.*, 2012; Shah *et al.*, 2014). The incidence is increasing worldwide over the years with high mortality rate. More than one million new breast cancer cases were reported which accounts for 25% of all cancer types (Ferlay *et al.*, 2015).

According to Globocan, the standardized incidence rate of breast cancer was 43.1 per 100,000 population while the mortality rate was 12.9 per 100,00 population. The highest incidence was in Belgium (111.9 per 100,000), followed by Denmark (105 per 100,000), Bahamas (98 per 100,000) and Netherlands (96 per 100,000). Continent wise, the incidence rate was highest in Northern America (91.6 per 100,000), followed by Western Europe (91.1 per 100,000), Middle Africa (26.8 per 100,000) and Eastern Asia (27 per 100,000) respectively (Ghoncheh *et al.*, 2016a). Among South East Asian countries, Singapore had a higher incidence rate (65.7 per 100,000) followed by Brunei (48.6 per 100,000) and Philippines (47 per 100,000) (Youlden *et al.*, 2014).

According to the National Cancer Registry Malaysia report from 2007 to 2011, breast cancer was the commonest cancer type in women in Malaysia with a total of 18,343 (17.1%) cases reported followed by colorectal (13.2%) and lung (10.2%). Breast cancer accounts for 32.1% of all cancer in females with life time risk of 1 in 30 individuals and an age standardized rate of 31.1 per 100,000 in Malaysia (Azizah *et al.*, 2016).

1.3 Breast cancer subtypes

Breast cancer is a heterogenous malignancy and has many subtypes with different biological behaviour, clinic-pathological features and molecular characteristics (Yersal and Barutca, 2014). There are four major molecular subtypes of breast cancer; Oestrogen (ER) positive/luminal, Human epidermal growth receptor 2 (HER2) positive (HER2-enriched), basal-like and normal breast-like. However, recent study on the gene expression profiling revealed that breast cancer can be divided further into luminal A (ER positive and/or Progesterone (PR) positive, HER-2 negative), luminal B (ER positive and/or PR positive, HER-2 positive), HER-2 over expression (ER negative, PR negative, HER-2 positive) basal-like (ER negative, PR negative, HER-2 negative, cytokeratin 5/6 positive and/or epidermal growth factor receptor 1 (HER-1) positive) as well as normal breast-like (Boyle, 2012).

1.4 Breast cancer risk factors

Several epidemiological studies have shown that genetic and environmental or interactions between these two factors play an important role in increasing the risk of breast cancer. Factors such as age, breast pathology, family history and genetic predisposition as well as environmental factors have been reported as risk factors for breast cancer development. However, other factors such as exposure to endogenous and exogenous hormone such as oral contraceptive pills as well as life style related factors also contribute to increased risk of breast cancer development (Abdulkareem, 2013; Gotzsche and Jorgensen, 2013; Shah *et al.*, 2014).

Age, gender and personal history: The risk of developing breast cancer increases with age as well as being a female gender. Breast cancer is uncommon before age of 20 years. A study reported a rate of 10/100,000 for breast cancer in women aged below 25 years and the risk increased up to 100 times in women by the age 45 years (Dumitrescu and Cotarla, 2005). Women have been reported to have a higher risk for breast cancer development as compared to men. However, men who are shown to express ER, PR and androgen receptors as well as men with Klinefelter's syndrome have been reported to have a higher chance of breast cancer development (Murphy *et al.*, 2006). Personal history such as diagnosed DCIS, stage IIB and hormone receptor negative are shown to pose a higher risk of breast cancer (Buist *et al.*, 2010). So, also women who do not breast feed, pregnancy at early age, late menarche and early menopause are also reported to be at higher risk for breast cancer development (Collaborative Group on Hormonal Factors in Breast, 2002; Ritte *et al.*, 2012).

Breast pathology: Proliferative breast lesion without atypia, including usual ductal hyperplasia, intraductal papillomas, sclerosing adenosis and fibroadenomas may increase the risk of breast cancer development. Some studies showed that women with atypia had 4.3 folds higher risk of developing breast cancer as compared to a normal individual (Hartmann *et al.*, 2005; Shah *et al.*, 2014).

Family history: History of breast cancer in the family especially among first/second degree relatives is one of the most important risk factors for the development of breast cancer (Martin *et al.*, 2010a). Women with strong a family history of breast cancer may inherit some gene mutation that might influence the risk of breast cancer

development. In general, women with positive family history show early onset of breast cancer, bilateral breast cancer, advanced stage, positive lymph node, negative hormone receptors and a poorer prognosis (Verkooijen *et al.*, 2006). Genetic factor is the most important factor that contribute to familial breast cancer development. A study showed that, women with inherited mutations in genes such as *BRCA*1, *BRCA2, TP53, PTEN, STK1* had a higher risk for development of familial breast cancer (van der Groep *et al.*, 2011). In fact, women with inherited mutations in *BRCA1* had 40% to 85% lifetime risk of developing breast cancer and 25% to 65% for risk of developing ovarian cancer. On the other hand, those who have inherited *BRCA2* mutation pose similar risk of breast cancer, with an ovarian cancer risk estimated to be 15% to 20% (Pruthi *et al.*, 2010).

Lifestyle and habits: Due to modernization, lifestyle habits are changing. Several epidemiological studies have shown that lack of physical activities, alcohol consumption, tobacco smoking, high fat diet, obesity and high cholesterol may increase the risk of breast cancer development (Khan *et al.*, 2010). Alcohol consumption and tobacco smoking consist of various genotoxic agents such as reactive oxygen species (ROS), polycyclic aromatic hydrocarbon (PAH) and heterocyclic amines (HCA) that will lead to DNA damage in the cell. On the other hand, lack of physical activities and high fat diet increase the ER level in the body and thus increase the risk of breast cancer development and growth.

Endogenous and exogenous exposure: Women who are exposed to ionizing radiation (medical diagnostic or therapeutic) and environmental carcinogens may have

higher risk of breast cancer. In addition, hormone replacement therapy (HRT) and usage of oral contraceptives are reported to increase the risk of breast cancer (Abdulkareem, 2013; Dumitrescu and Cotarla, 2005). Contraceptives pill contain the female sex hormones such ER and progestogens. These hormones prevent pregnancy by stopping the ovaries releasing eggs (called ovulation). However, these hormones can increase the growth of some breast cancers, which might explain why taking the pill slightly increases the risk of breast cancer.

1.5 Screening and diagnosis of breast cancer

Breast self-examination (BSE) and clinical breast examination (CBE) are the first line screening processes for breast cancer detection. Most clinicians encourage women to perform BSE monthly. These techniques will help to detect early signs of breast cancer including the presence of lump in the breast, a change of breast shape, dimpling of the skin, nipple discharge or presence of red scaly patch of the skin. Besides that, bony pain, malaise and loss of weight are other general signs of breast cancer.

Advanced technique using automated machines such as mammography, ultrasound and magnetic resonance imaging are also being used in the screening and diagnosis of breast cancer. Mammography is an important technique in early detection of non-palpable masses. However, routine mammography leads to undue stress and uncertainty in case of false positive results. Ultrasound helps clinician to screen breast cancers that could not be detected by mammography. However, MRI increase the modality in detection, assessment, staging and management of breast cancer (Shah *et al.*, 2014). In addition, mammogram, MRI scan, ultrasound and CT scan are also used

to determine tumour growth and the metastatic spread to the lymph nodes, liver or other organs (Veronesi *et al.*, 2005). For diagnosis, tissue biopsies are taken followed by staining and observation under a microscope. Based on the findings, the tissues, if cancerous will be classified according to the Tumour (T), Nodes (N), Metastasis (M) (TNM) system (see section 1.7).

1.6 Histological staging and grading

In breast cancer staging and grading, TNM system is used based on the size of tumour (T) and whether tumour has spread to the lymph nodes or not (N) or whether the tumour has metastasized to other parts of the body (M). Following breast cancer diagnosis, the patients are clinically staged using the American Joint Commission on Cancer (AJCC) (7^{th} Edition, 2009) guidelines, whereas tumour is histologically graded according to the Scaff Bloom and Richardson (SBR) histological system (Tables 1.1 and 1.2). Tumour grading compares breast cancer cells to the normal breast tissues and can be divided into three types; well differentiated (low grade), moderately differentiated (intermediate grade) and poorly differentiated (high grade) where the cells lose their features as compared to the normal breast cells.

Table 1.1: American Joint Commission on Cancer (7th Edition, 2009) guidelines - tumour node metastasis (TNM) classification

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget's)	Paget's disease of the nipple
T1	Tumour ≤ 20 mm in greatest dimension
T1mi	Tumour ≤ 1 mm in greatest dimension
T1a	Tumour > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumour > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumour > 10 mm but ≤ 20 mm in greatest dimension
T1	Tumour > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumour > 50 mm in greatest dimension
T4	Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or aedema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed (for example, previously removed)
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateralinfraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph node(s) with clinically evident

	level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateralinfraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)
Distant metastases (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0 (i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumour cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

Table 1.2: Clinical staging-American Joint Commission on Cancer (7th Edition, 2009) guidelines

Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0	N1mi	M0
	T1	N1mi	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	Mo
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1.1 and Table 1.2 were adapted from Shah *et al.,* (2014)

1.7 Management and treatment of breast cancer

Breast cancer is curable and treatable if detected early. The management and treatment of breast cancer depends on several factors including physical and biological characteristics of the disease, age, overall health and personal preferences of the patient. In treatment options, breast cancer is usually treated with surgery, chemotherapy, radiotherapy, hormonal and targeted therapies (Kabel and Baali, 2015)

Surgery is the main treatment in breast cancer and depends on the type and stage of tumour. Patients will undergo lumpectomy or mastectomy. Lumpectomy is a surgical procedure in which only the part of the breast containing the cancer is removed, whereas mastectomy is a surgery in which the entire breast is removed, including all the breast tissue and sometimes other nearby tissues. On the other hand, a surgery such as sentinel lymph node biopsy (SLNB) or axillary lymph node dissection (ALSD) can also be done to determine the possibility of whether breast cancer has spread to axillary lymph nodes.

Chemotherapy is a treatment where cancer cells are killed using single or combination of cytotoxic drugs either via intravenous or oral route. Usually, chemotherapy is administered to patients before, after or in place of surgery based on the stage of cases. Neoadjuvant chemotherapy is given to the patients before surgery to reduce or shrink the tumour size before undergoing surgery. On the other hand, adjuvant chemotherapy is given after the patients undergo surgery to improve the disease-free survival (DFS) and overall survival (OS) rates.

Radiotherapy uses high energy rays such as X-ray or other particles to kill cancer cells or to stop the cancer cell from growing. There are two types of radiation given to breast cancer patients 1) external beam radiation and 2) internal radiation. Radiotherapy will be given to breast cancer patients following breast conserving surgery, mastectomy or when cancer cells has spread to other parts of the body.

Hormonal therapy is beneficial for breast cancer patients who are positive for oestrogen and progesterone receptor hormones. The therapy is given after or sometimes before surgery. Oestrogen has been known as an important factor for cancer cell growth and spread. Hormone therapy acts by lowering oestrogen levels or by stopping the oestrogen production, thus reducing the risk of cancer recurrence. It is recommended to breast cancer patients who are hormone positive (ER positive and/or PR positive) where tamoxifen is one of the drugs used in hormonal therapy acting by blocking the oestrogen receptors on breast cancer cells.

Researchers have developed a new drug that can be targeted or specific to the targeted cells or protein by blocking the growth and spread of cancer cells. Targeted therapy is generally less likely than chemotherapy to harm normal, healthy cells. Breast cancer patients who are positive for human epidermal growth factor-2 (HER2) are normally recommended for targeted hormone treatment.

1.8 Breast cancer prevention

Cancer prevention is the way to stop or reduce the risk of cancer development. One of the best ways to combat cancer is to prevent it from occurring by identifying and controlling the factors that may increase the risk for breast cancer development. As stated earlier, genetic and environmental factors or combinations of these two factors are closely related with higher risk of breast cancer.

By routine practice of physical activity and healthy diet, one can reduce the risk of breast cancer. Physical activities can increase the rate of metabolism and oxygen uptake. It can also can increase metabolic efficiency and capacity and help to reduce blood pressure, insulin resistance as well as obesity. Increased intake of vegetables, fruits with high antioxidant and phytochemicals in diet and avoiding fatty foods, cigarette smoking as well as alcohol may also help lower the risk of breast cancer.

Besides that, systematic screening and surveillance programmes could be addressed in order to fight against as well as reduce the mortality of breast cancer. Individuals who have family history of cancer, exposure to environmental carcinogens and common symptoms of breast cancer as mentioned earlier, should be more alertful and should take prudent steps to practise healthy lifestyles by monitoring their healths and undergoing routine medical check-up and cancer screening programmes. Breast cancer is treatable and curable if detected early. Early detection and early treatment may help to reduce the incidence and mortality rate of breast cancer.

1.9 Present study: Problem statement

Triple negative breast cancer (TNBC) is one of the breast cancer subtypes, characterized by lack of ER, PR expressions and no amplification of the HER-2 receptors. This subtype accounts for 15-20% of all breast cancer cases. TNBC is typically associated with high histological grade and stage, aggressive tumour phenotype showing only partial response to chemotherapy with lack of clinical established therapies (Kaplan *et al.*, 2009). Moreover, TNBC is strongly associated with distant recurrence, visceral metastases and death when compared to other breast cancer types (Bauer *et al.*, 2007; Dent *et al.*, 2007). TNBC accounts for 15%-20% of the 500,000-breast cancer associated female deaths worldwide, each year and is considered as an international public health issue (Cancer Genome Atlas, 2012). In TNBC, recurrence often occurs within 1 to 3 years of diagnosis, while 5 years mortality rates appear to be increased following initial diagnosis. The median survival of advanced TNBC is at best 12 months, much lower than the median survival observed in other advanced breast cancer subtypes (Anders and Carey, 2009).

Due to lack of target receptors (ER, PR and HER2) and lack of specific targets for treatment, patients diagnosed with TNBC do not benefit from hormonal or targeted therapy and has a high probability of having early tumour relapse following diagnosis. There has always been continuous search for novel therapeutic targets. Although effective tailored therapies have been developed for patients with hormone positive and HER-2 positive disease, TNBC patients are unlikely to benefit from currently available systemic therapy. At Hospital Universiti Sains Malaysia (Hospital USM) and several other centres, TNBC patients are treated with Taxane, Adriamycin and

Cyclophosphamide (TAC) chemotherapy regimen. TNBC seems to be particularly chemosensitive to taxanes and anthracyclines which is part of the standard regimens used for high risk patients. Since this subtype is associated with high risk of recurrence and metastasis, adjuvant chemotherapy using TAC regimen is given for early TNBC cases after the patients undergo primary surgical resection of their breast tumour and axillary nodes. Nevertheless, TNBC is generally susceptible to chemotherapy initially where early complete response does not correlate with overall survival. The risk of relapse within 3-5 years is higher than other breast cancer subtypes (Cheang *et al.*, 2008; Hudis and Gianni, 2011).

TNBC is particularly lethal when it recurs. Drug resistance or disease recurrence is a major clinical manifestation and is the principle cause of TNBC related deaths. Due to tumour heterogeneity and interindividual differences in TNBC patients, the efficacy and toxicity of TAC chemotherapeutic drugs vary across individuals. As a result, a significant number of TNBC patients fail to respond or acquire resistance to the introduced TAC chemotherapeutic agents that usually leads to a relapse and worsening of prognosis. It has been shown that one out of two patients will fail to respond to initial treatments or will rapidly acquire resistance to chemotherapeutic drugs (O'Driscoll and Clynes, 2006). Since TNBC lacks specific target and clinical therapies, the availability of markers or signals that reliably differentiates between TAC chemotherapy sensitive and resistant patients are required to improve the chemotherapy.

In predicting the disease progression, the prospect of recurrence and treatment response, traditional clinical risk factors such as tumour size, patient's age, regional lymph node spread, and ER receptor status are commonly used (Early Breast Cancer Trialists' Collaborative *et al.*, 2008). However, the information derived from all these parameters are often unreliable in identifying who will respond better with TAC chemotherapy from others and who may end up with poor outcomes and recurrences. The causes for TNBC recurrence remain unknown. There is no acceptable module to investigate drug resistance and disease recurrence in TNBC. Hence, there is a pressing need to identify markers for TAC chemotherapy response in TNBC patients. This study aimed to investigate few putative genetic markers for their possible role in determining the TAC chemotherapy response, recurrence risk and treatment outcomes.

1.9.1 Genetic determinants of variable chemotherapy response and recurrence risk

Once the chemotherapeutic drugs enter a patient's system and transits through the body to interact with cancer cell, the drugs interact with a diversity of molecular entities. During this process, patient's genetics, especially germline-based variations can affect the pharmacokinetics of chemotherapeutic drugs and thus influence the bioavailability of these drugs. Pharmacokinetic variability also accounts for suboptimal therapeutic drug levels which results in decreased response. The factors accounting for suboptimal drug levels include variable metabolizing activities as well as different transporter activities. Genetic variations in the form of single nucleotide polymorphism (SNPs) that affect gene expression or function in individuals can cause interindividual differences in the metabolism and disposition of medications. Genetic variations in

genes encoding drug metabolizing enzymes and drug transporters could affect expression of corresponding proteins and can impact pharmacokinetics of chemotherapeutic drugs in patients and thereby may influence the intracellular delivery, effectiveness and response to those drugs.

To date, genomic assays may be helpful in differentiating interpatient variability in identifying breast cancer patients who are likely to benefit (or not) and also in predicting disease recurrence. Genetic and epigenetic factors have been shown to be attributable to TNBC chemoresistance (O'Reilly *et al.*, 2015; Ouyang *et al.*, 2014). Differences in genetic variation among breast cancer patients have been identified to be important factors that contribute to differences in treatment response. SNPs of pharmacogenes might lead to absence or altered enzyme activity and show an impact on individual's response or resistance to the treatment (Dumont *et al.*, 2015). SNPs may affect drug toxicity and efficacy in a variety of ways. For example, SNPs located in coding sequence of a gene may result in amino acid change that may alter protein function when compared to wildtype protein.

Drug transporters regulate the influx and efflux of drugs in cells. Genetic polymorphisms of transporters can have profound impact on drug disposition, drug efficacy and drug safety and thereby impact pharmacokinetics (Franke *et al.*, 2008). Of particular interest has been on the pharmacogenetic relevance due to genetic variation in efflux transporters of the ATP-binding cassette (ABC) subfamily member B1 (*ABCB1*). As a transporter, *ABCB1* has a broad affinity spectrum for different anticancer drugs including docetaxel, paclitaxel, doxorubicin etc.

Metabolism of a drug can also contribute to therapy resistance. The cytochrome P450 (CYPs) enzymes facilitate the metabolism and elimination of nearly 50% of all chemotherapeutics drugs. In the metabolism of the drugs used in TAC chemotherapy regimen, the *CYP3A4, CYP3A5* and *CYP1B1* play major role. Genetic variations in the above CYP genes also can have profound effect on enzymes activity and thereby contribute to the variations in response to the chemotherapeutic drugs used for TNBC patients.

Because genetic polymorphisms may affect the expression and activity of encoded proteins, it is a key covariate that is responsible for variability in drug metabolism, drug transport and for determining pharmacodynamics of drug effects. Cytochrome P450 (*CYP1B1, CYP3A4* and *CYP3A5*) are drug metabolism enzymes and *ABCB1* is drug efflux transporter that have been widely studied. SNPs in these drug metabolism and transport genes that may alter protein function are suggested to play an important role in determining the effectiveness of drugs and also in determining why some individuals are responsive or resistant to chemotherapeutic drugs. Since no reports are available on the impact of genetic variations in these drug metabolising or transporter genes on TAC chemotherapy response in TNBC patients, it was of interest to investigate whether genetic variations [SNPs] in *CYP1B1* [142 C>G (rs10012), 4326 C>G (rs1056836) and 4390 A>G (rs1800440)], *CYP3A4* [878 T>C (rs28371759)], *CYP3A5* [6986 A>G (rs776746)], *ABCB1* [1236 C>T (rs1128503), 2677 G>T/A (rs2032582) and 3435 C>T (rs1045642)] have any impact in modulating TAC chemotherapy response and treatment outcome in TNBC patients.

Gene expression also plays an important role in chemotherapeutic response. For example, mRNA regulation is a process where the production of specific gene is increased or decreased. Deregulation of gene expression might play an important role in chemotherapeutic response. Alteration in drug metabolizing and transporter genes may reduce or increase the gene expression, and thus inactivate (or activate) the gene function. Hence, it was aimed to determine the mRNA expression levels of *CYP1B1, CYP3A4, CYP3A5* and *ABCB1* in cancerous as well as normal adjacent tissues and to correlate the gene expression levels with TAC chemotherapy response and treatment outcome in TNBC patients.

Another aspect is epigenetic factors which also play important role in pharmacological response of chemotherapeutic drugs. Epigenetic factors such as microRNAs (miRNAs) represents a class of naturally occurring small non-coding RNA molecules. Through down regulating the expression of target genes, miRNAs play an important role in various biological processes including the proliferation, metastasis and chemoresistance of TNBC (Bockhorn *et al.*, 2013). The silencing mechanism by which miRNAs regulate the expression of other genes might be one of the key factors regulating the cell specific expression as well as individual differences in the gene expression. Because of their high stability and specific expression pattern during tumorigenesis and progression, miRNAs have become attractive candidate biomarkers for cancer diagnosis and prognosis and novel target for cancer treatment. It has been shown that alterations in miRNA expression properties can be used to estimate and monitor the success of different therapeutic modalities (Zheng *et al.*, 2010) and hence

miRNA expression are attractive novel aspects which can explain interindividual differences in drug response. Therefore, it was of interest to study few selected miRNAs with possible implications in modulating TAC chemotherapy response, predicting TNBC recurrence and treatment outcome. This study aimed to investigate the expression levels of selected miRNAs (miR-21, miR-27b, miR-34a, miR-182, miR-200c and miR-451) in cancerous and normal adjacent tissues and to correlate the miRNA expression levels with TAC chemotherapy response and treatment outcome in Malaysian TNBC patients.

It was hypothesized that, an individual's genomic profile especially genetic variations such as SNPs, expression of mRNAs and miRNAs within tumour samples may be able to identify characteristics that reflect tumour behaviour, response to treatment, disease progression and treatment outcome. Even though several studies have focused on the role of drug transporter and metabolism genes in genetic susceptibility to cancer, very little progress has been made in the identification of genetic and pharmacogenetic markers in modulating chemoresistance in TNBC patients. Therefore, the availability of genetic determinants that reliably differentiate between the chemotherapy regimen-sensitive and resistant patients would improve breast cancer therapy. Currently, there are no genetic determinants available to predict the response of TNBC patients to TAC chemotherapy before initiation of treatment. It was hoped that this study which focuses on epigenetic (selected miRNAs expression) and pharmacogenetic alterations in drug metabolizing and drug transport enzymes (SNPs and mRNA expression) in TNBC patients undergoing (TAC) chemotherapy regimen might generate new information in this direction.

1.9.2 Objective(s) of the study

The main objective of this study was to investigate the impact of selected SNPs of *CYP1B1, CYP3A4, CYP3A5* and *ABCB1,* their mRNAs expression and expression levels of selected miRNAs in modulating TAC chemotherapy response and treatment outcome among TNBC patients.

The specific objectives were:

1. To investigate the genotype, allele and haplotype frequencies of the following polymorphisms (*CYP1B1* 142 C>G (rs10012), 4326 C>G (rs1056836) and 4390 A>G (rs1800440), *CYP3A4* 878 T>C (rs28371759), *CYP3A5* 6986 A>G (rs776746), *ABCB1* 1236 C>T (rs1128503), 2677 G>T/A (rs2032582) and 3435 C>T (rs1045642) in TNBC patients undergoing chemotherapy with TAC and determine the association of the genotypes and haplotypes of the investigated SNPs with chemotherapy response in TNBC patients.
2. To investigate the mRNA expression level of *CYP1B1*, *CYP3A4*, *CYP3A5*, *ABCB1* in cancerous and non-cancerous tissues of TNBC patients and to determine their association with clinicopathological variables and chemotherapy response
3. To investigate the expression levels miRNAs (miR-21, miR-27b, miR-34a, miR-182, miR-200c and miR-451) in cancerous and non-cancerous tissues of triple negative breast cancer tissue patients and determine their contributory role in modulating chemotherapy response in TNBC patients.

4. To correlate the SNPs genotype pattern, mRNA and miRNA expression levels as well as clinicopathological variables with the relapse-free survival and overall survival of TNBC patients and evaluate their clinical significance.
5. To evaluate whether all the above parameters could serve as predictive biomarkers of chemotherapy response and treatment outcome in TNBC patients undergoing TAC chemotherapy.

1.9.3 Hypothesis

Alternate hypothesis: There is association between candidate SNPs and mRNA expressions of *CYP1B1, CYP3A4, CYP3A5* and *ABCB1* and expression of selected miRNAs with TAC chemotherapy response in TNBC patients.

CHAPTER 2

LITERATURE REVIEW

2.1 Triple negative breast cancer (TNBC)

Breast cancer is a heterogenous disease encompassing a variety of morphological and clinical distincts. As discussed in Chapter 1, breast cancer can be divided into five subtypes; luminal A, luminal B, HER-2 over expression, basal like and normal breast-like. TNBC is defined by the lack of ER, PR expression and no amplification of HER-2 (Elias, 2010). The term TNBC is referred to the immunohistochemically classified breast cancer showing absence of ER, PR and HER-2 protein expressions whereas the basal like subtype is defined by gene expression microarray analysis. TNBC is often but not always basal-like breast cancer (lack of ER, PR and HER-2 expressions), although there is a substantial overlap between TNBC tumour and basal like breast cancer (BLBC). There is significant heterogeneity within these largely overlapping subtypes (Turner *et al.*, 2010). Seventy-five (75%) of BLBC is TNBC subtype and 70% of TNBC is BLBC (Carey *et al.*, 2006). On the other hand, most of BRCA-1 mutant breast cancers are both TNBC and BLBC (Figure 1.3).

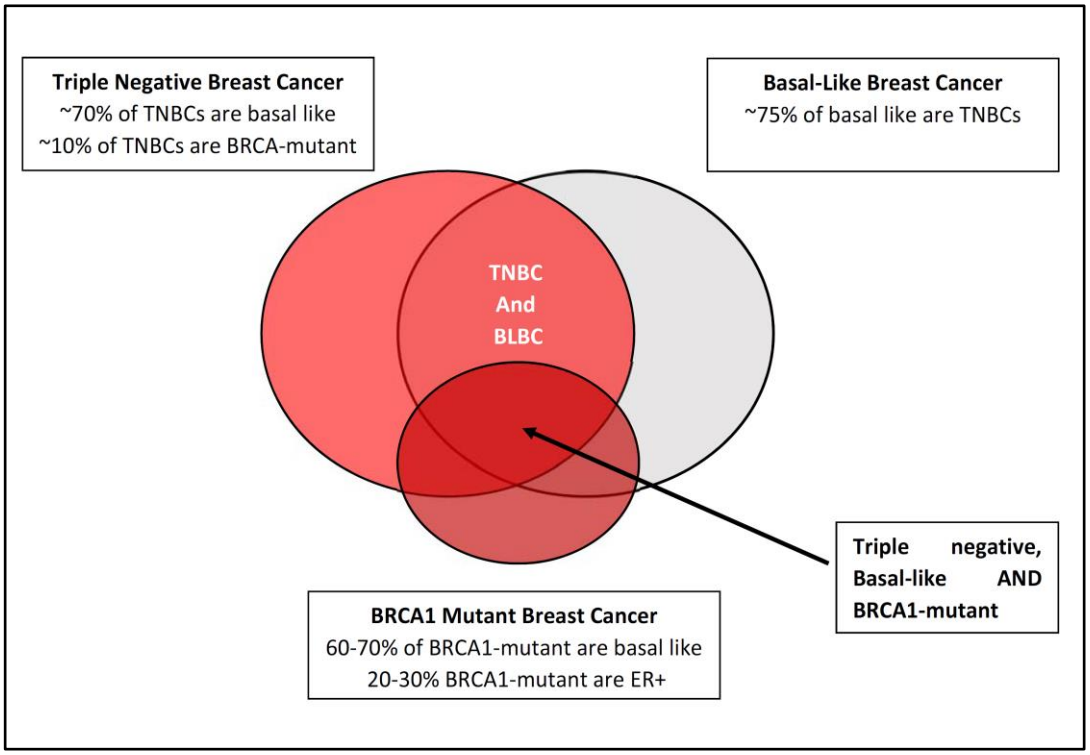

Figure 2.1: Similarities of TNBC, BLBC and BRCA-1 breast cancer (adapted from Hulsen *et al.*, 2008)

2.1.1 Incidence of TNBC

Worldwide, the incidence of TNBC represents approximately 15-20% of newly diagnosed breast cancer cases (Bauer *et al.*, 2007) where 170,000 of TNBC cases are diagnosed annually. The incidence of TNBC is higher in African-American women than other ethnic groups (Whites and Hispanics) (Clarke *et al.*, 2012). The African-American women had two-fold higher (4.1 per 100,000) incidence of TNBC than White (2.2 per 100,000) or Hispanic (2.1 per 100,000) (Amirikia *et al.*, 2011).

In Malaysia, the incidence of TNBC has been reported to range from 12.3% to 17.6% of the total breast cancer cases (Kanapathy Pillai *et al.*, 2012; Tan *et al.*, 2009). In other

population, the incidence of TNBC reported was 11.2% in Canada (Dent *et al.*, 2007), 13.0% in Singapore (Thike *et al.*, 2010), 16.3% in United Kingdom (Rakha *et al.*, 2007), 14.0% in Japan (Nishimura and Arima, 2008), 20.4% in China (Qiu *et al.*, 2016a) and 19.9% in India (Patil *et al.*, 2011). This indicates the disparity in TNBC incidence between the different ethnic groups in different countries.

2.1.2 Risk factors associated with TNBC

Some of the risk factors associated with TNBC development has already been mentioned in Chapter 1. However, there are several other risk factors reported to be associated higher risk of TNBC. TNBC frequently affects younger women. A study has shown that women under the age of 40 years had two-fold higher risk of being diagnosed as TNBC than women over 50 years of age. In addition, the age at diagnosis for TNBC is 5-10 years younger than non-TNBC patients (Newman *et al.*, 2015; Trivers *et al.*, 2009). Moreover, TNBC is reported to be more common in black women as compared to Whites (Trivers *et al.*, 2009). In addition, African-American women had two times higher risk of getting TNBC compared to Whites and Hispanics (Amirikia *et al.*, 2011).

TNBC is overlapped with BRCA1 mutant breast cancer subtype. Thus, women with *BRCA1* mutations have more than 50.0% chance of developing TNBC (Zhang *et al.*, 2012). A study by Gonzalez-Angulo *et al.* (2011) indicated that 20.0% of TNBC patients have positive *BRCA1* mutations. Another study on Mexican TNBC patients showed that 23.0% of the patients had *BRCA1* mutations (*BRCA1* ex9-12del) (Villarreal-Garza *et al.*, 2015). On the other hand, methylation at the promoter region