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Al-Quds University

Breast Cancer in Palestine: Expression of the Protein Activation Induced Cytidine Deaminase (AID)

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M.Sc. Thesis

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Thesis Approval

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Dedication

To my mother and father…

To my soul mate Yara and Omar…

To my Husband Ayman Al Mu'ti…

And to the rest of my family…

Without their encouragement, support and inspiration nothing could be accomplished.

Manar Mahmoud Rahhal

Declaration

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: ----------

Manar Mahmoud Mustafa Rahhal

Date: January 2, 2017

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Abstract

Breast Cancer is the most common cancer among women worldwide with an estimated more than one million new cases every year, and it is the leading cancer-related cause of death among women. In Palestine, breast cancer is the most common malignancy in total population and the most common adult female malignancy according to the Cancer Registry centers (CRC) and Palestinian Ministry of Health (MOH).

Activation induced cystidine deaminase (AID) is a member of APOBEC family of cystidine deaminase which deaminate cytosine in DNA leading to uracil. AID is an essential enzyme normally expressed in B cells. AID enzyme is necessary in diversifying the immune repertoire by two main processes; the first one is Class Switch Recombination (CSR), the second process is Somatic Hypermutation (SHM) which allows the immune system to adapt its response to any threats during the lifetime of an organism. Aberrant expression of AID causes genomic instability and induces mutations in multiple genes by favoring chromosome translocations and point mutations in both B and non-B cells, thereby stimulating cancer formation. AID expression has been reported in numerous cancers of non-B-cell origin, including breast, prostate, stomach, liver, and lung cancer.

This study aims to screen the expression of AID protein in tissue sections of breast cancer in patients from Palestine and to determine the correlation between AID protein expression and the clinico-pathological data of these patients. To achieve this, paraffin blocks of 69 breast cancer with their clinico-pathological data were collected retrospectively, between years (2009-2013) from the pathology department of Beit Jala Hospital. The expression levels of AID protein were examined in these cases by Immunohistochemistry (IHC) analysis.

Our results showed an aberrant AID expression in 26.1% (18 of 69) of the breast cancer cases. No statistical correlation was observed between AID protein expression and clinico-pathological data of the patients such as age, tumor grade and tumor stage ($p>0.05$). Our study is the first one that examines AID protein expression protein in breast cancer tissue samples in Palestine and in the world. In conclusion, our study showed aberrant expression of AID protein in Palestinian breast cancer patients, and this study will shed the light on the cancer research in Palestine.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
AID	Activated Induced Cystidine Deaminase
AJCC	American Joint Committee of Cancer
APOBEC	Apolipoprotein B m-RNA-editing enzyme-catalytic polypeptide-like
BC	Breast Cancer
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
CSR	Class Switch Recombination
DAB	3', 3’ Diaminobenzidine
DNA	Deoxyribonucleic Acid
DW	Distilled Water
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ES	Embryonic Stem cell
F	Female
H&E	Hematoxylene and Eosin
HER-2	Human Epidermal growth factor receptor 2
HSM	Hyper Somatic Mutation
H. pylori	Helicobacter Pylori
Ig	Immunoglobulin

IHC	Immunohistochemistry
IL	Interleukin
IDC	Invasive Ductal Carcinoma
MRI	Magnetic Resonance Imaging
miRNA	Micro RNA
NF-KB	Nuclear Factor Kappa-light-chain enhancer of Activated B cells
NLS	Nuclear Localization Signal
PR	Progesterone Receptor
RNA	Ribonucleic Acid
SHM	Somatic Hypermutation
TBS	Tris Buffer Saline
TNF	Tumor Necrosis Factor

Chapter One: Introduction

1.1. Breast Cancer:

Cancer has always been a big challenge to human health; not only it reduces the life quality but also increases mortality. Breast cancer (BC) is the most common cancer among women worldwide and the second most common cause of cancer related mortality in women due to recurrence and metastasis (Limame et al., 2014). Breast cancer is a heterogeneous malignant proliferation of epithelial cells lining the milk ducts (Liu et al., 2015). During the last decade, research has focused on the molecular biology of this disease. All cancer types including breast cancer involve genetic alterations that lead to loss of function in tumor suppressor genes and/or gain of function in oncogenes (Eroles et al., 2011).

1.1.1 Epidemiology of Breast Cancer:

BC is the most common cancer among women worldwide with an estimated 1.38 million new cases diagnosed in 2008 (Ferlay et al., 2010).

In Palestine, BC is the most common cancer with an incidence rate of 16.9 % from all reported cancer cases. BC is the most abundant in females (Abdeen, 2006), while colon cancer is the second (9.9%) from all reported cancers. Stomach cancer was in third place among females with rate (9.0%) of the all reported cases as shown in figure 1 (Ministry of Health, PHIC, Health Annual Report Palestine, 2014).

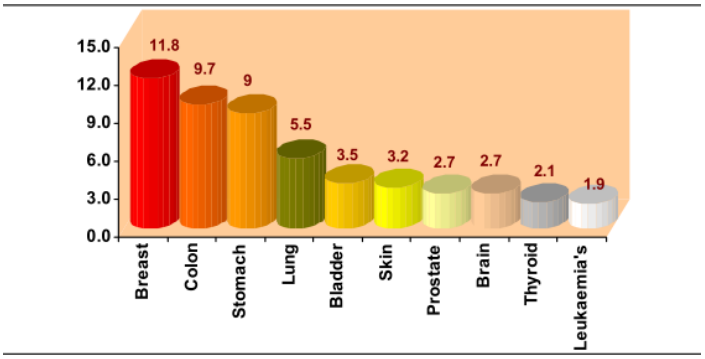


Figure 1: Most Common Cancer Cases among females in West Bank, Palestine, 2011.

(Ministry of Health, Palestinian Health Information Center PHIC, Health Status in Palestine, 2011)

As for mortality rates, the most common cancer mortality in Palestine is bronchus and lung cancer (15.4%) in males. Among females breast cancer was the first leading cause of death

(21.1%) with a mortality rate of 5.2 per 100.000 females. (Abdeen, 2006; Ministry of Health Palestinian Health Information Center PHIC, 2002).

1.1.2 Risk Factors of Breast Cancer

Breast cancer is a multifactorial disease; many factors influence the occurrence of BC. The major risk factor is gender. Although BC affects both males and females, the incidence for women is much higher; females are at 100 fold higher risk than males (Kreiter et al., 2014). Breast cancer incidence increases with age; the older the woman, the higher her risk, doubling about every 10 years until the menopause. It is rare before the age of 25 years old, and increasing between the ages of 30-49 and after 50years old. Family history increases breast cancer risk, women with first degree relatives having the disease like (mother, sister or daughter) have double the risk to get BC. Moreover, genetic predisposition has an important role as 15% of breast cancer patients report family history of breast and ovarian cancer. Gene defects affect breast cancer risk. For example, *BRCA1* and *BRCA2* genes are the most significant genes associating strongly with breast cancer risk. Mutated *BRCA1* and *BRCA2* genes affect biological functions including DNA repair. Other gene mutations may increase the breast cancer risk like: *ATM, P53, CHEK2, PTEN, CDH1, STK11*. Life style factors affect the breast cancer risk like taking birth control pills, hormonal therapy after menopause, age at menopause, breastfeeding, obesity, alcohol drinking, smoking, physical activity and radiation exposure (McPherson et al., 2000; Shah et al., 2014; Howell et al., 2014; Yip et al., 2014).

1.1.3 Types of Breast Cancer

According to the American Cancer Society most breast cancer cases are epithelial in origin, and are called carcinomas which arise from the epithelial cells that line breast ducts and lobules. Other types called Sarcoma which starts in the muscle or fat cells or connective tissues. Breast tissue is made up of ducts and lobules so breast cancer is classified depending on its histological appearance into ductal and lobular carcinomas as shown in figure 2.

The most common types of breast cancer are named according to the parts of the breast in which they start**:**

In situ Ductal Carcinoma:

Found in the early stages of breast cancer, it is treatable since it is not invasive, but if undetected or treated it can start to spread to other parts of the breast.

Invasive Ductal Carcinoma (IDC):

Invasive ductal carcinoma is the most common type of breast cancer, also called "infiltrative ductal carcinoma", in this type the cancer cells formed in the milk ducts and start to spread into other parts of the breast or to other organs.

Lobular Carcinoma:

Begins in the lobes, or glands which produce milk in the breast. The lobes are located deeper inside the breast, under the ducts. About 8% of breast cancers are lobular. If the cancer is LCIS (lobular carcinoma in situ) that means the cancer is limited within the lobe and has not spread.

There are other types like Inflammatory Breast cancer which is the least common but usually invasive and most aggressive, and Metastatic breast cancer when cancer cells start to spread to body organs such as lungs, bones, liver and brain (Bombonati and Sgroi, 2011) Other histological types of BC are rare and include mucinous, tubular, comedo, inflammatory, medullary, and papillary carcinoma. These types of breast cancer also differ in their clinical and tumor characteristics (Li et al., 2005)

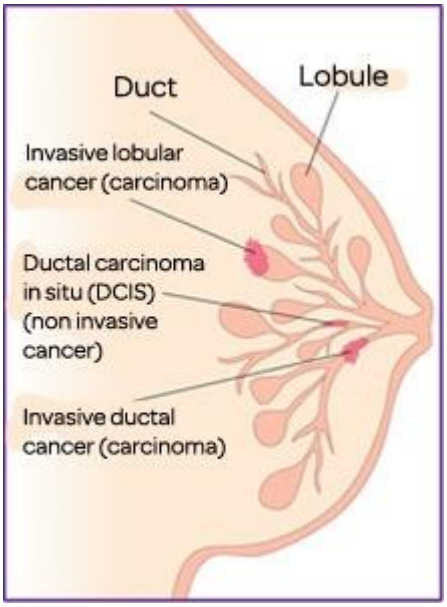


Figure2: Breast Cancer Types. (Breast Cancer Care Organization)

1.1.4 Stages of Breast Cancer:

There is a system of categorizing cancer into stages, based on three factors. The staging system most commonly used is the TNM which means (Tumor size, Node involvement, Metastasis) system. It is suggested by the American Joint Committee of Cancer (AJCC).

TNM staging system is classified according to the primary tumor characteristics (T1-T4), the involvement of lymph nodes (N0-N3) and the presence or absence of metastasis (M1-M0) as shown in the figure 3 below.

Tumor size **T**	Tumor size < 2 cm 	Tumor size 2-5 cm 	Tumor size > 5 cm 	Tumor extends to skin or chest wall 
Lymph Nodes **N**	**N0** No lymph node metastasis	**N1** Metastasis to ipsilateral, movable, axillary LNs	**N2** Metastasis to ipsilateral fixed axillary, or IM LNs	**N3** Metastasis to infraclavicular/ supraclavicular LN, or to axillary and IM LNs
Metastasis **M**	**M0** No distant metastasis	**M1** Distant metastasis	LNs= Lymph Nodes; IM= Internal Mammary	

Table 1 : TNM staging of Breast Cancer adopted from AJCC.

1.1.5 Signs and Symptoms of Breast Cancer:

According to the American cancer society the most common sign of breast cancer is the lump, usually detected on a screening mammogram before it can be felt by the woman. Other symptoms include changes in the breast like thickening, swelling, skin irritation and nipple abnormalities such as changing position, shape or rash around the nipple. Late signs appears when cancer grows or spreads to other parts of the body like bone pain, nausea, headache, weight loss, and cough.

1.1.6 Diagnosis of Breast Cancer:

Early diagnosis can lead to successful treatment of breast cancer. Molecular imaging is the most powerful technique in clinical diagnosis of breast cancer (Alcantara et al., 2014). BC can be detected using the followed tests:

1- Physical examination is very important, to detect skin changes such as; dimpling, erythema, nipple changes, asymmetry and obvious masses (Shah et al., 2014).

2- Diagnostic imaging have central role in the diagnosis, treatment, and staging of patients with breast cancer. Recently mammogram is the most common method for BC diagnosis, it is used to produce fine detail images for the breast to detect early breast cancer, it has been developed over the past years to be able to use lower doses of radiation (Esserman et al., 2002).

3- Ultrasound complement mammogram is a very common diagnostic tool in young women who have low risk of breast cancer; also it is the first diagnostic technique in pregnant and breastfeeding women (Alcantara et al., 2014). It is useful for identifying suspicious areas to be biopsied to confirm the diagnosis of breast cancer (Yamauchi et al., 2012). Recently used in BC screening for women > 40 years old.

4- Magnetic resonance imaging (MRI). It may reveal a mass, architectural distortion, or calcifications in the breast (Yamauchi et al., 2012). It uses magnetic fields to measure the tumor size and how much the tumor has grown after a woman has been diagnosed with BC, the MRI sensitivity of invasive ductal carcinoma is nearly 100%. Breast MRI is useful in some specific situations, including; obese women, women with dense breasts and for cancers that are invasive and adjacent to the chest wall, to evaluate tumor extension and muscle involvement if that will help determine surgical approach. (Li et al., 2014).

5- Surgical tests like biopsy which is the removal of small amount of breast tissue for examination under a microscope and analyzed by a pathologist can make definite diagnosis. It is used as traditional diagnosis for impalpable breast cancer, and the results help to guide treatment recommendations. (Liberman et al., 1996).

After diagnosis the patients will have one of the following BC types:

- Endocrine receptor-positive (estrogen or progesterone receptors)
- HER2-positive. Human epidermal growth factor receptor-2.
- Triple positive: positive for estrogen receptors, progesterone receptors, and HER2

- Triple negative: not positive for estrogen receptors, progesterone receptors, and HER2.

1.1.7 Treatment of Breast Cancer:

Breast Cancer is potentially curable if detected in the early stages. The treatment goal is to control the cancer locally and prevent its spread (Cardonick, 2014). Historically, single-modality treatment to cure BC was not successful, the majority of patients developed recurrent or metastatic disease within two years. Therefore, combinations of adjuvant systemic chemotherapy, surgery, and radiation therapy have led to an improved prognosis (Yamauchi et al., 2012 ; Yip et al., 2014).

The appropriate adjuvant therapy is determined by many factors like tumor size, grade, measures of proliferation, presence of lymphovascular invasion and human epidermal growth factor receptor 2 (HER-2) overexpression (Cianfrocca and Gradishar, 2005). Surgery is an option that should always be considered regardless age of the patient because it has been shown to offer advantages in terms of survival and local disease control (Swaminathan et al., 2012).

Endocrine therapy reduces the recurrence and mortality for hormone receptor positive patients. Radiation helps to get rid of loco-original microscopic disease in the breast, chest wall, skin and lymph nodes, it is used also to treat metastatic breast cancer that has spread to other areas like bones or brain (Rampurwala et al., 2014).

Two main adjuvant therapy modalities are cytotoxic chemotherapy and endocrine therapy (hormone receptor blocking therapy). Both adjuvant treatment modalities improve the overall survival in hormone receptor positive breast cancer patients (Westbrook and Stearns, 2013 ; Miller et al., 2014).

1.1.8 Molecular Markers of Breast Cancer:

Molecular markers are important tools for understanding patient clinical outcome and to ensure that cancer patients receive optimal treatment and to determine the future therapy plans. Most of the molecular markers have been both prognostic and of great predictive value (Taneja et al., 2010).The most common classical molecular markers used for BC screening and diagnosis are receptors which are proteins that help cells communicate with the outside. In breast cancer, if one of these receptors is overproduced it will trigger breast cells to grow and divide rapidly, which eventually leads to the development of a tumor or cancerous growth.

Hormone receptor: Estrogen receptor (ER) and progesterone receptor (PR) are strong predictive factors for response to endocrine therapy, they are assessed by immunohistochemistry. About 70-80% of all breast cancers are ER-positive and 60-65% are PR –positive (Weigel and Dowsett, 2010; Shah et al., 2014).

Human epidermal growth factor receptor-2 (HER-2): The oncogene HER2 was first identified to be an indicator of patient's prognosis. HER2 overproduction stimulate breast cancer cells to grow and divide rapidly leading to tumor growth as shown in figure 3 (Fitzgibbons et al., 2000; Taneja et al., 2010).

HER2 is a predictive marker that predicts the response to certain therapies which target the HER-2/neu receptor. Patients with overexpression of HER2 (HER2 positive), unfortunately are more likely to suffer from relapse and tend to have a worse overall survival (Fitzgibbons et al., 2000; Taneja et al., 2010).

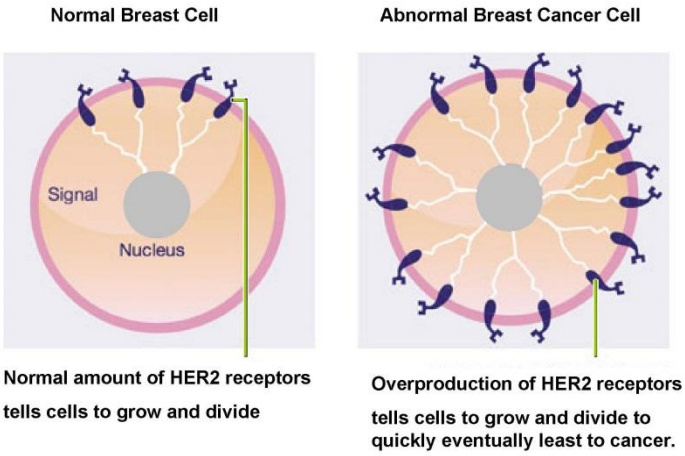


Figure 3: HER2 overexpression in Breast Cancer (WordPress.com)

Most breast cancers have one or more of these receptors overexpressed. However one type, called triple negative breast cancer does not have any of them. Triple negative breast cancers seem to be more difficult to treat and have a higher chance of recurrence (Phipps et al., 2011).

Ki-67: Nuclear antigen proliferative marker, common as prognostic marker for early stage breast cancer patients, because it plays an important role in cell proliferation. Ki-67 overexpression in more than 20-50% of the cells indicates that breast cancer patients are at high risk of recurrent disease. (Esteva and Hortobagyi, 2004; Taneja et al., 2010). Ki-67

used as prognostic factor provides useful information for the therapeutic decisions in breast cancer patients (Luporsi et al., 2012).

1.1.9 Breast Cancer Molecular Heterogeneity

Breast cancers exhibit tumor heterogeneity; it is reflected in the wide morphological and molecular variation, with a spectrum of histological features and molecular pathological markers that are useful in predicting clinical outcome and determining the appropriate treatment plan (Esserman et al., 2011).

The heterogeneity of cancer cells introduces significant challenges in designing effective treatment strategies to yield higher efficacy therapeutic approaches; Hormonal receptor status; Estrogen receptor (ER)/Progesterone receptor (PR) positivity defines which tumor can be targetable by hormonal therapy. Likewise (HER2) amplification or overexpression determines if the tumor will be targetable with HER2 antagonists. Tumors that are negative for ER, PR and HER2 (Triple Negative) are not amenable to these targeted therapies, and often related to poor prognosis (Kwei et al., 2010).

1.1.10 Genetic Alterations of Breast Cancer:

Genetic background plays an important role in tumor susceptibility, because the major cause of all types of cancer is genetic alterations. Many genes contribute in breast cancer development when they are genetically altered, the most common genes when mutated increase the risk of developing breast cancer are: breast cancer susceptibility gene 1 (*BRCA1),* breast cancer susceptibility gene 2 (*BRCA2*), *P53, PTEN, CHEK2*, *ATM*, and *STKII*. In addition to these genes SNPs in *TGF-B1, CASP8* and *PGR* associated strongly with breast cancer risk (Shukla et al., 2014).

The two major genes associated with tumor susceptibility in breast are *BRCA1* and *BRCA2* as shown in Figure 4 (Wooster and Webber., 2003). Many genetic and epigenetic changes are involved in breast cancer development and progression such as somatic gene mutations, copy number aberrations, and miRNA expression (Byler et al., 2014).

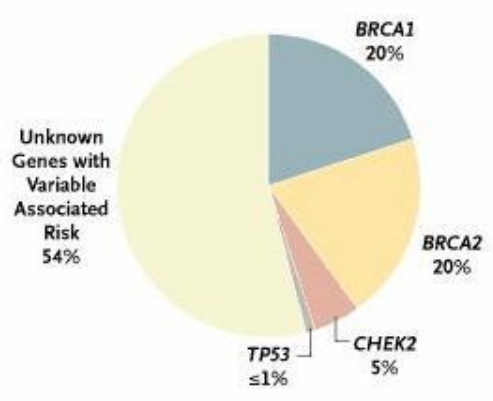


Figure 4: The Genetics of Breast Cancer (Wooster and Webber, 2003).

1.1.10.1 Loss of Tumor Suppressor Genes:

The most common tumor suppressor genes which are associated with Breast cancer include *P53, BRCA1, BRCA2* and *CHEK2* genes.

High life time risk is associated with mutations in the high penetrance genes like *p53, BRCA1, BRCA2* and *CHEK2* genes:

- *P53* is a tumor suppressor gene that is produced in response to DNA damage, resulting in cell cycle arrest until damage is repaired or apoptosis takes place. It is considered the "Guardian of the cell", mutations in this gene decreases its activity, missense mutations in *TP53* are more frequent in high-grade spontaneous breast carcinomas suggesting that early *p53* mutations might be one of the critical and decisive events in the development of breast cancer (Walerych et al., 2012).
- *BRCA1* and *BRCA2* are tumor suppressor genes located on chromosome 17 and 13 respectively. They are important for ensuring the stability of the genetic materials in the cells because they produce tumor suppressor proteins to help repair damaged DNA. Mutations or alterations in *BRCA1* and *BRCA2* increase the risk of Breast and Ovarian cancer (Larsen et al., 2014). Breast cancer risk estimates ranging from 59%-87% for *BRCA1*, and 38%-80% for *BRCA2* mutations (Betty A.Mincey, 2003)
- The *CHEK2* gene provides instructions for making a protein called checkpoint kinase 2 (CHK2). This protein acts as a tumor suppressor so it regulates cell division by preventing cells from keeping growing and dividing in an uncontrolled way. The CHK2 protein is activated in response to DNA damage; it interacts with several other proteins, including tumor protein TP53 to stop cell division and determine whether a cell will repair the damage or enter apoptosis (Desrichard et al., 2011). Mutations in the *CHEK2* gene have been identified in some cases of breast cancer. A well

characterized mutation is a deletion of a single nucleotide at position 1100 in the *CHEK2* gene (known as 1100delC). The 1100delC mutation is a breast cancer predisposition allele that leads to the production of an abnormally short, nonfunctional version of the CHK2 protein. So the cells can divide without control leading to develop cancerous tumors (Nevanlinna and Bartek, 2006; Desrichard et al., 2011).

1.1.10.2. Expression of Oncogenes:

Activation of oncogenes contribute to the development of cancer, the most common oncogenes that have been associated with breast cancer are HER2, c-myc and Cyclines:

HER2 oncogene: Human epithelial receptor 2 gene is located on chromosome 17 and encodes a transmembrane tyrosine kinase growth factor. HER2 overexpression has been shown to play an important role in the development and progression of certain aggressive types of breast cancer like invasive breast cancer. It is present in approximately 20–30% of breast cancer tumors. HER2 overexpression is associated with a more aggressive disease, higher recurrence rate, and shortened survival (Mitri et al., 2012)

c-MYC oncogene: A regulator gene that codes for a transcription factor. It is involved in cellular proliferation and apoptosis. Amplification and overexpression of MYC oncogenes are associated with breast cancer in about 15%-25% of breast cancer (Osborne et al., 2004).

Cyclines and cell cycle modulators: Cyclines are regulators of cell cycle and proliferation process. Cyclin D1 and cyclin E play an important role in the progression of the cell from G1 to S phase, cyclin D1 is overexpressed in 40%-50% of invasive breast cancer (Osborne et al., 2004).

1.1.10.3 Chromosomal Aberrations

Chromosomal aberrations are one of the genetic abnormalities which have a role in the development of breast cancer. These aberrations contain known oncogenes and tumor suppressor genes (Kytola et al., 2000). For example, the amplification of MYC and CCND1 (which encode cyclinD1) plays a key role in developing drug resistance.

There are many well established chromosomal abnormalities described in literature such as numerous gains and losses, including:

- Recurrent DNA amplifications of many oncogenes; at 8p12 (FGFR1), 8q24 (MYC), 11q13 (CCND1), 12q13 (MDM2), and 17q12 (HER2) (Kwei et al., 2010; Korkola and Gray, 2010).
- Loss of specific regions of the chromosomes, such as deletion which has an important role in inactivation of tumor suppressor genes like *PTEN*, *BRCA1* (17q21) deletion, *BRCA2*, and *p53* (Albertson et al., 2003).Another common copy number deletion polymorphism in the APOBEC3 gene cluster located on chromosome 22 that has been associated with an elevated risk of breast cancer (Nik-Zainal et al., 2014).
- The most common cytogenetic aberrations in breast cancer involve chromosome arms 1q, 3p, and 6q, and translocation in chromosomes 8, 1, 17, 16 and 20 (Kytola et al., 2000).

Aberration in chromosome 8 is very common in many solid tumors including loss of 8p and gain of 8q, and involved in various translocations with other chromosomes. These alterations reflect the importance of chromosome 8 in the pathogenesis of breast cancer because it contains important oncogenes like *c-MYC* (Rummukainen et al., 2001).

1.2 Activation induced cytidine deaminase (AID)

Activation induced cytidine deaminase AID is a member of APOBEC family of cytidine deaminase which deaminate cytidine in DNA leading to uridine (Figure 5-b). APOBEC is the Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (Takai et al., 2011; Michel Cogne., 2013). In humans, APOBEC family includes 11 members: APOBEC1, APOBEC2, series of seven APOBEC3 genes (A-C, DE, and F-H) , APOBEC4 and AID as shown in Figure (6) (Goila-Gaur and Strebel, 2008; Metzner et al., 2012).

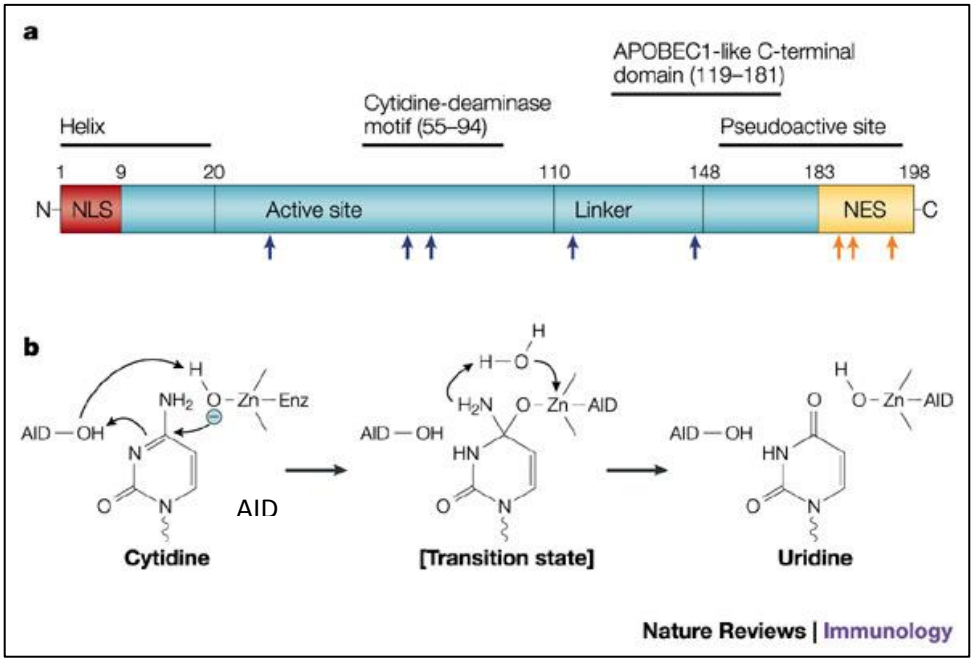


Figure 5: Activated Induced Cystisine Deaminase. (a) The primary structure of Activation induced cystidine deaminase AID. **(b)** Mechanism of cystidine deamination by AID enzyme (Chaudhuri and Alt, 2004).

APOBEC family proteins contain characteristic domain structure, the catalytic domain on the N- terminal and the pseudocatalytic domain on the C-terminal. The proteins share conserved zinc-binding motif so the catalytic domain is a zinc dependent cytidine deaminase domain that is essential for cytidine deamination (Huthoff and Malim, 2005; Chiu and Greene, 2008 ; Goila-Gaur and Strebel, 2008)

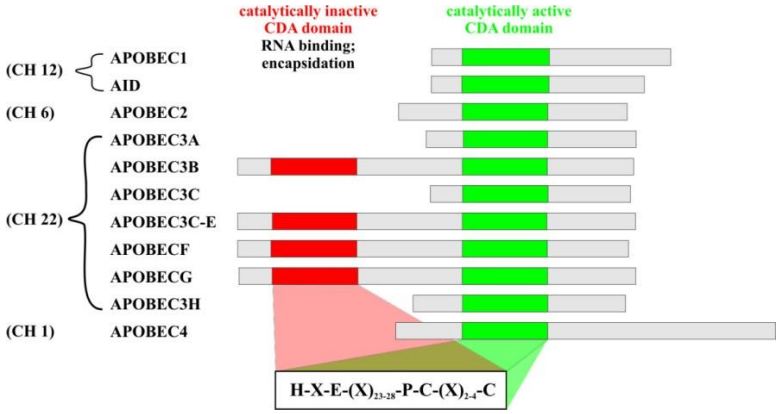


Figure 6: Human APOBEC proteins. Members of the APOBEC family contain either one or two CDA domains. Proteins are aligned based on their (CDA) shaded in green. Catalytically inactive domains are shaded in red. The consensus sequence for the CDA domains is shown at the bottom. Chromosomal association is shown on the left (Goila-Gaur and Strebel, 2008).

AID has been identified since 1999 by Honjo and colleagues, and initially thought to be an RNA-editing enzyme. It is encoded by Acida gene located on chromosome 12 (Seok-Rae Park, 2012).

AID is a mutator enzyme that mutates DNA and RNA. It is an essential enzyme in B cells differentiation process, antibody maturation and diversification in vertebrate adaptive immune system (Mechtcheriakova et al., 2012).

Recently, the structure of AID protein has been demonstrated (Figure 7). All AID models contain a core structure including a central beta sheet with four or five beta strands surrounded by six alpha helices with Zn-coordinating residues (King et al., 2015).

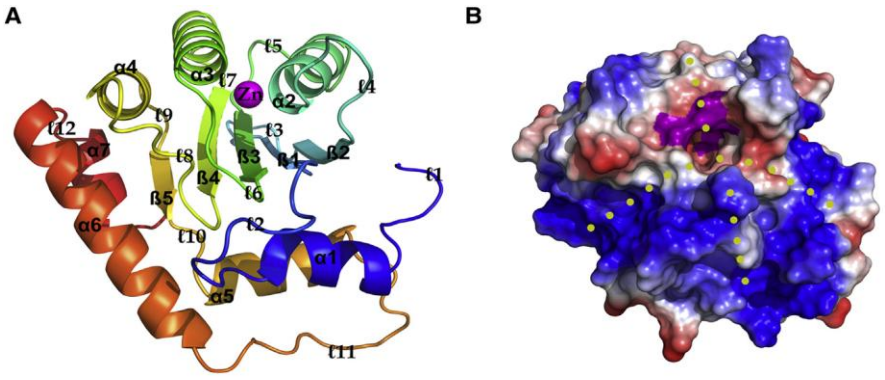


Figure 7: Three Dimensional Model of AID Protein. (A) Representative ribbon model of AID. N to C terminus progression is shown in color blue to red, with catalytic pocket zinc shown in purple. **(B)** Model of General surface topology of AID (King et al., 2015).

1.2.1 The Physiological role of AID

Our immune system has defense mechanisms against a wide range of foreign antigens and pathogens with a broad diversity. DNA cytosine deamination by AID enzyme is a natural physiological event in the adaptive immune system (Pettersen et al., 2014). AID was initially thought to be an RNA-editing enzyme, but the discovery that it could mutate *Escherichia coli* DNA provided insight into its role as a DNA mutator enzyme (Conticello, 2008; Chandra et al., 2015).

AID is expressed only in activated B cells under physiologic conditions. It is the key regulator of secondary antibody diversification mediating two main processes. The first process is Class Switch Recombination (CSR). In this process AID initiates the introduction of double strand DNA breakage in the heavy chain of the immunoglobulin leading to change it from one class to another, such as from the isotype IgM to the isotype IgG for antibody diversification (Franchini et al., 2013 ; Moris et al., 2014).

The second process is Somatic Hypermutation (SHM) which allows the immune system to adapt its response to any threats during the lifetime of an organism. Somatic hypermutation involves a point mutation at the variable regions of immunoglobulin genes (Mechtcheriakova et al., 2012; Franchini et al., 2013; Moris et al., 2014).

AID deficiency is responsible for the development of a rare immunodeficiency syndrome known as "Hyper-IgM Syndrome". The syndrome is characterized by increased levels of antibody IgM with absence of IgG, IgA and IgE resulting in high susceptibility to foreign pathogens and bacterial infections (Revy et al., 2000; Mechtcheriakova et al., 2012; Moris et al., 2014).

Although AID expression in germinal center B cells which undergo CSR and SHM is at high levels, it is also found in other cell types such as oocytes, embryonic stem (ES) cells, and in estrogen-induced breast tissue. Its function in these cells remains to be elucidated. However, AID function is not limited to antibodies diversification, and evidence is accumulating to suggest a role in epigenetic reprogramming. AID knockout mouse model revealed that AID has a role in DNA demethylation during germ cell reprogramming (Moris et al., 2014). AID, along with the related cytidine deaminase APOBEC1, can convert 5-methyl C in single stranded DNA to thymidine in vitro. This observation led to the proposal that these enzymes could be the elusive vertebrate DNA cytosine demethylase. AID could initiate demethylation by a damage and repair mechanism similar to that used in SHM (Fritz and Papavasiliou, 2010).

1.2.2 The Role of AID in Cancer

Genetic changes such as nucleotide alterations and chromosomal translocation in oncogenes and tumor suppressor genes, have an essential role in cancer formation and development (Takai, 2011). Most of the lymphomas diagnosed in the world arise from mature B cells and are characterized by the presence of chromosome translocations that involve one of the Ig loci and a proto-oncogenes (Yebenes, 2008).

AID may function as a genome-wide mutator by mutating multiple genes. Studies show evidence that AID acts as DNA mutator in *Escherichia coli* this provided insights into its mechanism of action, thus, if AID is expressed outside the immune system it can induce cancer in various tissues (Paulkin, 2009). Aberrant expression of AID cause genomic instability and mutating multiple genes by favoring chromosome translocations and point mutations in both B and non-B cells, thereby stimulating cancer formation. Thus AID could have a strong contribution to human malignancy, including solid tumors. AID expression has been reported in numerous cancers of non-B-cell origin, including breast, prostate, stomach, liver, and lung (Park, 2012; Mechtcheriakova et al., 2012 ; Moris et al., 2014 ; Petterson et al., 2014).

Recent sequencing of large numbers of human cancer genomes showed that mutations at cytosine residues, particularly C to T transitions, are the most prevalent mutations in human cancer, highlighting the enzyme AID as a potential source of mutagenesis. Other factors, including expression levels for uracil-DNA glycosylase may modulate genomic uracil levels and can be also introduced by coupling with replication of U/G mismatches but aberrant AID levels have the strongest effect leading to genes mutagenesis (Kotani et al., 2005; Petterson et al., 2014).

In mice, AID expression was necessary for translocations between Ig loci and proto-oncogenes, a hallmark of several human B-cell lymphomas. For example deregulated expression of AID has been proved to trigger *c-myc*/*Igh* translocations (a common trait in Burkitt's lymphoma) and *c-myc/miR-142* found in B cell leukemia (Conticello, 2008; Moris et al.,2014). In contrast, AID knockout mice have fewer translocations and fewer mutations in genes linked to B cell tumorigenesis (Petterson et al., 2014).

Constitutive or ubiquitous AID expression also leads to cancer development that is characterized by point mutations in oncogenes like *PIM1*, *MYC, p53*, Bcl-6, *RhoH/TTF (ARHH),* and *PAX5* (Chiu., 2009 ; Takai., 2011 ; Mechtcheriakova et al 2012 ; Moris et al., 2014). It has recently been reported that aberrant expression of AID in gastric epithelium

leads to the accumulation of nucleotide alterations in the *p53* gene. So AID Aberrant expression in human cells increases the mutation rate of *p53* by around 10 folds (Borchert, 2011).

This associations between AID with cancer has been well established and it is an indication on how tight intracellular regulation of AID enzyme is needed to minimize the chances of alterations in genomic DNA leading to cellular transformation (Conticello, 2008; Chiu, 2009).

1.2.3 Regulation of AID Expression

The identification of AID as a potential mutation inducer indicates the importance of its regulation to avoid dramatic side effects and genomic instability. AID is under the control of transcriptional, post-transcriptional and post-translational levels of regulation (Aoufouchi et al., 2008; Borchert et al., 2011; Cogne, 2013; Rebhandl et al., 2015).

Transcription factors including Pax5, HoxC4 and NF-KB control the AID promoter to yield high levels of AID expression in the B cell lineage. HoxC4-binding site located in the promoter region upregulates AID transcription. In addition to these enhancers, E2F- and c-Myb binding sites in region 2 of the AID promoter function as silencers that negatively regulate AID promoter activation. NF-κB and STAT6 are major transcription factors in the CD40, IL-4, and TGF-β signaling pathways mediated by toll-like receptors and cytokines, which are associated with inflammatory reactions, therefore activation of NFkB is important for its ability to induce AID expression (Dedeoglu et al., 2003; Huong Le et al., 2013; Chandra et al., 2015). Mutations in CD40 gene cause hyper IgM (HIGM) syndrome which is characterized of very low levels of serum IgG, IgA and IgE as a consequence to the loss of CSR process that results from AID activation (Xu et al., 2007; Frasca et al., 2008).

Microbes and pathogens induce AID expression by activating intracellular transduction pathways such as CD154 and IL-4 (Xu et al., 2007; Cogne, 2013). AID expression is induced in gastric epithelial cells by Helicobacter pylori (*H. pylori*) infection through the NF-κB pathways suggesting that inflammatory signals associated with infection by pathogens are involved in inducing AID (Huong et al., 2013).

Chronic inflammation pathophysiology associated with the transcriptional factor NF-κB which is activated by various proinflammatory cytokines. NF-κB plays a key role in the upregulation of AID expression, and many studies suggest that activation of NF-κB in

epithelial cells under many inflammatory conditions might induce AID, leading to genetic instability (Figure 8) (Shimizu et al., 2012).

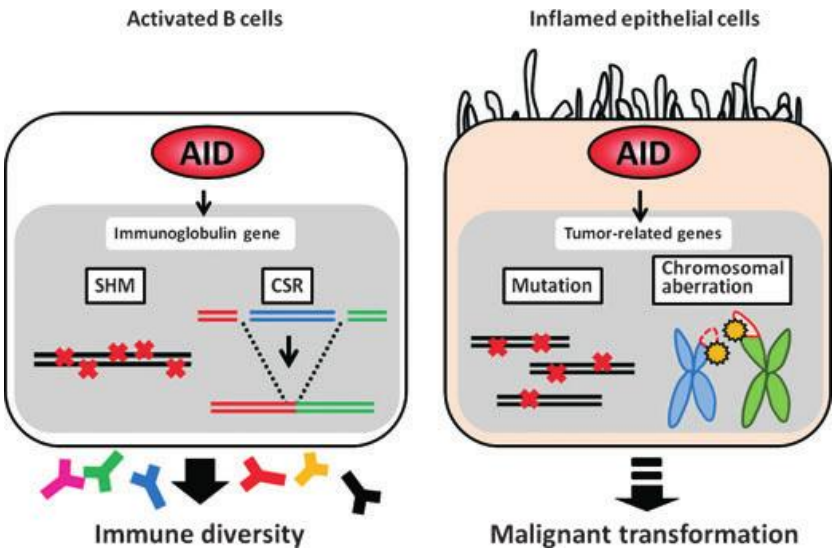


Figure 8: Activation-induced cytidine deaminase (AID) function under physiological and inflammatory conditions. (Left picture) Under physiological conditions, AID is expressed only in activated B cells and is a key molecule for generating immune diversity by inducing (SHM) and (CSR) in immunoglobulin genes. (Right picture) Under inflammatory conditions, AID is aberrantly expressed in epithelial cells. AID can induce somatic mutations and chromosomal aberrations in tumor-related genes, contributing to malignant transformation (Shimizu et al., 2012).

AID transgenic mice develop gastric neoplasms suggest that aberrant AID expression in gastric epithelial cells contributes to cancer development by the accumulation of somatic mutations. AID is induced in response to *H. pylori* infection or proinflammatory cytokine like TNF-alpha stimulation via the NF-B signaling pathway and is capable of contributing to the generation of somatic mutations in various tumor-related genes such as *TP53*. Thus, inflammation-mediated AID expression plays a key role in genetic instability and cancer developing (Figure 9) (Marusawa, 2008).

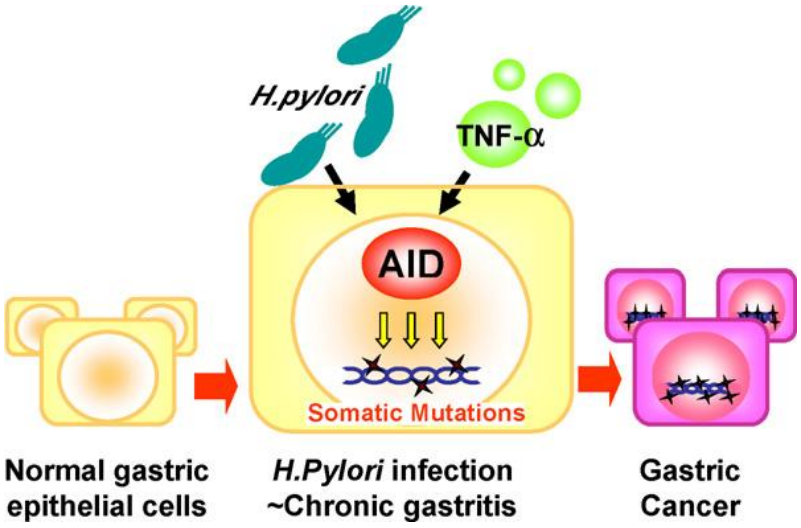


Figure 9: AID links chronic inflammation to gastric cancer development by its mutagenic activity (Marusawa, 2008).

AID is post-transcriptionally regulated by several mechanisms including microRNA regulation, phosphorylation, nucleo-cytoplasmic shuttling and ubiqitination, all of which play important roles in regulating AID and function in SHM and CSR (Xu et al., 2007 ; Basu et al., 2009). Phosphorylation of AID is necessary for its activation, protein kinasa A (PKA) phosphorylates messenger of AID on Serine38 in cells including activated B cells. Recent studies show that drug-induced PKA inhibition decrease CSR while PKA activation-by deletion of PKA negative regulatory subunit-increase CSR through AID activation (Basu et al., 2009; Cogne et al., 2013; Yi Hu et al., 2014).

As DNA deaminase, AID would perform its function in the nucleus, after that it must be retained to the cytoplasm. Nuclear localization signal (NLS) at the amino terminus of human AID regulates the dynamic shuttling of AID between the cytoplasm and the nucleus. Subcellular localization is an important step in regulating AID that affect its stability because AID has a significantly shorter half-life in the nucleus than in the cytoplasm (Xu et al., 2007; Orthwein et al., 2010; Cogne, 2013; Yi Hu et al., 2014).

The correlation between the endocrine system and the immune system is well documented. Estrogen has stimulatory effect immune response. Studies show that AID can be activated by estrogen in immune and non-immune cells. This provides an evidence of how estrogen can alter the immune system and induce oncogenic DNA alterations (Pauklin et al., 2009). Estrogen mediating genome instability via the activation of AID may provide a novel molecular mechanism that is important for breast cancer pathology (Pauklin et al., 2009; Mechtcheriakova et al., 2012).

Estrogen directly and indirectly activates AID expression; this can lead to immune hyper-stimulation. Estrogen receptor (ER) binds the AID promoter directly, or indirect via activation of transcription factors that enhance AID expression (Incorvaia et al., 2013). Therefore estrogen is able to induce a DNA mutator in a variety of hormonally responsive cells (Figure 10). These findings might explain why women are more susceptible to autoimmune diseases and cancer in particular breast cancer (Orthwein and Noia, 2012). These studies brought the light to this research since 70% of BC are estrogen receptor positive, estrogen can mediate genome instability via the activation of AID and other DNA deaminases (Persyamy et al., 2015).

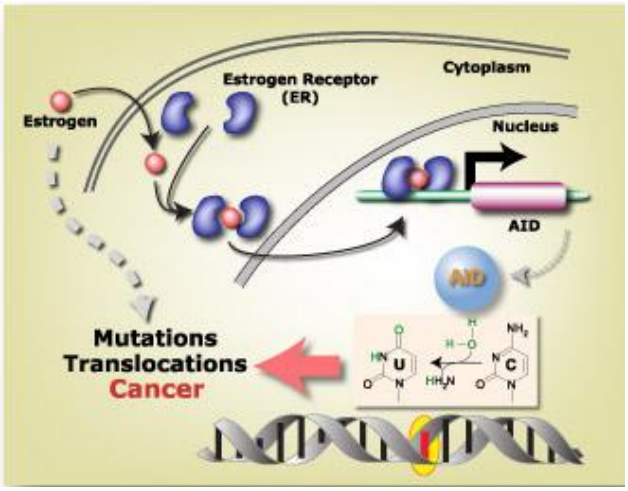


Figure 10: Model of AID activation by Estrogen leading to DNA damage. Extracellular estrogen enters the cell, binds to the estrogen receptor dimer (ER), is transported as a ligand complex into the nucleus, binds near the AID promoter, and activates AID transcription (Petersen-Mahrt et al., 2009).

Recent evidence suggests that one level of AID regulation comes from microRNA (miRs). These short noncoding RNA regulates many genes by repressing translation or directing mRNA destruction through partial sequence complementarity to 3 untranslated regions (3UTR) of target mRNA. MiR-155 repress AID and the disruption of the miR-155 increase AID-induced *c-MYC* translocation, this may explain why disruption of miR-155 is associated with Burkett's lymphoma. It is characterized by AID-induced mutation and a translocation between *c-MYC* proto-oncogene and the IgH loci (Dorsett et al., 2008; Borchert et al., 2011).

1.3 Objectives:

Recently, researches have shown the aberrant AID expression in different types of cancer. This causes genomic instability thereby stimulating cancer formation. In Palestine one study was reported in 2013 showed that AID may be involved in a subset of lung cancer and since then no studies are reported (Data unpublished).

The aim of this study is to evaluate for the expression of AID protein in breast cancer taken from patients in Palestine and to determine the correlation between AID protein expression and clinico-pathological data of the patients, such as age, BC type, BC grade, BC stage and invasiveness. This is the first study that examines the expression of AID protein in BC tissue samples by IHC in Palestine and worldwide.

Chapter Two: Materials and Method

2.1. Materials

Reagents and buffers used in IHC and H&E staining are listed in table 2 below.

Table 2: Materials for IHC and H&E staining

Material	Company	Catalog#
Xylene	Loba Chemie	Cas#: 1330-20-7
Ethanol	Carlo Ebra	64-17-5
Citrate buffer pH=6	Invitrogen	Ref#: 005000
Hydrogen peroxide 3%	MP Biomedicals, USA	Article#: 194057
Pap –pen	Invitrogen	00-8899
TBS buffer	Sigma	T5912-1L
Primary AID Ab	e-Bioscience	14-5959
Cass block	Invitrogen	0-8120
Secondary anti-Rat Ab	Histofine	414311F
DAB chromagen	Cell-Marque	957D-31
DAB buffer substrate	Cell-Marque	957D-32
Hematoxylene `	Sigma	GHS3128-4L
Mounting Medium	Fluka	03989-10ML
Super Frosted Plus Slides	Fisher brand	12-550-15
Eosin Y	Sigma	E6003-25G

2.2. Methods

2.2.1 Sample collection:

Archived paraffin embedded blocks of 69 breast cancer samples were collected retrospectively from the Pathology Department at Beit Jala Hospital in Bethlehem city between years (2009 - 2013). Breast cancer cases were accompanied with their pathological reports obtained from the hospital records archive. The pathological reports contain data about the patients including: Age, gender, data of specimen collection, tumor grade and stage. The study design was approved by the Faculty of Medicine at Al-Quds University and Beit Jala Hospital.

2.2.2. Tissue Sectioning:

For light microscopy, the microtome is used to cut three micrometer-thick tissue sections, since it is the ideal thickness for IHC. The tissue sections are mounted on a super frosted plus glass microscope slides. These slides provide maximum adhesive protection and thus the tissues won't fall off the slides. The slides are charged to create a strong bond between fresh, frozen or formalin fixed paraffin embedded tissue sections and glass slides. Each case block was cut into two sections; one section was prepared for H&E staining for histological diagnosis and evaluation, and the other section for IHC staining.

2.2.3. Standard Staining (H&E):

Standard stain Hematoxylene and Eosin was performed for each breast cancer case for histological diagnosis and evaluation by the pathologist, and as reference for each IHC stained slide to see the original tissue, and to confirm the tumor stage and grade when there is missing data in the patients reports. The protocol was done as shown below in table 3 (Avwioro, 2011).

Table 3: H&E Staining Protocol

Treatment	Time
Xylene 1	2 min
Xylene 2	2 min
Ethanol 100%	1 min
Ethanol 80%	1x5sec
Ethanol 70%	1x5sec
Tap water	3 min
Hematoxylene	2-4 min
Tap water	Wash
Eosin	5-6 min
Tap water	3 min
Ethanol 70%	1x5sec
Ethanol 80%	1x5sec
Ethanol 100%	I min
Xylene 1	2 min
Xylene 2	2 min
Mounting solution and cover glass	

2.2.4. Immunohistochemistry (IHC)

Immunohistochemistry has become a crucial technique and widely used in medical research and diagnosis. IHC currently has many clinical applications in diagnosis, prognosis, therapeutic decision making, and pathogenesis. The principle of IHC bridges three disciplines: Immunology, histology, and chemistry. The fundamental concept of this technique is the demonstration of antigens within tissue sections by means of specific antibodies. Antigen-antibody binding is demonstrated with a colored histochemical reaction that is visualized by number of ways. In the most common an antibody is conjugated to an enzyme, such as peroxidase that can catalyze a color-producing reaction

or the antibody can be tagged to a radioactive element, and can be visible using light or fluorescent microscopy. In contrast to many detection techniques, IHC allows localization of an antigen in tissue sections thereby dramatically enhancing diagnostic interpretation and understanding of the pathogenesis (Ramos-Vara and Miller, 2014; Rizzardi et al., 2012)

All tissue sections mounted on superfrosted slides were treated according to the protocol of immunohistochemistry as in table 4 (Ramos-Vara, 2005).

IHC staining controls are required for validation of staining results to ensure that every IHC test is performed correctly and the immunostains are working. Positive tissue control is tissue with the specific antigen that is known to show positive reaction with the antibody, the purpose of using positive control is to ensure correct staining. Negative tissue control is tissue without the specific antigen present, and the purpose is to document specificity of the staining (Chan et al., 2000; Packeisen et al., 2002).

In this project the positive control used for AID antibody was sections from tonsil tissue since tonsils are the first line of defense against pathogens they express high levels of cytoplasmic AID. These control slides are kindly provided from Beit Jala Hospital.

Table 4: Immunohistochemistry protocol.

Treatment	Time	Notes
Xylene 1	20 min	Deparafinization
Xylene 2	10 min	Deparafinization
Ethanol 100%	3x3 min	To remove dissolved paraffin wax and xylene
Ethanol 96%	3min	Rehydration
Ethanol 80%	3min	Rehydration
DDW	3x5sec	Washing to rinse off Ethanol
Citrate buffer (pH6)	20 min, medium power , in microwave	To enhance Antigen exposure
DDW	3x2 min	Washing
3% Hydrogen peroxidase	10 min	Block endogenous Peroxidase
DDW	3x1 min	Washing
Surround circle the tissue		By using Pap pen
TBS buffer	3x2min	Washing
Primary AID antibody	Overnight	Incubated at 4C, diluted 1:50,
TBS buffer	3x5min	Washing
Secondary Anti Rat-Ab	30 min	2 drops (0.1 ml)
TBS buffer	3x5min	Washing
DAB chromagen	20 min	"substrate chromagen"
DDW	5sec	Washing
Hematoxylene	30 sec	Counter stain
Tap water	5 sec	Washing
Ethanol 96%	2 min	Dehydration
Ethanol 100%	2 min	Dehydration
Xylene 1	2min	
Xylene2	2min	
Mounting solution and cover glass		

At the beginning of the IHC protocol slides must be deparrafinized and rehydrated since incomplete paraffin removal can cause poor staining of the section, therefore xylene and graduated concentration of alcohol were used.

When using a new antibody in IHC protocol, the antibody must be calibrated to find the optimal staining conditions. Each antigen has a preferred method of antigen retrieval and each antibody has an optimal dilution.

Fixation process of the slides lead to conformational changes such as the formation of methylene cross-links which masks the antigens and cause very poor staining by IHC. Antigen retrieval by heat is necessary to the hydrolysis of methylene bridges and unmasks the antigens in order to allow the antibodies to bind, so as to get good and efficient stain for the antigen. The two methods of antigen retrieval are heat mediated (also known as heat-induced epitope retrieval (HIER) and the enzymatic method (Paulsen et al., 2015).

For performing heat induced antigen retrieval proper buffer should be used (e.g., citrate, Tris) with various pH (3–10), but for most antigens citrate buffer (pH 6.0) will give satisfactory results and good cell morphology when compared with buffers with higher pH or solutions containing EDTA (Ramos-vara, 2005).

Heat mediated antigen retrieval can be done using a pressure cook, microwave or hotplate. In this research the microwave was used. AID antigen was calibrated by using different levels of powers at different timing; at High power 350-375 degrees for (15, 20 min) and at Medium power 300-350 degrees for (15, 20 min), the best results were obtained at Med power for 20 minute with Citrate buffer (pH 6).

The sensitivity of an immune reaction mostly depends on the detection system used. The Ag-Ab reaction cannot be seen with the light microscope unless it is labeled. Therefore, reporter molecules are attached to the Abs to allow visualization of the immune reaction. There are a variety of labels used, including fluorescent compounds, enzymes, and metals. The most commonly used labels are enzymes (e.g., peroxidase, alkaline phosphatase, glucose oxidase). Enzymes in presence of a specific substrate and a chromagen will produce a colored precipitate at the site of the Ag-Ab binding (Ramos-vara, 2005). Detection systems are classified to direct and indirect methods. Direct methods are one-step process with a primary Ab conjugated with a reporter molecule. The method lacks sufficient sensitivity for the detection of most antigens. The need for more sensitive Ag detection develop a two-step method, the indirect method in which the first layer of Abs is unlabeled and called the primary antibody, but the second layer, raised against the primary Ab, is labeled and called the secondary antibody. The sensitivity of this method is higher

because it is resulting in a strong signal and the number of labels per molecule of primary Ab is higher, increasing the intensity of reaction (Ramos vara, 2005).

In this research indirect detection system was done, the primary antibody is monoclonal Anti-Human/Mouse Activation Induced Cystidine Deaminase (the concentration is 0.5 mg/ml), it recognizes human and mouse AID. Calibration for the accurate dilution of the AID antibody was done in a previous study on the same AID protein done for lung cancer tissue sections, and the optimal staining result was obtained by the dilution 1:50 Ab into Cass block. Cass block is a universal blocking agent for reducing nonspecific background staining in immunolabeling techniques, and works well as a diluting reagent for primary antibodies. Calibration for the accurate slides incubation of the primary AID Ab was done (One hour incubation at room temperature and overnight incubation at 4^0 C) and the perfect results were obtained at overnight incubation for primary AID Ab. The amount of Ab for each slide was 2.6 microliter.

Unwanted background staining due to endogenous enzyme activities is no longer a problem in immunohistochemistry (Buchwalow et al., 2011). That's why we treat the slides with 3% Hydrogen Peroxide (H_2O_2) to block the endogenous peroxidase activity which may lead to false positive results, since we are using 3', 3' Diaminobenzidine DAB as a substrate for peroxidase enzyme labeled on the secondary Ab.

The secondary Ab used in this experiment is labeled with horse radish peroxidase (HRP), so we used DAB substrate chromagen for detection system (2.6 microliter for each slide), it enables the antibody- antigen complex to be viewed easily under the light microscope. It is oxidized by peroxidase (HRP) and gives dark brown color. Slides were incubated with DAB at different timing; 15 min, 20 min, 30 min, and the best results were obtained at 20 min.

Evaluation of AID protein expression in the BC treated slides was done by the pathologist in Beit Jala Hospital Dr. Riyad Shrem. The intensity of cytoplasmic expression classified according to the three grade scale (0: no staining, 1+: focal positive staining, 2+ moderate positive staining and 3+ very strong positive staining) (Rizzardi et al., 2012).

2.2.5. Statistical Analysis:

Statistical analysis was done to find if there is a significant correlation between AID protein expression in Breast Cancer tissues with the clinico-pathological data of BC cases. Pearson's chi-squared test was used, and converted to P-value. If the P-value <0.05 it was considered to be statistically significant, the statistical program SPSS was used.

Chapter Three: Results

3.1. Study Samples:

The general clinical and pathological data of Breast Cancer cases are summarized in Table 5; including age, BC type, BC stage and Tumor Grade.

IHC was performed on 69 BC cases and on control slides from tonsil tissue.

For further confirmation 10 slides were chosen randomly and IHC was repeated and showed similar results.

Table 5: Clinico-pathological Data and AID Expression for the BC Cases.

Slide #	Block #	Age	BC Type	BC Stage	Tumor Grade	AID Exp.
1	1655-12	68	IDC	T4NxMx G3	Poorly d.	**0**
2	1945-12	48	IDC	T2N2Mx	Moderately d.	**+1**
3	186-10	40	IDC	T1N2Mx G2	Moderately d.	**0**
4	2557-12	57	IDC	T2N3Mx G2	Moderately d.	**+1**
5	2347-12	37	IAC	T2NoMx G3	Poorly d.	**0**
6	2637-12	38	IDC	T2N3Mx G2	Moderately d.	**+2**
7	2818-12	57	IDC	t2N3Mx G2	Moderately d.	**0**
8	2817-12	41	IDC	T3N2Mx G2	Moderately d	**+1**
9	1019-12	64	IDC	T2N0Mx G2	Moderately d	**+1**
10	1286	56	IDC	T3N2Mx G2	Moderately d.	**+1**
11	3545-12	53	IDC	T2N1Mx G2	Moderately d.	**0**
12	219-09	44	IDC	TxN2Mx	Moderately d	**+1**
13	640-09	72	IDC	TxN1Mx G2	Moderately d.	**0**
14	556-12	43	IDC	T1N1Mx G2	Moderately d.	**0**
15	1114-12	29	IDC	T4N2Mx G2	Moderately d.	**0**
16	2649-12	45	IDC	T2N0Mx G2	Moderately d.	**0**
17	3299-12	29	IDC	-	-	**0**
18	402-11	71	IDC	T2N0Mx G2	Moderately d.	**0**
19	646-11	77	IDC	T2N0Mx G2	Moderately d.	**0**
21	1226-11	32	IDC	TxN3Mx	Moderately d.	**0**
22	1527-11	49	IDC	T2N2Mx G2	Moderately d.	**0**
23	1618-11	55	IDC	T2N0Mx G2	Moderately d.	**0**
24	1631-11	43	IDC	TxN3Mx G2	Moderately d.	**0**
25	2174-11	64	IDC	T2N0Mx G2	Moderately d.	**0**
26	2756-11	41	IDC	T3N2Mx G3	Poorly d.	**0**
27	2977-11	54	IDC	-	-	**0**
28	3007-11	60	IDC	T1N0Mx G2	Moderately d.	**0**
29	3369-11	47	IDC	T3N3Mx G3	Poorly d.	**0**
30	3488-12	42	IDC	G2	Moderately d.	**0**

31	183-09	40	IDC	TxN3Mx	Moderately d.	**0**
32	1308-09	86	IDC	-	-	**0**
33	3147	45	IDC	-	-	**0**
34	1887-09	75	IDC	TxN2Mx	Moderately d	**+1**
35	2195-09	46	IDC	-	-	**0**
36	2145-09	39	IDC	TxN0Mx	Moderately d.	**0**
37	2224-09	54	IDC	T2N1Mx	Moderately d.	**+1**
38	2353-09	49	IDC	T4N0MX G2	Moderately d.	**0**
39	2686-09	59	IDC	-	-	**0**
40	2712-09	69	IDC	TxN1Mx	Moderately d.	**0**
41	2783-09	52	IDC	T2N0Mx G2	Moderately d.	**+1**
42	2843-09	71	IDC	T2N0Mx	Moderately d.	**0**
43	2859-09	34	IDC	G2	Moderately d.	**0**
44	1655-12	68	IDC	T4NxMx G3	Poorly d.	**0**
45	2995-09	43	IDC	TxN2Mx	Moderately d.	**+1**
46	3830-09	34	IDC	T0NxMx	Moderately d.	**0**
47	3402-09	52	IDC	T1N1Mx	Moderately d.	**0**
48	3387-09	54	IDC	T1N1Mx	Moderately d.	**0**
49	3313-09	45	IDC	T2N3Mx G2	Moderately d.	**+1**
50	502-10	33	IDC	T3N3Mx	Moderately d.	**+1**
51	978-10	49	IDC	T2N1Mx G3	Poorly d.	**+2**
52	1410-10	50	IDC	T2N2Mx G2	Moderately d.	**0**
53	185-10	53	IDC	T4N2Mx G2	Moderately d.	**0**
54	1988-10	47	IDC	T2N1Mx G2	Moderately d.	**+1**
55	1671-10	61	IDC	T2N2Mx G2	Moderately d.	**+1**
56	2689-10	42	IDC	T1N1Mx G2	Moderately d.	**0**
57	780-10	31	IDC	T2NxMx G2	Moderately d.	**0**
58	2048-10	46	IDC	T2N2Mx G2	Moderately d.	**0**
59	1728-10	58	IDC	-	-	**0**
60	1183-10	63	IDC	T4N3Mx G3	Poorly d.	**0**
61	2616-10	46	IDC	T3NxMx G2	Moderately d.	**0**
62	514-10	77	IDC	-	-	**0**
63	1618-10	49	IDC	T3N3Mx G2	Moderately d.	**0**
64	2546-10	34	IDC	T4N3Mx G2	Moderately d.	**0**
65	774-10	46	IDC	T2N3Mx G2	Moderately d.	**0**
66	1164-10	35	IDC	T4N3Mx G2	Moderately d.	**0**
67	3540-10	38	IDC	-	-	**0**
68	2967-10	55	IDC	T3N1Mx G2	Moderately d.	**+1**
69	3576-10	46	IDC	-	-	**+1**
71	2487-10	36	IDC	-	-	**0**

- IDC: Invasive Ductal Carcinoma
- Gender not involved because all BC cases are for Females patients

3.2 Clinico-pathological data summary for BC cases:

As seen in table 6; 100% of the patients were females. Ages of the patients range from 29 and 86 years, with an average of 49.5 years old.

In this study 100% of the patients have Invasive Ductal Carcinoma also called Infiltrative Ductal Carcinoma.

According to the national cancer institute there are three grades of BC:

- Grade 1 (well differentiated grade): Cancer cells look similar to normal cells and grow very slowly.
- Grade 2 (moderately differentiated grade): Cancer cells look more abnormal and faster growing.
- Grade 3 (poorly differentiated grade): Cancer cells look very different from normal cells and grow quickly.

Tumor Grade ranges between moderately differentiated 91%, and poorly differentiated 9%.

Table 6: Summary of Clinico-pathological Data of BC cases.

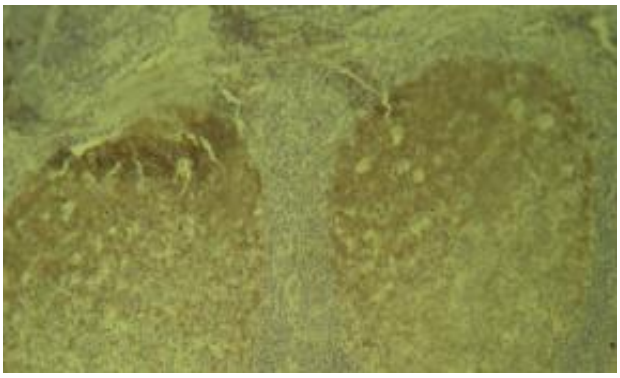
Patient characteristics		Frequency (%)
Gender	*Male* *Female*	0 (0%) 69 (100%)
Age	*Range* ≥*60* <*60* *Average*	29-86 14 (20.3%) 55 (79.7%) 49.5
BC type:	*IDC* *Insitu DC*	69 (100%) 0 (0%)
Tumor Grade	*Poor d* *Mod d* *Well d*	4 (9 %) 44 (91 %) 0
Tumor Stage	T0 Tx TI T2 T3 T4	1 (2%) 9 (16%) 6 (11%) 24 (43%) 8 (14%) 8 (14%)
Tumor Stage	N0 Nx N1 N2 N3	12 (21%) 5 (9%) 11 (19%) 15 (26%) 14 (25%)

3.3 IHC Evaluation and Statistical Analysis for the BC cases

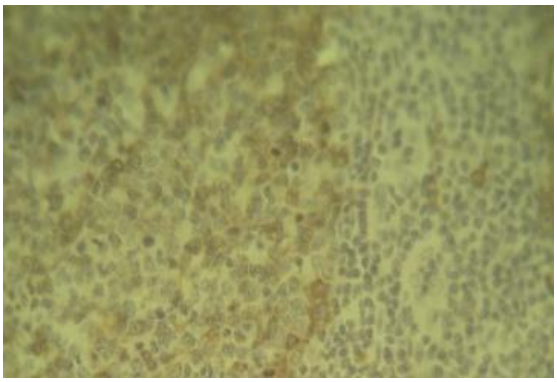
Positive controls which are tonsils tissue sections were stained in brown with cytoplasmic localization (Figure 11-a) At 10X magnification, and (Figure 11-b) at 100X magnification.

Figure 11-a: Cytoplasmic AID Expression in Positive Control at 10x magnification.

Figure 11-b: Cytoplasmic AID Expression in Positive Control at 100x Magnification



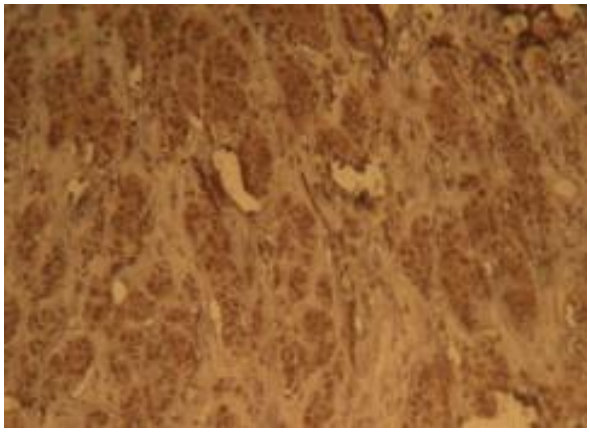
a



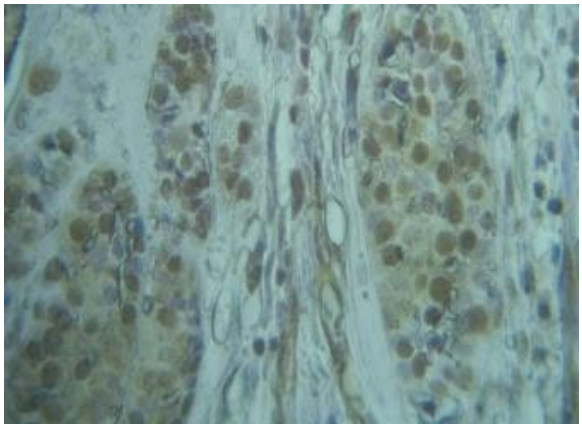
b

AID protein expression was detected in 26% (18 of 69) of BC cases with cytoplasmic localization (Figure 12).

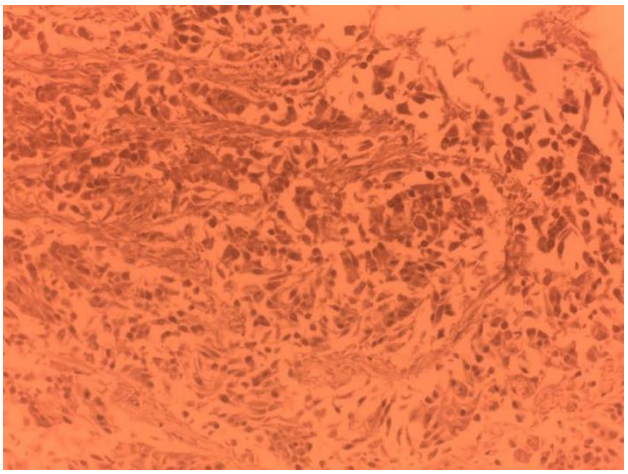
Figure 12: Positive AID expression in BC tissues at 10x magnification (a), (c), (d), at 100x magnification (b).



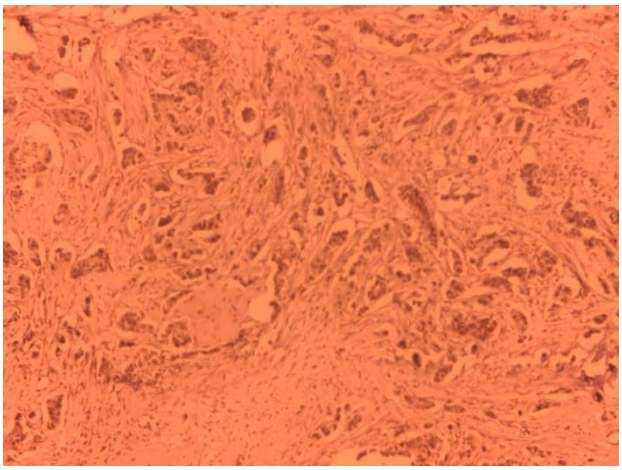
a



b



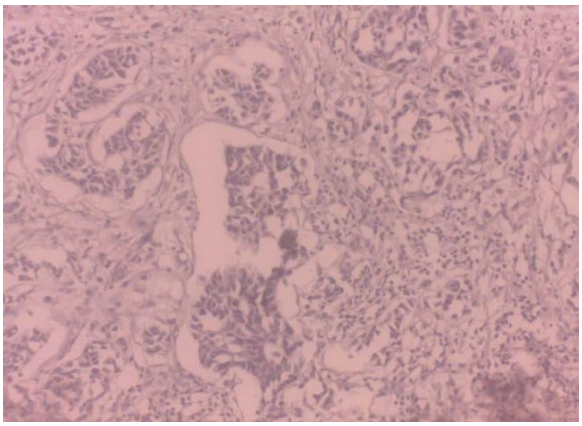
c



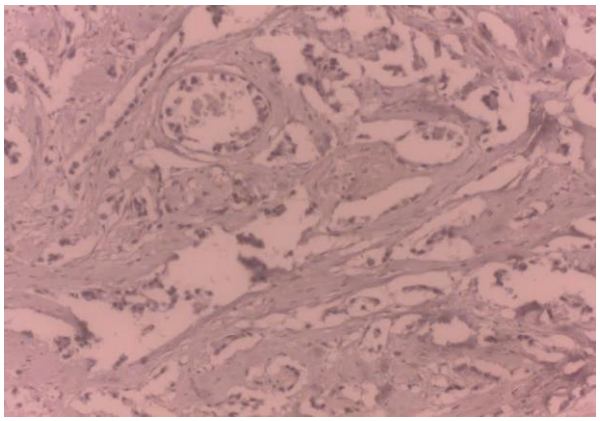
d

Negative AID expression tissues was in 51 slides of 69, results were as shown in figure 13.

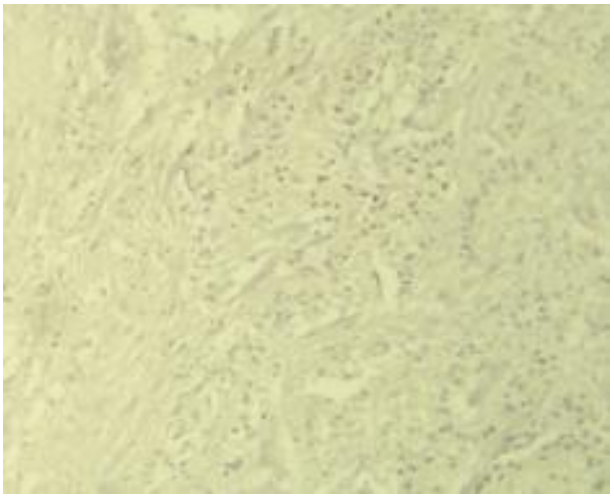
Figure 13: Negative AID Expression in BC Tissues.



a



b



c

Table 7 below describes the correlation between AID protein expression and Clinico-pathological parameters, 79.7% of the positive cases were more than or equal to 60 years, 20.3% were below the age of 60 years. However, no significant difference was obtained between the two groups of age and AID expression (p=0.747). Moreover, 68.6% and 12 % of moderately and poorly differentiated tumor showed AID protein positive, respectively. No significant difference was found between BC tumor grade and AID expression (p=0.081). On the other hand, all tumor stages showed AID protein positive; stage T2 was the dominant with a percentage of (36.2%), but no significant difference was observed between the tumor stage and AID expression (p=0.907). Since we are studying Breast cancer all the cases were females so p value can't be found and also for the BC type since all the cases are the same BC type. Some cases did not have complete clinical data, and were not included in the table.

Table 7: Correlation between AID Protein and Clinico-pathological Parameters.

Variable/AID	0	+1	+2	Total	% of Cases	X^2	P value
Gender						Ø	Ø
M	0	0	0	0	0%		
F	51	16	2	69	26%		
Age						0.584	**0.747**
<60	40	13	2	55	27%		
≥60	11	3	0	14	21%		
BC Type						Ø	Ø
IDC	51	16	2	69	26%		
INDC	0	0	0	0	0%		
Tumor Grade						5.025	**0.081**
Well d.	0	0	0	0	0%		
Moderately d.	35	15	1	51	31%		
Poorly d.	6	0	1	7	14%		
Tumor Stage						8.966	**0.907**
Tx	6	3	0	9	33%		
T1	7	0	0	7	0%		
T2	15	8	2	25	40%		
T3	5	3	0	8	38%		
T4	7	0	0	7	0%		
Nx	5	0	0	5	0%		
N1	7	3	1	11	36%		
N2	7	7	0	14	50%		
N3	10	3	1	14	29%		
N0	10	2	0	12	17%		

- X^2: Chi square test. P-value≤ 0.05, AID protein expression versus Clinico-pathological parameters.

Hormone receptor status:

Estrogen receptor (ER), progesterone receptor (PR) and HER-2 receptors are found on breast cells. Overexpression of these receptors are called hormone-receptor positive in the pathology reports. Table 8 show the hormone receptor status for 29 cases of BC (Data available only for these cases). Estrogen-estrogen receptor complexes directly bind to the AID promoter and enhance AID transcription, leading to AID expression induction.

Table 8: Hormone receptor status.

	Case #	AID Expression	ER status	PR status	HER-2 status
1-	41	+	-	-	+
2-	49	+	-	+	-
3-	50	+	+	+	+
4-	51	+	+	+	-
5-	54	+	+	+	
6-	55	+	+	-	-
7-	3	-	+	+	-
8-	11	-	-	-	
9-	14	-	-	-	-
10-	16	-	+	+	
11-	18	-	+	+	-
12-	19	-	+	+	
13-	22	-	-	+	-
14-	26	-	-	-	-
15-	28	-	-	-	-
16-	29	-	+	+	-
17-	46	-	+	+	
18-	47	-	-	+	
19-	52	-	+	+	-
20-	53	-	+	+	
21-	59	-	-	-	-
22-	60	-	-	-	
23-	62	-	+	+	
24-	63	-	+	+	
25-	64	-	-	-	
26-	65	-	-	+	-
27-	66	-	+	+	+
28-	67	-	-	+	
29-	71	-	+	+	-

Chapter Four:

4.1 Discussion

Activation induced cytidine deaminase (AID) is expressed in germinal center B cells, it is essential in the affinity maturation of Immunoglobulin chains during B cell differentiation, through two processes: SHM and CSR which take place in secondary lymphoid tissues (lymph nodes, tonsils, and spleen) (Kalchschmidt et al., 2016; Pefanis and Basu, 2015). AID supports acquired immune system diversification thus humans lacking AID are severely immunocompromised (Khair et al., 2015).

AID is a physiological tool to introduce DNA alterations and may function as a general genome-wide mutator enzyme. In addition to diversifying the immune repertoire, AID can also target non-Ig genes. It has been shown that abnormal AID expression in non-B cells contributes to human malignancy including solid tumors (Mechtcheriakova et al., 2012; Kanu et al., 2016).

The mutagenic potential of AID makes its expression and activity tightly regulated on different levels to minimize the risk of unwanted DNA damage. However, chronic inflammation and other factors have been shown to trigger aberrant AID expression in B cells and, importantly, in non-B-cell background. Under these circumstances, AID may also target non-Ig genes. These include cancer-related genes such as oncogenes and tumor suppressor genes. AID expression leads to increased mutation rate of *TP53*, and genomic stability genes (Mechtcheriakova et al., 2012; Casellas et al., 2016).

Aberrant expression of AID in somatic cells plays a critical role in carcinogenesis. Transgenic mice with overexpression of AID are exposed to tumorgenecity and had developed malignant T cell lymphomas and micro-adenomas in lung with frequent point mutations which appeared to be introduced by AID activity. Therefore, these studies suggested that deregulated AID could be responsible for human malignancy (Okazaki et al., 2003; Tatemishi et al., 2014). Several studies have reported that AID is aberrantly expressed in several cancer types such as hepatocellular carcinoma, lymphoma and leukemia (Park, 2012), pancreatic tumorigenesis (Sawai et al., 2015), gastric cancer, colorectal cancer (Nonaka et al., 2016), breast cancer (Harris, 2015; Rebhandl et al., 2015; Munoz et al., 2013; Babbage, 2006) and lung cancer (Shinmura et al., 2011). These observations suggest that aberrant AID expression causes mutations in various oncogenes or tumor-suppressor genes and these mutations induce tumor formation.

In this regards, immunohistochemistry (IHC) is widely used in cancer research including breast cancer, because it is an important complementary tool for the routine diagnosis of cancer and for the identification of the different histological types and prognostic factors. IHC is also used to categorize patients in order to ensure appropriate and specific treatment and therapy response (Capelozzi, 2009).

In the present study, we examined the expression of AID protein in breast cancer tissue sections derived from Palestinian patients paraffin embedded blocks using IHC technique. AID immunostaining was localized mainly in the cytoplasm in accordance with previous studies (Shinmura et al., 2011; Marusawa., 2008; Endo et al., 2008; Babbage et al., 2006). Our results revealed that AID protein is overexpressed in 26.1% of BC cases (18 of 69).

In consistent with a study reported by Babbage et al showed that AID protein is over expressed in 36.4% (35 of 96) of the cell lines examined (Babbage et al., 2006). Protein level of AID was not detected in normal breast tissue since AID protein is not expressed under physiological conditions in normal tissue other than tonsils and lymph nodes for antibody diversification through CSR and SHM mechanisms (Perez-Duran et al., 2007). Our results showed no significant correlation between AID protein expression and clinico-pathological parameters; age, tumor stage and tumor grade.

A Study by Babbage et al 2006 reported the expression of AID in three cell lines of breast cancer. The number of cells positive for AID was 35 out of 96, so the frequency of single cells expressing AID in the breast cancer cell lines is 36.4%. That study focused on the immunoglobulin variable (V) region gene analysis using well-defined breast cancer cell lines. In five of six identified V region genes, somatic mutations were apparent, for this somatic mutation there is an absolute requirement for AID enzyme expression. Interestingly, transcripts for AID were found constitutively in all six breast cancer lines examined at a level comparable with B-cell lymphoma. It has also been suggested that the aberrant mutations and genomic instability associated with aberrant AID expression. These findings suggested that AID expression in breast cancer cells could be a tumor-associated feature and essential for mutational and switch activity (Babbage et al., 2006).

Another recent study applied Real-time RT-PCR for 151 breast cancer cell lines, the results confirmed that AID is expressed in ER+ and ER− breast cancer, as well as in the majority of the examined breast cancer cell lines. AID has an important role as a regulator of ER-mediated gene expression in BC (Periyasamy et al., 2015).

Recent whole-genome sequencing of breast cancer has yielded genome-wide mutational signatures, one of which is consistent with the DNA mutation profiles associated with cystidine deamination. AID expression is frequently elevated in breast and other cancers, it can promote C-to-T mutations in breast cancer cells, these findings has led to AID overexpression in breast cancer could aid tumor initiation and progression by driving somatic mutations in cancer. Other studies showed that cystidine deamination is likely responsible for the breast cancer hypermutation (Periyasamy et al., 2015; Taylor et al., 2013).

Several studies have shown that AID overexpression can induce DNA damage responses resulting in the appearance of classical markers such as double-stranded breaks, and elevated levels of mutation (Harris, 2015). In our study to explain the tumorigenic activity of AID among these patients, further studies are still needed to examine the up regulation of AID and its tumorigenic effect inducing mutations in tumor suppressor genes and oncogenes. The transcriptional regulatory elements for AID have been localized to four regions, there are conserved binding sites for at least 19 transcription factors within these regions, both activating and repressive factors. Activating factors such as NF- κ B, Stat6, CREB, E2A , Pax5 and HoxC4 are known to regulate the AID gene locus by binding to AID promoter causing AID transcription induction (Rebhandl et al., 2015; Park, 2012; Stavnezer, 2011). Recent studies showed that NF-KappaB is constitutively activated in a variety of solid tumors including breast, prostate, lung, cervical, and pancreatic cancer (Wu et al., 2015; Chen et al., 2012). Several proinflammatory cytokines, including TNF-α and IL-1β, play important roles in the pathophysiology of chronic inflammatory disease. AID expression is strongly induced in response to proinflammatory cytokine stimulation via NF-κB activation (Hoesel and Schmid, 2013; Endo et al., 2007), leading to mutations on oncogenes such as *KRAS* and *cMyc* (Sawai et al., 2015).

Steroid hormones like estrogen are thought to be oncogenic for breast. It can influence the development and growth of the majority of breast carcinomas through their binding to steroid hormone receptors estrogen receptor ER (Karamouzis et al., 2011). Estrogen-estrogen receptor complexes directly bind to the AID promoter and enhance AID transcription, leading to AID expression induction. However, other studies suggested that estrogen-estrogen receptor complexes directly bind to the HoxC4 promoter, and not to the AID promoter. Activating HoxC4 transcription leads to induce AID expression in response to inflammations and infections (Orthwein and Noia, 2012; Park, 2012). Estrogen induced AID expression by >20 fold, therefore we can suggest that estrogen-induced autoimmunity

and oncogenesis may be caused by AID as a DNA instability factor (Pauklin et al., 2009). Estrogen mediating genome instability via the activation of AID may provide a novel molecular mechanism that is important for breast cancer pathology (Pauklin et al., 2009; Mechtcheriakova et al., 2012).

Chronic inflammation also leads to aberrant AID expression and increase AID expression which contributes to cancer development by inducing genetic alterations in epithelial cells. The development of hepatocellular carcinoma (HCC) caused by hepatitis virus infection and gastric cancer caused by *H. pylori* infection, are representative examples of inflammation-associated carcinogenesis. Inflammation pathophysiology is associated with the transcriptional factor NF-κB which is activated by various proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, and viral/bacterial infection. Recent studies indicated that the mutagenic activity of AID may cause inflammation-associated carcinogenesis in various tissues (Takai et al., 2011; Shimizo et al., 2012). Other studies provide strong evidence that the inflammation-induced AID expression is required for epithelial to mesenchymal transition (EMT) in breast cancer cells. EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain invasive properties allowing them to migrate easily (Rebhandl et al., 2015).

Women who are exposed to some sources of environmental pollution such as pesticides, fertilizers, dusts are at higher risk of breast cancer. Environmental pollution is a major cause of chronic inflammation; exposure occurs at certain workplaces and in the general population from foods. One of the environmental pollutant is known as Cd (the Ames-negative carcinogen). Cd has been shown to induce AID gene expression in somatic cells via the activation of NF-κB. It is reported that Cd activates NF-κB and also estrogen receptor ER which enhances AID transcription as we mentioned before (Tatemishi et al., 2014).

Aberrant expression of AID in autoimmune diseases causes the somatic mutations and dysfunction of tumor suppressor genes like *TP53*. Several studies showed that AID was up-regulated in non-lymphoid tumor cells such as breast cancer, cholangiocarcinoma, hepatoma and colorectal cancer cells (Takai et al., 2009). Moreover, the somatic mutations of *TP53* found in these cancer cells appeared to be a direct target of AID. These studies suggest a possible mechanism by which the aberrant expression of AID induces somatic mutations in *TP53*, leading to tumor formation (Igarashi et al., 2010).

Infections with some bacteria and viruses play a role in developing cancer, one of their mechanisms is by increasing AID expression. *Helicobacter pylori*, a class 1 carcinogen for human gastric cancer, affects AID expression, thereby contributing to the accumulation of mutations in tumor-related genes. Aberrant AID activity may therefore be a novel link between infection and carcinogenesis (Marusawa and Chiba, 2010). Some viruses, especially *human papilloma virus* HPV, have been suspected to play an etiological role in breast cancer. Many molecular studies indicated that HPV DNA is present at a high frequency in BC samples but is rare in normal breast tissues. HPV could trigger aberrant expression of AID in breast epithelial cells which results in genomic instability and mutations in TP53 tumor suppressor gene (Matsumoto et al., 2007; Nagata et al., 2014; Ohba et al., 2014).

This study highlighted the importance of the DNA editor, AID, in the cellular events leading to genetic mutations during the development of breast cancers. We demonstrated for the first time that aberrant AID protein expression is found in BC cells in Palestinian patients in agreement with other studies. It is the first study done in Palestine and worldwide to examine the AID protein expression in breast cancer tissues by IHC technique. Here, a possible suggestion for AID activation in these BC cases is that estrogen my binding of estrogen receptor on AID promoter and induces its expression, since we found that most AID positive cases are estrogen receptor positive.

The limitations of this study is there is a small number of BC cases to work with and the lack of clinical data for some patients like the hormone receptor status, and fresh tissue samples were not available, so cooperation with researchers and the hospitals is of great importance

In summary, AID may represent a new marker for breast cancer. AID inhibition may decrease the rate of tumor evolution and stabilize the targets of existing therapeutics (Burns et al., 2013). In this study possible suggestion for AID activation in these BC cases is that estrogen may induce AID expression by binding to its promoter and enhancing its transcription, leading to AID expression induction. Inflammation may also play an important role in activating AID and contributing to BC development through NF-κB pathway. Activated AID induces mutations in tumor suppressor genes and oncogenes. So AID could provide genetic fuel for cancer development and metastasis. AID may be a prime therapeutic target because it is rarely expressed in most normal tissues, and it is a dominant-acting enzyme with an active site that may be druggable (Harris, 2015). Further studies are needed to verify this hypothesis.

In conclusion, our findings showed that aberrant AID expression may be involved in some BC patients. No correlation was detected between AID expression and age, tumor stage or tumor grade. AID may have a diagnostic and prognostic utility in the future. More studies are needed to examine AID induction, regulation and mechanism of action within BC. Further studies are required to examine whether the elevated levels of AID could contribute to the development of metastases in BC.

4.2 Recommendations

Little is known and much remains to be learned about the molecular identification and mechanism of induction of AID in breast cancer. To support our results, further molecular studies on AID expression using large scales of BC patients (100 cases or more) are needed.

In Palestine, many obstacles are existed in the research field including: the difficulty of having fresh tissue from patients to apply different molecular techniques such as RT-PCR and western blot. Moreover, the lack of clinical data in the patients files which makes the statistical analysis of the obtained data for clinical research inadequate. Joint cooperation between researchers, hospitals and pathologists are recommended.

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دراسة جزيئية عن مرض سرطان الثدي في فلسطين: فحص مستوى البروتين

Activation Induced Cytidine Deaminase (AID)

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الملخص

سرطان الثدي (Breast Cancer) هو مرض خبيث ينتج عن النمو غير الطبيعي لخلايا الثدي.
عالميا يعتبر سرطان الثدي الاكثر شيوعا بين النساء و هو ثاني سبب رئيسي للوفاة و معدل الاصابة بلغ امرأة من 8 نساء بمعدل 12.2%. في فلسطين سرطان الثدي من أكثر أنواع السرطانات شيوعاً وهو في المرتبة الاولى بين السرطانات المؤدية للوفاة و معدل الاصابة به 11.8% بحسب احصائيات وزارة الصحة الفلسطينية لعام 2011.

بروتين (AID) من عائلة أنزيمات ال (APOBEC) يلعب دورا هاما في جهاز المناعة وبناء الاجسام المضادة و هو البروتين الوحيد الذي دوره الطبيعي في الجسم هو خلق تنوع في جينات الاجسام المضادة في الخلايا البائية في الغدد اللمفاوية و نتيجة هذا التنوع في الجينات يحصل التنوع في الأجسام المضادة لمكافحة أكبر عدد من الجراثيم التي تدخل جسم الانسان و تعزيز جهاز المناعة. البروتين (AID) يلعب دورا هاما في جهاز المناعة وبناء الاجسام المضادة الا ان هذا البروتين قد يستهدف جينات أخرى غير التي يستهدفها بشكل طبيعي من أجل تقوية جهازا المناعة و هذه الجينات تلعب دورا هاما في نشأة السرطان. العديد من الدراسات التي أجريت مؤخرا أثبتت ارتفاع مستوى البروتين (AID) في الخلايا في أنواع متعددة من السرطانات مثل سرطان المعدة و الكبد و الامعاء و الثدي و الرئة و غيرها.

الهدف من هذه الدراسة, هو فحص مستوى هذا البروتين في عينات لمرضى سرطان الثدي. لذلك قمنا بجمع 69 عينة من مرضى سرطان الثدي من مستشفى بيت جالا, بين الأعوام (2009– 2013) و قمنا بفحص مستوى البروتين (AID) من خلال تقنية ال (IHC) Immunohistochemistry , و هذه الدراسة هي الأولى على مستوى العالم و في فلسطين اللتي تفحص مستوى بروتين (AID) في عينات من نسيج مرضى سرطان الثدي بواسطة IHC .

أظهرت النتائج ان البروتين (AID) موجود عند 26% من عينات مرضى سرطان الثـــدي التـــي تـــم فحصـــها. و اظهرت نتائج الاحصائية عدم وجود علاقة بين مستوى هذا البروتين مع المتغيرات الطبية للمرضى مثل العمـــر و مستوى السرطان و درجته و هذه النتائج تشكل الخطوة الأولى في فهم علاقة هذا البروتين في نشأة سرطان الثدي و فتح آفاق جديدة للبحث العلمي في مجال سرطان الثدي على المستوى الجزيئي في فلسطين. و لتحقيق ذلك أوصـــي بالتعاون المتكامل بين المستشفيات و الباحث العلمي لإنجاح البحث و تزويده بكافة العناصر الضرورية.