MECHANISM OF APOPTOTIC CELLS CLEARANCE BY MACROPHAGE IN MCF7 CELLS TREATED WITH METHANOL EXTRACT OF *Centella asiatica* (MECA)

ABDUL WAHAB ALIYU

UNIVERSITI SAINS MALAYSIA

January 2019

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by

ABDUL WAHAB ALIYU

Thesis submitted in partial fulfilment of the requirements

for the degree of

Master of Science (Biomedicine) Mixed Mode

January 2019

ACKNOWLEDGEMENT

All praise are due to Allah, the most gracious, the most merciful. I am immensely grateful to Almighty Allah for giving me the privilege and honour to accomplish this study. I would like to use this medium to forward my sincere regards to those who have markedly contributed to the success of this study. My uncountable appreciation goes to my supervisor, Assoc. Prof. Dr Hasmah Abdullah for providing me with profound academic support through close supervision and encouragement despite her tight schedules. Similarly, my special regards goes to my co-supervisor in the person of Assoc. Prof Dr Rapeah Suppian for her commitment toward the success of this work. I will continue to remain grateful to both of you and continue to derived benefits from your mentorship. My appreciation goes to Assoc. Prof. Dr Few Ling Ling, the coordinator of Biomedicine Mixed Mode for her kind support especially during the time of my application for admission to this programme. Same goes to Assoc. Prof. Dr See Too Wei Cun and other staff of this programme for their support in one way or the other. My apreciation is incomplete without mentioning the effort of my mentor in the person of Prof, A . U Zezi the former Dean of Pharmaceutical Science G. S. U, Sir, I really appreciate your support always. My gratitude goes to my entire family, especially my mother, Hajiya Nana and elder brother Usman Aliyu for their prayers and encouragement until completion of this study. My unique appreciation goes to my beloved wife Fatima Muhammad and my little son Abdul Khaliq for their patients, emotional supports and prayers before and during the course of this struggle. At the end, my profound acknowledgement goes to the Gombe State University and Tertiary Education Trust Fund (Tetfund) for proving me with the sponsorship to undertake this Master programme.

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LIST OF SYMBOLS

A	Alpha
B	Beta
˚C	Degree Celsius
Γ	Gamma
G	Gram
H	Hour
L	Liter
M	Molar
M	Micro
%	Percentage
x *g*	Times gravity
V	Voltage
x	Times
±	Plus or minus
<	Less than

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APAF1	apoptotic protease-activating factor 1
APS	Ammonium per-sulphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
Bax	Bcl-2 associated X protein
Bcl-2	B- cell lymphoma 2
BSC	Biosafety cabinet
CD	Cluster of differentia
CTL A4	Cytotoxic T lymphocytes associated antigen 4
CO_2	Carbon dioxide
COX	Cyclooxygenase
CX3CL1	Chemokine ligand 1
DCs	Dendritic cells
ddH_2O	Deionized distilled water
dH_2O	distilled water
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxy ribonucleic acid
E-cadherin	Epithelial cadherin
ECM	Extra cellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor

EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extra signal regulated kinase
FBS	~~Fetal bovine serum~~
FDA	Food and drug administration
GAS6	Growth arrest-specific 6
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HER2	Human epidermal growth factor receptor 2
HmGB1	Highmobility group box 1
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitrogen
JNK	c-Jun N-terminal kinase
J774A.1	Mouse BALB/C macrophage
kDa	Kilo Dalton
LAG-3	Lymphocyte activation gene-3
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCF7	Michigan cancer foundation-7
MHC	Major histocompatibility complex
MECA	Methanol extract of *centella asiatica*
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
MFG-E8	Milk fat globule-EGF factor 8 protein

ml	Milliliter
mm	Millimeter
MMP	Matrix metalloproteinases
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PD-L	Programmed death-ligand
Pen-Strep	Penicillin streptomycin
pH	Potential hydrogen
PR	Progesterone receptor
ProS1	protein S
PtdSer / PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SPSS	Statistical package for social science
TAMs	Tumor associated macrophages
TAP	Transporters Associated with Antigen Processing
TBS	Tris buffer saline
TBST	Tris buffer saline tween 20
TEMED	Tetramethyl ethylene diamine
TGF-β	Transforming growth factor better
TIM-3	T cell immunoglobulin and mucin domain-3
TLR	Toll like receptor

TNF α	Tumor necrotic factor alpha
Tregs	Regulatory T cells
UK	United Kingdom
USA	United Stat of America
UTP	Uridine triphosphate
VISTA	V-domain Ig suppressor of T cell activation

MEKANISME PENGHAPUSAN SEL APOTOTIK OLEH MAKROFAJ DALAM SEL MCF7 YANG DIRAWAT DENGAN EKSTRAK METANOL *CENTELLA ASIATICA* (MECA)

ABSTRAK

Apoptosis adalah satu proses perkembangan yang dinamik dan merupakan bahagian penting homeostasis dalam haiwan multiselular. Ia berlaku dalam tisu normal dan juga dalam sel yang tidak normal seperti sel kanser payudara apabila terdedah kepada drug kemoterapeutik sitotoksik. Kegagalan penghapusan sel-sel kanser yang mengalami apoptosis pada pesakit menyebabkan komplikasi berkaitan pasca-kemoterapi seperti autoimun. Kajian awal telah membuktikan keupayaan nilai terapeutik ekstrak metanol daripada *Centella asiatica* (MECA) untuk mengubahsuai sel kanser payudara apoptotik oleh makrofaj tetapi mekanisme yang terlibat tidak dicirikan. Oleh itu dalam kajian ini, mekanisme induksi apoptosis dan penghapusan sel kanser payudara apoptotik oleh makrofaj dikaji secara *in vitro*. Dalam kajian ini, sel kanser payudara manusia (MCF7) sahaja atau dalam kultur bersama dengan sel makrofaj murin J774.1A pada nisbah kepelbagaian jangkitan (MOI) 1: 2 telah dirawat dengan kepekatan IC_{50} MECA (13.2µg /ml) dan dinilai untuk induksi apoptosis dan efferositosis sel apoptosis menggunakan SDS-PAGE dan analisa Western blot. Hasil menunjukkan bahawa, MECA menyebabkan apoptosis dalam MCF7 melalui peningkatan ekspresi protein proapoptosis, Bax. Tanpa disangka, protein Bcl-2 juga meningkat berbanding kawalan negatif. Yang menariknya, MECA meningkatkan ekspresi MCF7 apoptotik oleh J774.1A melalui pengaktifan protein ERK1 / 2 yang penting dalam makrofaj transeksual yang dirawat MECA berbanding dengan kumpulan yang tidak dirawat. Hasilnya menunjukkan ekstrak tumbuhan dengan potensi antikanser dan imunomodulator seperti MECA dapat berfungsi sebagai

sebatian utama yang berpotensi untuk pembangunan agen alternatif bagi rawatan kanser payudara tanpa atau minimum kesan sampingan.

MECHANISM OF APOPTOTIC CELLS CLEARANCE BY MACROPHAGE IN MCF7 CELLS TREATED WITH METHANOL EXTRACT OF *CENTELLA ASIATICA* (MECA)

ABSTRACT

Apoptosis is a dynamic developmental process and is an integral part of homeostasis in higher multicellular animals. It takes place in normal tissue and is induced in abnormal cells such as breast cancer using cytotoxic chemotherapeutic agents. Defective clearance of apoptotic cancer cells in patients lead to post-chemotherapy related complication such as autoimmunity. Previous preliminary data have shown the therapeutic value of methanol extract of *Centella asiatica* (MECA) in modulating the clearance of apoptotic breast cancer cells by macrophage but the mechanism involved was not characterized. Thus in this current work, the mechanism of apoptosis induction and clearance of apoptotic breast cancer cells by macrophage were investigated using *in vitro* test system. In this study, human breast cancer cell lines (MCF7) alone or in co-culture with murine macrophage cell line J774.1A at multiplicity of infection (MOI) ratio of 1 : 2 were treated with IC_{50} value of MECA (13.2µg/ml) and evaluated for apoptosis induction and efferocytosis of apoptotic cells using SDS-PAGE and western blot analysis. The result demonstrated that, MECA induced apoptosis in MCF7 through significantly increase proapoptotic Bax expression. Unexpectedly, the Bcl-2 expression also increased as compared to negative control. Interestingly, MECA enhances clearance of apoptotic MCF7 by J774.1A via significant activation of ERK1/2 proteins in MECA treated transfected macrophage compared to untreated group. This is a good sign of minimal post-chemoptheraphy complications. In conclusion, plant materials with both anticancer and immunomodulatory potentials

such as MECA could served as potentials lead compounds for the development of alternative agents for treatment of breast cancer with minimal or no adverse effect.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Breast cancer (BC) is ranked as second most frequently occurring malignant disease across the world with high level of cancer related death annually (Liu and Ho, 2018). Treatment of breast cancer include targeting apoptosis with radiotherapy and chemotherapy to prevent reoccurrence and metastatic dissemination of the disease. However, these approaches lead to marked release of apoptotic cells which increase the burden of apoptotic cells clearance on macrophage and was shown to potentiate the proliferation of viabletumor cell by 40% (Reiter *et al.*, 1999).

Rapid and effective removal of these apoptotic cells is necessary to prevent breaking of immune tolerance of the body system and thus maintain homeostasis (Pinheiro *et al.*, 2017). On the other hand, delay and defective clearance leads to various disorders including autoimmune syndromes and chronic inflammation, a great contributing factor to tumorigenesis (Coussens and Werb, 2002; Yoon *et al.*, 2015). Clearance of apoptotic (both normal and cancerous) cell is the responsibility of innate immune cells called professional phagocyte exemplify by macrophage and dendritic cell via a process called efferocytosis. However non professional neighbouring cells can also perform efferocytosis such as epithelial cell and fibroblasts (Poon *et al.*, 2014).

Drug therapy related problems associated with chemotherapy such as chemoresistance, post-chemotherapy rheumatism have paved the way for searching of other alternatives (Amiri and Rafiei, 2010; Yang *et al.,* 2013). Hence, natural product of plant origin have been receiving attention towards targeting various cancers including cancer of the breast because of their dual anticancer and immune modulation effects coupled with their higher therapeutic value and wider margin of safety (Baraya *et al.*, 2017). A good example is *Centella asiatica* which have shown various biological activities including antimicrobial, neuroprotective, anticancer and immune modulation (Roy *et al.*, 2013). *C. asiatica* have been found to trigger chemotactic movement, phagocytosis and cellular mediated cytotoxicity in human neutrophils against *Candida albicans* (Mali and Hatapakki, 2008). Methanol extracts of *C. asiatica* (MECA) has been shown to induced apoptosis in breast cancer (MCF-7) (Babykutty *et al.*, 2009).

Macrophages exert efferocytosis of apoptotic cells by phagocytosis which mediates anti-inflammatory and immunosuppressive responses (Das *et al.*, 2014). Extracellular signal regulated kinase (ERK) was shown to be involved in regulation of macrophages function including efferocytosis (Jehle et al., 2006; Linton et al., 2016)). ERK1/2 have been shown to mediate phagocytosis of apoptotic neurons by microglia, a nervous tissue resident macrophage (Fu *et al.*, 2014).

Cross talk between Rac and Ras signalling in phagocytic removal of cells undergoing programmed death have been reported by the work of (Osada *et al.*, 2009). Their investigation unveiled that activation of MAPK (ERk and P38) by engulfment adapter protein (GULP) upstream of PdtSer – SR-BI complex formation, leads to

phosphorylation of Rac1 which in turn mediate cytoskeletal repositioning and thus engulfment of apoptotic debris.

Defective clearance of apoptotic cells is the main cause of chronic inflammation and autoimmunity which cause morbidity and disability following chemotherapy of BC. Search for active compound of plant origin with both anticancer and immunomodulatory potentials is increasing and MECA is considered as a promising candidate. Preliminary findings of our group shows that MECA modulates efferocytosis of apoptotic MCF7 cells by macrophage. While ERK signalling is known to mediate efferocytosis, its modulation in macrophage by MECA is not known. Therefore, mechanism of MECA enhanced clearance of apoptotic MCF-7 by macrophage needs to be elucidated.

1.2 Objectives of the study

1.2.1 General objective

To elucidate the mechanism of efferocytosis of MECA treated MCF-7 cells by macrophage

1.2.2 Specific objectives

1. To determine the expression level of pro/anti- apoptotic proteins (Bax /Bcl2) in MCF7 cell treated with MECA by SDS-PAGE and western blot analysis.
2. To determine the expression profile of ERK1/2 proteins in MCF-7 MECA stimulated macrophage by SDS-PAGE and western blot analysis.

1.3 Hypothesis

1. MECA induces apoptosis via mitochondrial pathway. As oberverd in prior experimentation by our research group, MECA induced apoptosis in MCF7. Here we hypothesised that, it could be as a result of damage to DNA probably caused by this plant which may lead to cell cycle arrest for the genetic material to be repaired. If repair process fails due to profound damage to DNA , activation of apoptotic genes may result . This could lead to intrinsic induction of apoptosis which involves participation of mitochondria.

2. MECA modulates efferocytosis of apoptotic MCF-7 cells by macrophage through extracellular signal regulated kinase pathway. ERK is well known for its roles in mediating macrophage functions. Our previous finding shows that MECA enhance clearance of apoptotic MCF7 . Here, we hypothesised that, it could be via modulation of ERK signalling pathway.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease which is defined by progressive and non-stop overgrowth of abnormal malignant cells and tissues. It start at one point in the body but if not control, can spread to other parts, thus overtaking the normal cells of the body with consequential formation of tumor cells which in turn lead to death (Shareef *et al.*, 2016). Cancer is non-communicable diseases and can affect any part of the body. It is cause by many factors, notably among them is damage to the DNA. Defect in tumor related DNA repair mechanism, lead to passage of damage DNA through cell cycle check point. The consequences is development of mutation and genomic instable condition in the newly formed daughter cells. Theses changes confer the daughter cells with cancer characteristics (Bray *et al.*, 2018; Lord and Ashworth, 2012).

2.1.1 Global cancer incidence

With respect to disease causing morbidity and fatality, cancer emerged as the most often reported culprit across the world. As of the year 2015, 17 and half million cases of cancer were recorded across the globe, and the number is projected to rise up by nearly 70% in 20 years to come (Fitzmaurice et al., 2017). Cancer related mortality rate in African continent was estimated to be nearly half (7.2 %) the death rate inAmerica which was found to be 15.8%. Asia was having the highest percentage of cancer-related death with 54.9%. This figure is more than doubled the outcome obtained in the Europe (21.5%)(Ferlay et al., 2015) (Figure 2.1).While the most frequently encountered cancer (about 1.6 million cases) in men was prostate cancer, the number one killer in men was

cancer affecting respiratory tract including bronchial, tracheal and pulmonary cancers (1.2 millions deaths). For female population, breast cancer was the most frequently diagnosed (2.4million) and the leading cause of mortality. On the other hand cancer-related death have shown declining pattern a decade prior to 2015 for gastric oesophageal and chronic myeloid leukaemia (Fitzmaurice et al., 2017).

Its is a serious course for concern that, breast cancer is ranked as number two in the list of commonly occurring cancer throughout the globe and markedly results in profound mortality per twelve-monthly (Liu and Ho, 2018)

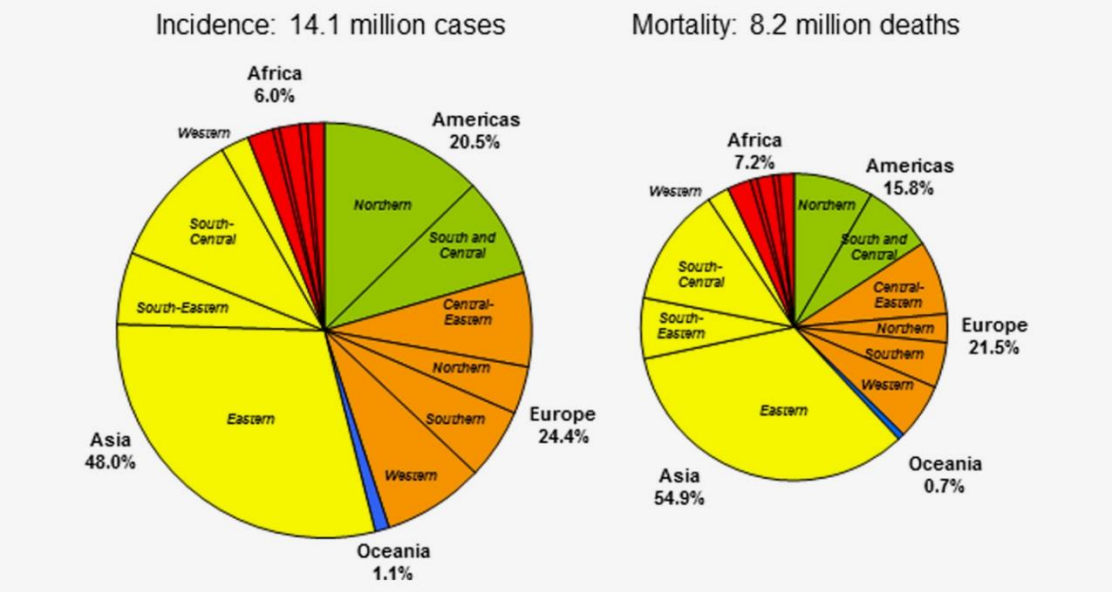

Figure 2. 1 Incidence of cancer continentally adapted from**.** Ferlay et al., (2015)

2.1.2 Breast Cancer

2.1.3 Breast cancer incidence in Malaysia

In both male and female, breast cancer is ranked first in terms of incident cases but third with regard to number of cancer-related death among Malaysian population as of the year 2015 (Fitzmaurice *et al.*, 2017). In female population, breast cancer incidence differ from one ethnic group to another across the ethnicity of Malaysia. Chinese race is topping the list with respect to breast cancer incidence with 59.9 per 100 000 followed by the Indian (54.2 per 100 000) while the Malays ranked the least (34.9 per 100 000) according to three years (2003-2005) report of National Cancer registry. During the year 2012, 5410 recent breast cancer cases were diagnosed and the Age Standardized Rate (ASR) was 38.7 per 100 000 in the same year according to GLOBACAN estimation (Yip *et al.*, 2014). A female in this region has 5 in 100 probability of developing the disease during the course of her life (Dahlui *et al.*, 2011)

2.1.4 Classification of breast cancer

Breast cancer is subdivided at molecular level into different classes base on the pattern and expression profile of biomolecules which included estrogen receptor (ER), progesterone receptor (PR) and human endothelial growth factor receptor 2 (Her2) (Liu and Ho, 2018) into Triple negative, normal-like, Luminal A, HER-2 type, Claudin-low and Luminal B. Table 2.1 gives summary of the distribution of these biomarkers across breast cancer subtypes. Lumina A shows marked expression of ER and PR relative to Lumina B with shows weaker expression of these biomolecules. On the other hand Her 2-type expressed only Her2 receptor while triple negative expressed none hence its name. Like basal-like (triple negative), claudin-low does not expressed any of these biomarker and differ with triple negative in the expression of E-cadherin (lower in

claudin-low) (Mehrgou and Akouchekian, 2016). Molecular classification of breast cancer patients provides room for individualized treatment of these patient and helps in determine the prognosis of this condition in respective cases of breast cancer (Schnitt, 2010)

Table 2. 1 Molecular classification of breast cancer

Molecular subtype	ER expression	PR expression	HER-2 expression
Luminal A	++	++	-
Luminal B	+	+	++
Triple negative	--	-	-
normal-like	++	++	-
HER-2 type	-	-	++
Claudin-low	-	-	-

Key:

++ = strong positive

+ = weak positive

- = negative

2.1.5 Contributing risk factors for breast cancer

Risk factors contributing to the development of breast cancer can be grouped into factors related to life style such as alcoholism, cigarette smoking, consumption of poor diet, food additives and obesity. Second group fall under drug related factors such as oral contraceptives and injectables hormones (Wu *et al.*, 2016). Some risk factors are naturally occurring like advanced aging, family history, frequent uninduced miscarriages and breast density, while others are environmental associated risk factors including occupational exposure to radiation and harmful chemicals (Mehrgou and Akouchekian, 2016).

Profound breast density alone is a powerful contributing risk for the development of larger cancer of the breast with lymph nodes involvement. The risk of encountering cancer of the breast in this case is about six times higher than in female with low dense breast (Duffy *et al.*, 2018). Cigarette smoking is well known for its implication as a factor in development of many diseases including various type of cancers such pulmonary, hepatic, gastric , ovarian, renal and pancreatic tumors (Anand *et al.*, 2008).

Disease condition such as metabolic disorders like in the case of type 2 diabetes mellitus has been implicated to be a potential risk of developing various diseases including breast cancers (Larsson *et al.*, 2007). The possible association between breast cancer and diabetes has been reviewed by Samuel *et al.* (2018), their findings indicated that, diabetic associated derangement in the composition of blood plasma such as high level of lipid, glucose and insulin provide ground for minimal long term inflammatory environment to set in which in turn alters cellular signalling pathways that couple with oxidative stress resulting from toxic effects of high glucose initiate or augment breast cancer growth (Figure 2.1).

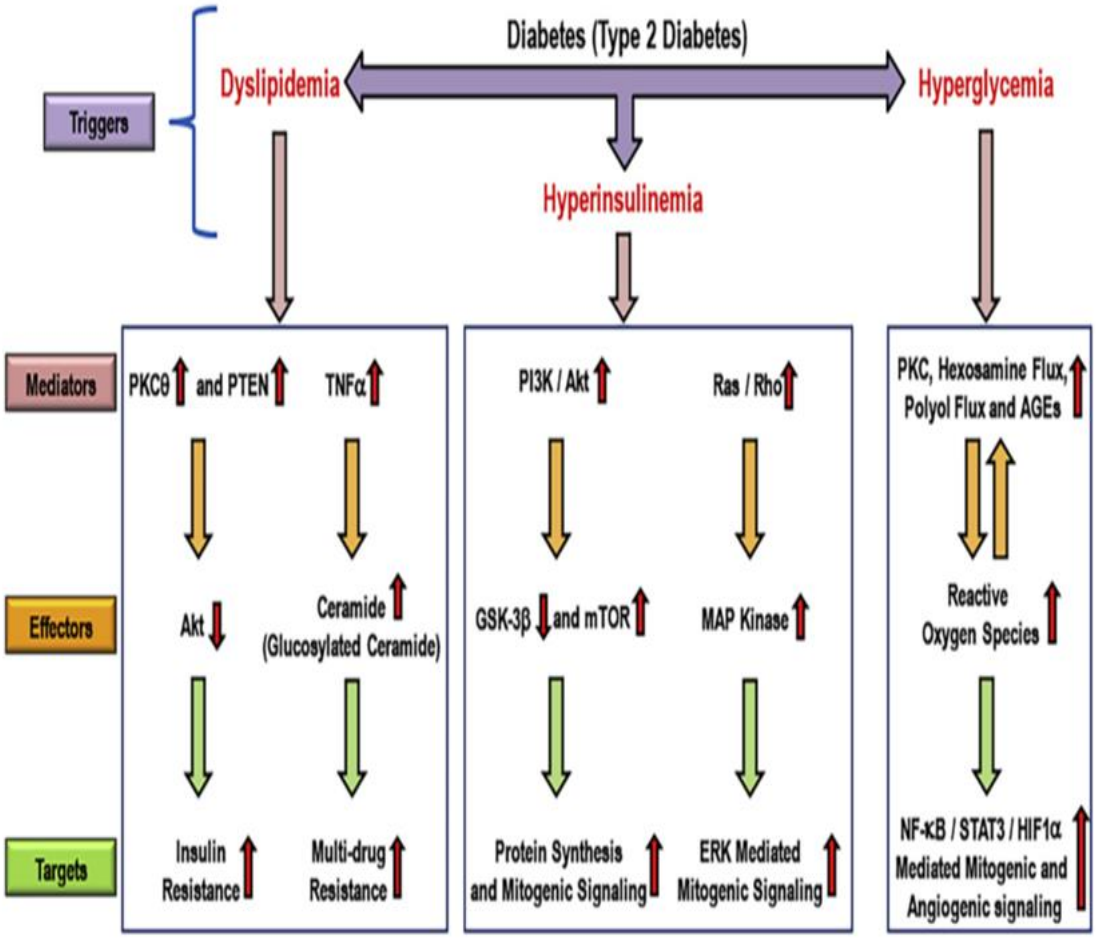

Figure 2.2 Signalling events linking diabetes to breast cancer development and progression. Samuel *et al.* (2018).

2.1.6 Diagnosis of breast cancer

One among other factors that profoundly contribute to the high level of breast cancer related morbidity and mortality, particularly in the third world countries is diagnosis-related problems (inappropriate diagnosis due to lack of adequate breast cancer diagnostic tools and skills) which may lead to misdiagnosed and or late diagnosis (Jemal *et al.*, 2011). Breast cancer diagnosis is a multistep approach starting with patient history taking to assess breast cancer associated risk factors (age, previous medication like contraceptives tablets and or injectables hormone, patient previous history of breast cancer or other cancers), followed by patient physical examination which is vital for the detection of breast cancer at its earlier stage. Imaging procedures are also employed base on the result obtained from patient history and physical investigation. The most commonly employed imaging techniques in breast cancer diagnosis include mammography, breast MRI and breast ultrasound scanning. However the choice of preferable method depends on patient characteristics (Shah *et al.*, 2014).

2.1.7 Management of breast cancer

Currently, the therapeutic approach to breast cancer cure is largely determined by several disease presenting factors like the grade and pathohistological type, stage and molecular subtype status. Other patient related factors such as financial capabilities, preferences and social status also influence treatment plan (Goldhirsch *et al.*, 2013). Therefore precise diagnosis is critical toward defining breast cancer patients according to their relevant clinical groups to pave the way toward actualizing personalized medicine. Many potential pharmaceutical active principles targeting biomolecular

pathways with minimal untoward effects are undergoing clinical testing to improve breast cancer patients quality of life on individual basis (Yersal and Barutca, 2014)

2.1.7.1 Surgery

Surgery remains the mainstay in the therapeutic management of all cancers including that of the breast, in terms of breast cancers, it is called mastectomy and it encompasses the surgical detachment of the breast, superimposing skin, nipple, areolar tissues and axillary node where axillary node is involved. However, the technical procedures involved in mastectomy have witnessed sharp evolutionary advancement (from radical mastectomy to modified radical and now simple mastectomy) due to high demand toward improving patient satisfaction and recent development in technology (Bland et al., 2018).

Incomplete removal (incomplete mastectomy/lumpectomy) is termed breast-conserving therapy (BCT) and is employed at the early manifestation of breast carcinoma. However, the more sophisticated approach is utilized at the later stage of breast cancer which involve the entire removal of the breast. The norms requires BCT to be supplemented with radiation therapy (RT) and this have massively caused the decline in the incidence of breast cancer relapse and breast cancer related mortality (Bodai and Tuso, 2015

2.1.7.2 Radiotherapy

Radiotherapeutic management of breast cancer involves localized irradiation of breast tumor cells with high intensity X-rays with the clinical goal of causing impairment in the helical structure of DNA which in turn can cause the cancerous cells to loose its

apoptotic evading machineries. Concomitant administration of chemotherapy and radiotherapy (chemo-radiation) produced synergistic effects due to chemotherapy enhanced sensitization of tumor cells to radiation induced death (Smith and Prewett, 2017). Radiation treatment of breast cancer have demonstrated positive clinical outcome by decreasing the risk for local relapse by 50% and has shown to be critical for the success of breast cancer cure long ago (Shah *et al.*, 2014).

Currently, breast cancer combination therapy involving ionizing radiation and immunotherapy is under extensive investigation due to promising abscopal effects (ability of localized ionizing radiation to stimulate generalized anti-tumor immunity) and coupled with difficulties in controlling metastatic breast cancer with surgery and radiation treatment alone. Pre-clinical findings demonstrated the potentials of radioimmunotherapy particularly in tumors with decrease immunogenicity such as breast cancers (Hu *et al.*, 2017). Preliminary laboratory testing conducted by Demaria *et al.* (2005) indicated that concomitant administration of Ipilimumab with ionizing radiation prevent pulmonary distal metastasis of breast cancer in rat model.

In a clinical trial conducted by Golden *et al.* (2015), abscopal effect was observed in about 36% (5 out of 14) of metastatic breast cancer patients receiving concomitant radio-chemotherapy and immunotherapy. However there were grade 3-4 associated untoward effects such as fatigue and haematological side effects that were seen in 6 and 10 patients respectively. Thromboembolic events (grade 4 adverse effects) was also observed in one patients

2.1.7.3 Chemotherapy

Chemotherapy one among other forms of systemic treatment of cancers refers to drug regimen that when administered, moves via blood circulatory system to reach it target site of action which in this case is cancer cells. Other forms of systemic therapy exist and include targeted and endocrine therapies.

The anticancer effects of chemotherapeutic agents is as a result of cell cycle stoppage and subsequent cell death. Some regimens interrupt cell when undergoing division (cell cycle specific) while others affect cells regardless of the phase at which the cells are (cell cycle nonspecific) **(**Smith and Prewett**,** 2017**).** The application of chemotherapy for treatment of different cancers including breast cancer lacking estrogen receptor expression , have been in clinical practice for more than five decades. An advantage of this treatment method lies in it applicability both at the earlier and later stage of breast cancers (stage 2 until 4).

Chemotherapy is given alone as a single regimen or in combine form containing more than one chemotherapeutics depending on patient case scenario. A clear example of a single regimen for breast cancer treatment is doxorubicin which was tested clinically since 1960s and was found to be clinically relevant and effective against breast cancers. The current trends employed the use of anthracyclines and taxanes for metastatic, ER negative and stage 2 HER2 responsive breast cancers and this has shown marked decrease in rate of relapse by up to 17% (Shah *et al.*, 2014). For combination regimental therapy, cyclophosphamide + methotrexate + fluorouracil is a good

example. However the benefits derived from combination treatment (survival gain) is at the detriment of patient quality of life (Carrick *et al.*, 2009).

Major setback retarding the popularity of chemotherapy in the management of breast cancer is the undesired effects induced by these agents which deteriorate the quality of life of patient under chemotherapy. These are exemplify by the damage to the organs like heart caused by cyclophosphamide and other agents that subsequently lead to cardiac failure in almost 30% of those who received this regimen. In addition to heart failure, doxorubicin and epirubicin (anthracyclines) also caused cardiomyopathy while taxanes caused ischemic heart disease and arrythmias (Bodai and Tuso, 2015). Other toxic effects of chemotherapeutics are renal failure, asthenia, infertility , onycholysis , anaemia, peripheral neuropathy hyper/hypotension and memory impairment (Smith and Prewett, 2017).

2.1.7.4 Hormonal therapy

Hormonal therapy is another form of systemic therapy employ for subset of breast cancer patient showing marked estrogen and or progesterone hormonal receptor proteins expression and are termed hormone receptor positive breast cancer patient. The extent of positivity in these individuals is indicated by the degree to which ER and PR are upregulated. Majority of cases in postmenopausal female and about half the number of premenopausal women show positive in hormone receptor status (Abraham and Staffurth, 2016).

Breast cancer hormonal therapy is multidimensional and can take up to three different approaches. It could be via inhibition of physiological function of ovaries (ovarian ablation) or through negative regulation of estrogen synthesis and release or via competitive antagonism of estrogen mediated biological response at estrogen receptor site (Ali *et al.*, 2016). Ovarian ablation can be achieved either through physical method involving permanent surgical removal ovaries, chemical method using gonadotropin releasing hormone mimicking agent (gosereline) or radiological approach that involved ablatio by irradiating the ovaries (Abraham and Staffurth, 2011)

Alternative breast cancer hormonal treatment using negative regulators of estrogen production and/or effects are in existence. However the most often used are tamoxifen and aromatase inhibitors. The most widely used competitive inhibitor of estrogen mediated breast cancer progression is Tamoxifen, a selective estrogen receptor modulator (SERM) that mediates its anti-breast cancer activity via antiestrogenic competitive blockade of estrogen-estrogen receptor complex formation. As far back as from 1970s, this agent has gained popularity due to its demonstrated ability of slowing down the associated risk of relapse by 41% and breast cancer-related death by greater than 30% annually in early stage hormone receptor positive disease following the five (5) course of a tamoxifen after mastectomy (Abraham and Staffurth, 2011; Bodai and Tuso, 2015).

Unwanted effects of tamoxifen is a serious course for concern in the adjuvant treatment of breast cancers. These effects are related to the complex nature of tamoxifen mode of activity that varies between different tissue distribution of estrogen receptor.

The most common ones are vaginal dryness, nocturnal sweating, depression, hot flashes and thromboembolism(Abraham and Staffurth, 2011; Bodai and Tuso, 2015)

2.1.7.5 Immunotherapy

The clinical goal of cancer immunotherapy is to sensitize the host defence mechanism to lunch long acting and a powerful immune reaction enough for effective degradation of invasive cancer cells but with little untoward effects. Hence the patient immune status (competency) need to be assessed before commencing this treatment modality in supplementing other approaches for the management of cancers (Finn, 2012). Molecular characterization of breast cancers and it surrounding microenvironment have demonstrated their various immune regulation properties. Apart from nodal status which is vital in defining breast cancer prognosis, HER2/neo (HER2) upregulation is another factor that influence breast cancer relapse and patient survival and thus represent target site for attack by immunotherapeutic regimen. Trastuzumab an anti-HER2 specific monoclonal antibody have been found to prevent relapse by 50%, and elongates survival period of breast cancer patient under this adjuvant breast cancer care (Schneble *et al.*, 2015)

Another way of controlling breast cancer using immune system is via modulation ofThymus cellular antagonistic check point. In this approach, anti-breast cancer immunity is achieved by preparing T cell to lunch anti-tumor attack against breast cancer specific antigens or by controlling immune cell regulatory signalling in such a way that anti-tumor response is favoured (Sanchez *et al.*, 2016). A classic example of this form of medication is typify by ipilimumab, a cytotoxic T lymphocyte

associated antigen 4 (CTLA4) antibody, Nivolumab, a programmed death 1 (PD-1) antibody and its ligand (PD-L1) (McArthur and Page, 2016)

2.1.8 Resistance to breast cancer treatment

A serious cause for concern in the therapeutic management of breast cancer is resistance not only to chemotherapy but also radiation therapy and immune therapy. Breast cancer chemoresistance lead not only to relapse but also distal metastasis.

Numerous research work have revealed the molecular mechanism associated with breast cancer resistance to cytotoxic agents. Among other mechanisms, expression of circulating miR-125b was fond to be linked with the development of chemoresistance (Wang *et al.*, 2012). Similarly, marked expression of biomarker such as CD44 coupled with lack of CD24 expression by breast cancer was found to be related to chemoresistance development. This was demonstrated by Li *et al.* (2008).

Among other factors linked to development of radioresistance, epithelial to mesenchymal transition (EMT) and cancer stem cells (CSCs) are of great concern clinically. Similarly, changes in tumor microenvironment such as loss of adhesive molecules like E-cadrine is among causative agent producing radioresistance (Marie-Egyptienne *et al.*, 2013; Theys *et al.*, 2011). In preclinical testing conducted by Duru *et al.* (2012), the mechanism by which breast cancer cells resist radiotherapy was unveiled. Their findings demonstrated that antiapoptotic signalling network mediated

by HER2 was profoundly contributing to resistance by breast cancer stem cells (BCSCs).

With respect to hormonal therapy, resistance to this form of treatment have limited the effectiveness in terms of tumor regression in ER+ patient with metastatic cases to only 30% with another 20% having long term stable cases. Amplification of EGFR/HER2 signalling was implicated as one of the culprit in producing resistance to endocrine therapy of breast cancers and blockade of the cross talk between ER and EGFR/HER2 path ways has been found to be preclinically and clinically relevant (Osborne and Schiff, 2011).

Regarding the most widely used hormonal agent (tamoxifen) multiple mechanism were identified such as: altered ERα expression, amplification of HER2, MAPK and PI3K signalling among others (Hayes and Lewis-Wambi, 2015).

Even though immunotherapy with trastuzumab in combination therapy have recorded profound success, tackling the threat of adaptive and acquired breast tumor resistance is still a problem (Schneble *et al.*, 2015). Oliveras-Ferraros *et al.* (2010) have demonstrated that resistance to trastuzumab was a result of HER2 upregulation in a basal-like molecular subtype thus producing basal/HER2 + resistance to trastuzumab. Table 2.2 gives the summary of mechanism involve in the development of immuno-resistance to breast cancer immunotherapy.

Table 2. 2 Mechanism of primary and adaptive resistance to immunotherapy adapted from Sharma et al.,(2017)

	Mechanism	Examples
TUMOR CELL INTRINSIC	Absence of antigenic proteins	Low mutational burden
		Lack of viral antigens
		Lack of cancer-testis antigens
		Overlapping surface proteins
	Absence of antigen presentation	Deletion in TAP
		Deletion in B2M
		Silenced HLA
	Genetic T cell exclusion	MAPK oncogenic signalling
		Stabilized b-catenin
		Mesenchymal transition
		Oncogenic PD-L expression
	Insensibility to T cells	Mutation in interferon gamma pathway signalling
TUMOR CELL EXTRINSIC	Absence of T cells	Lack of T cells with tumor antigen-specific TCRs
	Inhibitory immune checkpoints	VISTA, LAG-3, TIM-3
	Immunosuppressive cells	TAMs, Tregs

2.1.9 Test system for breast cancer

Drug research and subsequent development could not be realized without the availability of test model for diseases especially in the field of cancer research. Both in vitro and in vivo test system for cancer research have profoundly contributed toward leads discovery in pre-clinical testing of potential antiproliferative agents. In case of breast cancer research, numerous human breast cancer cell lines have been utilized for nearly eight decades. While BT-20 have been used since 1950s, 75% of all pharmacological screening of potential anticancer agents were carried out using MCF7, MDA-MB-231 and T-47D from 1970s until today (Holliday and Speirs, 2011). For In vivo breast cancer experimentation, xenografts and /or genetically engineered mouse (GEM) are used. While xenografts is obtained by transplanting human breast cancer cells into appropriate host such as mice, GEM model is produced via induction of mutation in genetic materials of mice in such a way that all pathological features of breast cancer developed in mice. The later and the former murine models have been used for long to determine contributing factors in the event of breast cancer invasiveness and to screen potential agent with anticancer activity (Richmond and Su, 2008)

2.2 Tumor microenvironment of breast cancer

An essential factor which influences tumor pathophysiology is it microenvironment as it provide ground for tumor cell growth, tumor cell defence, tumor progression and metastasis (Mbeunkui and Johann, 2009). Even though the component that made up of tumor cellular environment differ from one tumor type to another, many characteristics remained the same for all solid tumor. The component that made up of this cellular

environment are: immune cells, both blood and lymphatic vessels, fibroblasts, bone marrow-derived inflammatory cells and extracellular matrix (Joyce and Fearon, 2015; Turley *et al.*, 2015).

In the case of breast cancer microenvironment, high level of innate and adaptive immune cells infiltrate the breast cancer environ conferring breast cancer with either antitumor or pro-tumor immunity depending on their bidirectional interaction with one another(Gajewski *et al.*, 2013; Loi *et al.*, 2013).

2.2.1 Cancer and immune system relationship

The immune cells interact with one another and with other cell types to guard the body against various forms of xenobiotics while ensuring immunotolerance to self-antigens. Cancer cells though endogenous to the body need to be eliminated due to their harmful nature to other tissue including normal function of immune system at the detriment of the body system. To maintain normal tissue homeostasis, the immune cells need to be prepared toward neoplastic cells recognition and subsequent elimination thereby limiting neoplastic progression and metastasis thus producing durable responses (Sharma *et al.*, 2017).

Adaptive immunity and innate immunity are the two components of immune system with distinct cellular subtypes and different selectivity. Innate immunity is the first line of defence mechanism against pathogens and is conferred by macrophages, natural killer (NK) cells, basophils, dendritic cells (DCs), eosinophils, Neutrophils and mast cells which constitute cellular arm of innate immune system, physical,