بســـم الله الرحمن الرحيم

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Evaluation of E-Cadherin and Vimentin Expression as Prognostic Markers for Epithelial-Mesenchymal Transition and Tumor Aggressiveness in Breast Cancer

A Thesis Submitted for Partial Fulfillment for Requirement of M.Sc. Degree in Medical Laboratory Sciences, (Histopathology & Cytology)

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الآية

بسم الله الرحمن الرحيم

قال تعالى :

(وَلَقَدْ آتَيْنَا لُقْمَانَ الْحِكْمَةَ أَنِ اشْكُرْ لِلَّهِ ۚ وَمَنْ يَشْكُرْ فَإِنَّمَا يَشْكُرُ لِنَفْسِهِ ۖ وَمَنْ كَفَرَ فَإِنَّ اللَّهَ غَنِيٌّ حَمِيدٌ)

صدق الله العظيم

سورة لقمان الآية (12)

Dedication

To my mother, to her strength and patience

in her stubborn fight with cancer

Love and respect.

To my father,

brother,

Sisters,

I'm grateful for having you in my life.

To my beloved friends, wherever they are.

Acknowledgment

First of all, I am thankful and grateful to my God (Allah) for making me able to do this research.

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Finally, I'm wishing all the luck to my colleagues in the master program.

Abstract

This is a descriptive retrospective laboratory based study, conducted at El-rahma medical centre, during the period from March to July 2018. The study aimed to evaluate the expression E-cadherin and Vimentin as prognostic markers for Epithelial-Mesenchymal Transition (EMT) and tumor aggressiveness in breast cancer using immunohistochemistry.

Fifty six paraffin blocks were collected from archive for women patients previously diagnosed as breast cancer. Tissue Microarrays (TMAs) prepared from paraffin blocks, were cut by rotary microtome, and then stained by immunohistochemistry method (Dextran labeled polymer). The data obtained was analyzed using SPSS program version 22.0, Frequencies, mean, Chi-square test values and Pearson's correlations were calculated.

The age of patients ranged between 30 to 80 years with mean age of 51.1 years and standard deviation was 10.1. The study found that most patients were more than 40 years 45/56 (80%). The majority of the study cases were in age group of 50-59 representing 19/56 (33.9 %), followed by 40-49 representing 16/56 (28.6%), 60-69 representing 10/56 (17.9%), 30-39 representing 9/56 (16.1%) and 70-79 representing 2/56 (3.6%).

The histopathological diagnosis of cases revealed that, the majority of diagnosed samples were invasive ductal carcinoma (IDC) in 40/56 (71.4%), medullary carcinoma (MC) in 14/56 (25%) and colloid (mucinous) carcinoma (CC) in 2/56 (3.6%).

The histological grade of the study population showed, grade I in 6/56 (10.7%), grade II in 20/56 (35.7%) and grade III in 30/56 (53.6%).

The study showed that, E-cadherin expression was positive in 49/56 (87.5%), negative in 7/56 (12.5%). Positive E-cadherin expression was weakly expressed in

18/49 (36.7%), moderately expressed in 27/49 (55.1%) and strongly expressed in 4/49 (8.2%), this result revealed a significant correlation between E- cadherin expression with age and histological grade (p-value = 0.028 – 0.027) respectively, but the correlation with the histological type is not significant (p-value = 0.126).

Vimentin expression was positive in 55/56 (98.2%), negative in 1/56 (1.8%). Positive Vimentin expression was weakly expressed in 29/55 (52.7%), moderately expressed in 26/55 (47.3%) and strongly expressed in 0/55 (0%), this result showed a significant correlation between Vimentin expression with age and histological type (p-value = 0.016 – 0.004) respectively, but the correlation with the histological grade is not significant (p-value = 0.051).

The study revealed an inverse correlation between E-cadherin and Vimentin (r = -0.389) and strong significant correlation (p-value = 0.002).

The study concluded that, decreased expression of E-cadherin and increased expression of Vimentin was associated with Epithelial-Mesenchymal Transition (EMT) and tumor aggressiveness.

الخلاصة

أجريت هذه الدراسة المعملية الوصفية ، الإسترجاعية بمركز الرحمة الطبي خلال الفترة من مارس وحتى يوليو 2018. تهدف الدراسة لتقييم تعبير الكادرين الطلائي والفايمنتين كموسّمات تشخيصية للتحوّل الطلائي – الوسيطي والأورام العدوانيّة في سرطان الثدي باستخدام كيمياء الأنسجة المناعية.

جُمع ستٌ وخمسون قالب شمعي من الإرشيف لعينات نساء مرضى تم تشخيصهم مسبقاً بأنهم مصابين بسرطان الثدي. حُضّرت مصفوفات الأنسجة الصغيرة من قوالب الشمع وقُطعت باستخدام المشراح الدوّار وصُبغت بواسطة طريقة كيمياء الأنسجة المناعية (بوليمر الديكستران المعلَّم). واستُخدم برنامج الحزم الإحصائية للعلوم الاجتماعية ، النسخة 22 لتحليل البيانات. حُسبت الترددات ، المتوسط ، قيم إختبار مربع كاي وارتباطات بيرسون.

تراوحت أعمار المرضى بين 30 – 80 عام بمتوسط عمر 50.1 سنة وكان الإنحراف المعياري 10.1. أظهرت الدراسة أن معظم المرضى كانت أعمارهم أكثر من 40 سنة. كانت غالبية الحالات تحت الدراسة في الفئة العمرية من 50-59 والتي تمثل 19\56 (33.9%) ، تليها 40-49 التي تمثّل 16\56 (28.6%) ، 60-69 التي تمثّل 10\56 (17.9%) ، 30-39 يمثّلون 9\56 (16.1%) ، 70-79 التي تمثّل 2\56 (3.6%).

أظهر تشخيص الورم الخبيث للحالات أن غالبية العينات كانت سرطان الأقنية الغازية في 40\56 (71.4%) ، وسرطان النخاع التوسعي في 14\56 (25%) ، وسرطان ميوسيني في 2\56 (3.6%).

أظهر التمايز النسيجي لعينة الدراسة ، النوع الأول في 6\56 (10.7%) ، والنوع الثاني في 20\56 (35.7%) ، والنوع الثالث في 30\56 (53.6%).

أظهرت الدراسة أن تعبير الكادرين الطلائي كان إيجابياً في 49\56 (87.5%) ، سلبيا في 7\56 (12.5%). التعبير الإيجابي للكادرين الطلائي كان معبّراً بشكل ضعيف في 18\49 (37.3%) ، معبّراً بشكل متوسط في 27\49 (55.1%) ، وتم التعبير عنهُ بوضوح في 4\49 (8.2%) ، كشفت هذه النتيجة عن ارتباط معنوي بين تعبير الكادرين الطلائي مع العمر والتمايز النسيجي (قيمة p = 0.028 – 0.027) على التوالي ، لكن لم يكن هناك ارتباط معنوي مع النوع النسيجي (قيمة p = 0.126).

كان تعبير الفايمنتين إيجابياً في 55\56 (98.2%) ، سلبياً في 1\56 (1.8%). التعبير الإيجابي للفايمنتين كان معبّراً بشكل ضعيف في 29\55 (52.7%) ، معبّراً بشكل متوسط في 26\55 (47.3%) ، ومعبّراً عنه بوضوح في 0\55 (0%) ، كشفت هذه النتيجة عن ارتباط معنوي بين تعبير الفايمنتين مع العمر

والنوع النسيجي (قيمة p = 0.016 – 0.004) على التوالي ، لكن لم يكن هناك ارتباط معنوي مع التمايز النسيجي (قيمة p = 0.051).

كشفت الدراسة عن وجود علاقة عكسية بين الكادرين الطلائي والفايمننتين (r = -389) ، وارتباط معنوي قوي (قيمة p = 0.002).

خلصت الدراسة إلى أنّ انخفاض تعبير الكادرين الطلائي وزيادة تعبير الفايمننتين يرتبط بالانتقال الطلائي – الوسيطي وعدوانية الورم.

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List of abbreviations

ATM	Ataxia Telangiectasia Mutated
BRCA	Breast Cancer Gene
CC	Colloid (mucinous) Carcinoma
CEA	Carcinoembryonic Antigen
CECs	Circulating Endothelial Cells
CNB	Core Needle Biopsy
CT	Chemotherapy
DAB	3, 3 Diaminobenzidinetetrahydrochloride
DCIS	Ductal Carcinoma In-Situ
DM	Digital Mammography
EC	E-Cadherin
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
ET	Endocrine Therapy
EPCs	Endothelial Precursor Cells
FISH	Fluorescence In-Situ Hybridization
FNAC	Fine Needle Aspiration Cytology
HER2	Human Epidermal Growth factor Receptor 2
HICs	High-Income Countries
HRT	Hormone Replacement Therapy
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma

IHC	Immunohistochemistry
LCIS	Lobular Carcinoma In-Situ
LMICs	Low Middle-Income Countries
LNM	Lymph Node Metastasis
MBI	Molecular Breast Imaging
MC	Medullary Carcinoma
MET	Mesenchymal-Epithelial Transition
MRI	Magnetic Resonance Imaging
NF-1	Neurofibromatosis-1
NOS	Not Otherwise Specified
PBS	Phosphate Buffer Saline
PTEN	Phosphatase and Tensin
RT	Radiation Therapy
SPSS	Statistical Package of Social Sciences
STK-11	Serine-Threonine Kinase -11
TP53	Tumor Protein 53
TMA	Tissue Microarray
TNM	Tumor Node Metastasis

Chapter One

Introduction

1. Introduction

1.1 Overview

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012 [1], accounting for 25% of cancer cases and 15% of cancer-related deaths. The highest breast cancer incidence rates are in North America, Australia, New Zealand, and Northern and Western Europe. Breast cancer mortality rates are higher in many low middle-income countries (LMICs) than in high-income countries (HICs), such as those in sub-Saharan Africa, despite their lower incidence because of late stage at diagnosis and limited access to treatment [2]. International variation in breast cancer incidence rates reflects differences in the availability of early detection as well as risk factors [1].

Annual mortality rates among breast cancer patients were significantly greater in low middle-income countries in South Asia (3.06%) and Sub-Saharan Africa (2.76%), compared to high-income countries like the United States (1.69%). From 2005–2015, mortality in South Asia and Sub-Saharan Africa increased by 27.9% and 19.7%, respectively [3].

In high-income countries, breast cancer is often diagnosed at an early stage and the prognosis is good; in LMICs, however, breast cancer is more often diagnosed at a later stage, and survival is poorer. Five-year survival is 85% or higher in the United States, Canada, Australia, Israel, Brazil, and many Northern and Western European countries, whereas it is 60% or lower in many LMICs, such as South Africa, Mongolia, Algeria, and India [4].

More than 90% of breast cancer-related mortalities are caused not by the primary tumor, but by its metastases at distant sites [5].

In Sudanese population cases comprised 1255 women from central Sudan diagnosed with breast cancer and referred to and treated at Institute of Nuclear

Medicine, Molecular Biology, and Oncology, from January 1999 to December 2006 [6].

Studies on breast cancer in Sudan have been limited. The reasons for this include the lack of population-based cancer registry as well as lack of research resources (manpower and financial) [7].

Established factors that increase breast cancer risk include a family history of the disease, *BRCA*1 or 2 mutations, reproductive factors that influence endogenous estrogen exposure, alcohol drinking, physical inactivity, excess body weight, the use of exogenous hormones, and high-dose radiation to the chest, particularly at a young age [8, 9]. Recent prospective studies have also shown an association between smoking and breast cancer [10, 11]. On the other hand, breastfeeding has been reported to slightly reduce breast cancer risk [12], particularly estrogen and progesterone receptor-negative subtypes [13].

Breast cancer can be broadly categorized into in situ carcinoma and invasive (infiltrating) carcinoma [14]. The major invasive tumor types include infiltrating/ invasive lobular (ILC) or ductal (invasive ductal carcinoma [IDC]). ILC comprises up to 15% of all cases [15]. Infiltrating IDC is, by far, the most common subtype accounting for 70%–80% of all invasive lesions [16].

Based on gene expression profiling, breast cancer has been characterized into Luminal A, Luminal B, HER2 (human epidermal growth factor receptor 2)-enriched, and basal-like subtypes—each of which has been shown to have different prognoses [17].

Methods of breast cancer diagnosis include mammography, magnetic resonance imaging (MRI), molecular breast imaging (MBI), breast biopsy, immunohistochemistry (IHC), Fluorescence in situ hybridization test (FISH), blood based assay [18].

The main types of treatment for breast cancer are surgery, radiation therapy (RT), chemotherapy [19], endocrine (hormone) therapy (ET), and targeted therapy [20].

Epithelial-Mesenchymal Transition (EMT) is a physiological phenotypic shift in which epithelial cells break down cell-cell and cell-extracellular matrix connections and then migrate to other locations in the body [21].

EMT also may be involved in metastasis of epithelial cell malignancies [22]. In cancer cell EMT, E-cadherin protein and activity are decreased, causing disruption of cell-cell junctions and loss of cell polarity. At the same time, cancer cells that undergo EMT demonstrate increased expression of mesenchymal cell proteins such as vimentin [23].

E-Cadherin (EC) is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues [24]. The EC gene is located on chromosome 16q22.1 [25]. EC knockouts have been associated with nonviability and abnormal epithelial morphogenesis. Selective loss of EC can cause dedifferentiation and invasiveness in human carcinomas. In various cell lines, a reciprocal relationship has been shown between levels of EC expression and invasiveness [26].

Vimentin is an intermediate filament that is used as a marker of mesenchymal cells to distinguish them from epithelial cells [27]. Vimentin is expressed at sites of cellular elongation, and is associated with a migratory phenotype. Increased vimentin expression is frequently used as an EMT marker in cancer [28, 29]. There is a positive correlation of vimentin expression with augmented invasiveness and metastasis [30].

E-cadherin and vimentin are now regarded as major and conventional canonical markers of Epithelial-Mesenchymal Transition (EMT) [31].

The reports of previous studies showed that the elevated expression of vimentin contributes to the aggressive phenotype in invasive breast cancer. However, the role of E-cadherin in breast cancer biology might be unclear and

more complex. The aim of this study is to reveal the prognostic importance of the expression of E-cadherin and vimentin in breast cancer.

1.2 Objectives

1.2.1 General objective:

- To evaluate the expression of E-Cadherin and Vimentin as prognostic biomarkers for Epithelial-mesenchymal transition and tumor aggressiveness in Breast Cancer.

1.2.2 Specific objectives:

- To correlate the expression of E-cadherin and Vimentin with age of patients, histological type of breast cancer and tumor Grade.
- To correlate the expression of E-cadherin with the expression of Vimentin.

Chapter Two

Literature Review

2. Literature review

2.1 Scientific background:

Cancer of the breast is a serious, often fatal disease that most often occurs in women. Regular self-examination of the breast can lead to early detection of breast cancer and effective treatment. Most tumors of the mammary glands are benign, but those that are malignant can spread to other areas of the body and ultimately lead to death [32].

2.2 Structure and function of the breast:

Each breast is a hemispheric projection of variable size anterior to the pectoralis major and serratus anterior muscles and attached to them by a layer of fascia composed of dense irregular connective tissue [33]. The mammary (mam′ă-rē ; relating to breasts) glands are the organs of milk production and are located in the breasts [32].

Each breast has one pigmented projection, the nipple, that has a series of closely spaced openings of ducts called lactiferous ducts, where milk emerges. The circular pigmented area of skin surrounding the nipple is called the areola; it appears rough because it contains modified sebaceous (oil) glands. Strands of connective tissue called the suspensory ligaments of the breast (*Cooper's ligaments*) run between the skin and fascia and support the breast. A mammary gland consists of 15 to 20 lobes, or compartments, separated by a variable amount of adipose tissue. In each lobe are several smaller compartments called lobules, composed of grapelike clusters of milk-secreting glands termed alveoli embedded in connective tissue. Contraction of myoepithelial cells surrounding the alveoli helps propel milk toward the nipples. When milk is being produced, it passes from the alveoli into a series of secondary tubules and then into the mammary ducts. Near the nipple, the mammary ducts expand slightly to form sinuses called lactiferous sinuses, where some milk may be stored before draining into a

lactiferous duct. Each lactiferous duct typically carries milk from one of the lobes to the exterior [33].

The functions of the mammary glands are the synthesis, secretion, and ejection of milk; these functions, called lactation, are associated with pregnancy and child-birth. Milk production is stimulated largely by the hormone prolactin from the anterior pituitary, with contributions from progesterone and estrogens. The ejection of milk is stimulated by oxytocin, which is released from the posterior pituitary in response to the sucking of an infant on the mother's nipple (*suckling*) [33].

2.3 Histology of the breast:

Each breast consists of 15 to 25 independent units called breast lobes, each consisting of a compound tubulo-acinar gland. The size of the lobes is quite variable and the bulk of the breast is made up of a few large lobes that connect to the surface. Immediately before opening onto the surface, the duct forms a dilatation called the lactiferous sinus. Smaller lobes end in blind ending ducts that do not reach the nipple surface. The lobes are embedded in a mass of adipose tissue, subdivided by collagenous septa [34].

2.3.1 Inactive (resting) mammary gland:

Inactive mammary gland shows a large amount of dense irregular connective tissue and adipose tissue with small mammary gland lobules. The glandular tissue contains mainly intralobular ducts, which are lined by cuboidal epithelial cells and underlying myoepithelial cells. The resting mammary gland has only a few secretory alveoli, some undeveloped intralobular ducts, interlobular ducts, lactiferous sinuses, and lactiferous ducts [35].

2.3.2 Active (during pregnancy) mammary gland:

Active mammary gland shows large lobules and a relatively small amount of interlobular connective tissue. The glandular tissue contains many proliferated

alveoli and intralobular ducts. A large interlobular duct is located within the connective tissue [35].

2.3.3 The nipple:

The nipple is a small projection at the center of the breast. It is covered by thin skin and surrounded by the areola (pigmented skin) [35]. The nipple contains bands of smooth muscle, orientated in parallel to the lactiferous ducts and circularly near the base [34]. The nipple has many sensory nerve endings that receive stimulation during suckling [35].

2.4 Disorders of the breast:

2.4.1 Fibrocystic changes:

Fibrocystic changes are the most common breast abnormality seen in premenopausal women. Estrogenic therapy and oral contraceptives do not seem to increase the incidence of these alterations, and oral contraceptives may, in fact, decrease the risk [36]. The clinical presentation is variable, ranging from asymptomatic to mastalgia that is related to the menstrual cycle. Histologically, a wide range of lesions are seen within fibrocystic changes, including epithelial metaplasia, hyperplasia of benign or usual type, adenosis, cyst formation, inflammatory changes, and fibrosis. Apocrine metaplasia is common in fibrocystic changes [37]. Certain clinical features of fibrocystic change tend to distinguish it from cancer, but the only certain way of making this distinction is through biopsy and histologic examination. Although fibrocystic changes are benign, some features may confer an increased risk for development of cancer. Proliferative fibrocystic changes usually are bilateral and multifocal and are associated with increased risk of subsequent carcinoma in both breasts. Fibrocystic changes can be subdivided into nonproliferative and proliferative patterns [36].

2.4.1.1 Nonproliferative changes:

Cysts and Fibrosis: Nonproliferative changes are the most common type of fibrocystic lesions, characterized by an increase in fibrous stroma associated with dilation of ducts and formation of variably sized cysts [36].

2.4.1.2 Proliferative Change:

Epithelial Hyperplasia: Normal ducts and lobules of the breast are lined by two layers of cells; a layer of luminal cells overlying a second layer of myoepithelial cells. Epithelial hyperplasia is recognized by the presence of more than two cell layers. The spectrum of epithelial hyperplasia ranges from mild and orderly to atypical hyperplasias with features that resemble those of in situ carcinoma [36].

Sclerosing Adenosis: Is less common than cysts and hyperplasia but is significant because its clinical and morphologic features may mimic those of carcinoma. These lesions contain marked intra-lobular fibrosis and proliferation of small ductules and acini [36].

2.4.2 Inflammatory processes:

* *Acute mastitis*: develops when bacteria, usually Staphylococcus aureus, gain access to the breast tissue through the ducts [36]. Acute mastitis usually occurs at the postpartum period, when the lactating breast tissue is swollen, and sometimes the ducts are obstructed, with inspissation of the secretion. In addition, breast-feeding may cause trauma and cracks to the nipple, resulting in ascending infection of the commensals either originating from the skin or from the suckling baby's oral cavity. More severe cases would result in abscess formation. Histologically, there is infiltration of acute inflammatory cells, mostly neutrophils within the breast parenchyma. When there is abscess formation, significant necrosis is present with collection of necrotic debris and the exudates [37].

* *Chronic mastitis*: due to specific microorganisms is rare, and among these, granulomatous mastitis due to mycobacterium tuberculosis is probably the most common [38].

* *Mammary duct ectasia (plasma cell mastitis)*: is a nonbacterial chronic inflammation of the breast associated with inspissation of breast secretions in the main excretory ducts. Ductal dilation and eventual rupture leads to reactive changes in the surrounding tissue that may present as a poorly defined periareolar mass with nipple retraction, mimicking the changes caused by some cancers [36].

* *Fat necrosis*: is a specific type of inflammatory change that occurs in the breast due to trauma or surgical procedure. Traumatic injury to the breast tissue causes disruption of the adipocytes, resulting in the release of the lipid into the stroma, eliciting an inflammatory response [37].

2.4.3 Benign tumors of the breast:

2.4.3.1 Fibroadenoma:

Fibroadenomas are benign breast tumors composed of epithelial and stromal components [39]. Presenting as solitary painless, mobile, and well-defined nodules [37].

Women can present with these lesions at any age, but the tumors are most commonly diagnosed when the patients are in their 20s, an age when breast cancer is extremely rare. Perhaps for this reason, fibroadenomas have traditionally been thought to be unrelated to breast cancer. Fibroadenomas exhibit a wide range of cytologic and histologic patterns; the epithelial component can vary from an absence of hyperplastic activity to carcinoma in situ. It would thus be valuable if a subgroup of patients with fibroadenoma could be identified who are at a particularly high risk for breast cancer [39].

2.4.3.2 Phyllodes Tumor:

Phyllodes tumor is uncommon fibroepithelial neoplasm that resembles fibroadenoma grossly. Patients with phyllodes tumors usually are older than patients with fibroadenomas, and there may be a history of a rapidly growing mass [37].

2.4.3.3 Hamartoma:

Hamartoma may present as a soft palpable mass or as breast asymmetry, and is usually round to oval and lobulated. Histologically, it shows ducts, lobules, interlobular fibrosis, smooth muscle, and adipose tissue in varying proportions. This is a benign tumor and rarely recurs [37].

2.4.3.4 Intraductal papilloma:

Intraductal papilloma is a benign neoplastic papillary growth. It is most often seen in premenopausal women [36]. Papillomas can be divided into solitary or multiple. Solitary papilloma is usually located beneath the nipple, whereas the multiple papillomas are more peripherally located. The former is more likely to present as nipple discharge and the latter is usually asymptomatic [40].

2.4.4 Malignant tumors of the breast:

2.4.4.1 Carcinoma in situ:

Ductal carcinoma in situ (DCIS) is increasingly diagnosed as a non-palpable lesion. Newer classifications/grading always use nuclear grade as one of the de fining features for DCIS. Other histologic features being used are necrosis and the presence of tumor cell polarization – the organization of the nuclei around lumens within the tumor, resulting in rosette or cribriform structures [41, 42].

High-grade DCIS is easily differentiated from benign lesions, with the highly pleomorphic tumor cells present within the enlarged ducts associated with central comedo necrosis. The associated calcifications within the necrotic debris produce a characteristic casting or branching pattern in mammography. The ducts may be so

distended that aggregation of these ducts may become palpable. Low-grade DCIS, on the other hand, shows monotonous and uniform tumor nuclei that may sometimes be difficult to distinguish from benign epithelial hyperplasia [37].

Lobular carcinoma in situ has a uniform appearance. The cells are monomorphic with bland, round nuclei and occur in loosely cohesive clusters within the lobules [36]. The neoplastic cells are small and uniform, smaller than the ductal lesions, with higher nuclear cytoplasmic ratio, mild nuclear pleomorphism, rare mitoses, and occasional cytoplasmic vacuoles [43].

2.4.4.2 Papillary carcinoma:

Papillary carcinomas are uncommon malignant lesions, representing several different morphological entities, all possessing a common papillary architecture [37]. Intracystic or encapsulated papillary carcinoma is rare, usually occurring in older women, and may present as a breast mass. The characteristic feature of this tumor is the essentially absence of a complete layer of myoepithelial cells on the outside and the delicate nature of the papillary fronds [19]. Intracystic papillary carcinoma has a good prognosis, having better outcome than mixed intracystic papillary/nonpapillary tumors [19, 44].

2.4.4.3 Invasive carcinoma:

Invasive breast carcinomas are classified based on their histological features, and this classification also reflects their clinical behavior [37].

2.4.4.3.1 Invasive ductal carcinoma:

A majority (70% to 80%) of cancers fall into this group. This type of cancer usually is associated with DCIS and, rarely, LCIS. Most ductal carcinomas produce a desmoplastic response, which replaces normal breast fat (resulting in a mammographic density) and forms a hard, palpable mass. The microscopic appearance is quite heterogeneous, ranging from tumors with well-developed tubule formation and low-grade nuclei to tumors consisting of sheets of anaplastic

cells. The tumor margins typically are irregular. Invasion of lymphovascular spaces may be seen. About two thirds express estrogen or progesterone receptors, and about one third overexpress HER2/NEU [36]. Most IDCs are graded based on three microscopic features, namely, the degree of tubule formation, nuclear pleomorphism, and mitotic count. The tumor cells in higher-grade cancer are usually arranged in sheets or are dis-cohesive, showing very little tubule formation, whereas lower-grade cancer shows significant tubule formation generally. Nuclear morphology is also evaluated in terms of variation in nuclear size, regularity of the nuclear border, hyperchromasia, and prominence of nucleolus. Mitoses are usually counted per 10 high-power microscopic fields. A combination score on these three components reflects tumor grade and prognosis. Furthermore, hormone receptor assessment is also mandatory in tumor evaluation. ER, PR, and HER2 protein expression are routinely performed by immunohistochemistry, and the results carry prognostic and predictive significance [37].

2.4.4.3.2 Invasive lobular carcinoma:

Invasive lobular carcinoma represents 5–10 % of the invasive breast tumors [37]. Consists of cells morphologically identical to the cells of LCIS. Two thirds of the cases are associated with adjacent LCIS. The cells invade individually into stroma and are often aligned in "single-file" strands or chains. This growth pattern correlates with the presence of mutations that abrogate the function of E-cadherin, a surface protein that contributes to the cohesion of normal breast epithelial cells. Although most manifest as palpable masses or mammographic densities, a significant subgroup may exhibit a diffusely invasive pattern without a desmoplastic response and may be clinically occult [36].

2.4.4.3.3 Inflammatory carcinoma:

Inflammatory carcinoma is defined by the clinical presentation of an enlarged, swollen, erythematous breast, usually without a palpable mass. The underlying

carcinoma is generally poorly differentiated and diffusely infiltrative. Characteristically, carcinoma involves dermal lymphatic spaces. The resultant blockage of these channels leads to edema, resulting in the characteristic "inflamed" clinical appearance; true inflammation is minimal to absent. Many of these tumors metastasize to distant sites; the overall 5-year survival is under 50%, and understandably even lower in those with metastatic disease at diagnosis [36].

2.4.4.3.4 Medullary carcinoma:

Medullary carcinoma is a rare subtype of carcinoma, accounting for less than 1% of breast cancers. These cancers consist of sheets of large anaplastic cells with well-circumscribed, "pushing" borders. Clinically, they can be mistaken for fibroadenomas. DCIS usually is absent or minimal. Medullary carcinomas occur with increased frequency in women with BRCA1 mutations, although most women with medullary carcinoma are not carriers. These carcinomas uniformly lack the estrogen and progesterone receptors and do not overexpress HER2/NEU (a combination that often is referred to as triple-negative) [36].

2.4.4.3.5 Colloid (mucinous) carcinoma:

Mucinous carcinoma occurs commonly in postmenopausal women, presenting at the clinic with a palpable mass. The tumor is usually circumscribed and associated with radiologic microcalcification [37]. The tumor cells produce abundant quantities of extracellular mucin, which dissects into the surrounding stroma. On gross evaluation, the tumors usually are soft and gelatinous. Most express hormone receptors but do not overexpress HER2/NEU [36].

2.4.4.3.6 Tubular carcinomas:

Tubular carcinomas rarely present as palpable masses but account for 10% of invasive carcinomas. They usually are detected as irregular mammographic densities. On microscopic examination, the carcinomas consist of well-formed tubules with low-grade nuclei. Lymph node metastases are rare, and prognosis is

excellent. Virtually all tubular carcinomas express hormone receptors and do not show HER2/NEU overexpression [36].

2.4.5 Common Features of Invasive Cancers:

In all forms of breast cancer, local disease progression leads to similar physical findings. Invasive cancers tend to become adherent and fixed to the pectoral muscles or deep fascia of the chest wall and the overlying skin, with consequent retraction or dimpling of the skin or nipple. The latter is an important sign because it may be the first indication of malignancy. Involvement of the lymphatic pathways may result in localized lymphedema. In such cases, the skin becomes thickened around exaggerated hair follicles, giving an appearance known as peau d'orange ("orange peel") [36].

2.5 Epidemiology and Risk Factors of breast cancer:

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed and 521,900 deaths in 2012 (second most common cancer overall). This represents about 12% of all new cancer cases (14.1 million). Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females. Survival rates vary widely, optimistically heading toward a positive trend. Increased survival is due to the dramatic shift in the screening methods, early diagnosis, and breakthroughs in treatments [45].

2.5.1 Age:

Risk steadily increases throughout life, especially after menopause, peaking at roughly 80 years of age; 75% of women with breast cancer are older than 50 years of age, and only 5% are younger than 40 [36].

2.5.2 Geographic Variations:

Surprising differences in the incidence and mortality rates of breast cancer have been reported for various countries. The risk for development of this disease is significantly higher in North America and northern Europe than in Asia and Africa.

These differences seem to be environmental rather than genetic in origin, because migrants from low-incidence to high-incidence areas tend to acquire the rates of their adoptive countries, and vice versa. Diet, reproductive patterns, and nursing habits are thought to be involved [36].

2.5.3 Race/Ethnicity:

The highest rate of breast cancer is in non-Hispanic white women. However, Hispanic and African American women tend to develop cancer at a younger age and are more likely to develop aggressive tumors that present at an advanced stage. Such disparities between ethnicities are an area of intense study and currently are thought to be due to a combination of genetic differences and social factors, such as lifestyle choices and access to health care [36].

2.5.4 Personal history:

A personal history of breast cancer is also a significant risk factor for the development of a second ipsilateral or contralateral breast cancer. In fact, the most common cancer amongst breast cancer survivors is a metachronous contralateral breast cancer [46]. Factors associated with an increased risk of a second breast cancer include an initial diagnosis of DCIS, stage IIB, hormone receptor negative cancers, and young age [47].

2.5.5 Breast pathology:

Proliferative breast disease is associated with an increased risk of breast cancer. Proliferative breast lesions without atypia, including usual ductal hyperplasia, intraductal papillomas, sclerosing adenosis and fibroadenomas confer only a small increased risk of breast cancer development, approximately 1.5-2 times that of the general population [48]. Atypical hyperplasia including both ductal and lobular, usually incidentally found on screening mammography, confers a substantial increased risk of breast cancer. Women with atypia have an approximately 4.3 times greater risk of developing cancer compared to the general population [48, 49].

2.5.6 Family history:

A woman's risk of breast cancer is increased if she has a family history of the disease. A history of a sister with breast cancer also demonstrated an increased relative risk of 1.66 if the diagnosis was made prior to age 50 and a relative risk of 1.52 if diagnosed after age 50 compared to patients without a family history [50].

The highest risk is associated with increasing number of first degree relatives diagnosed with breast cancer at a young age (under age 50). Compared with women who had no affected relative, women who had one, two or three or more affected first degree relatives had risk ratios of 1.80, 2.93 and 3.90, respectively [51].

2.5.7 Genetic predisposition

Approximately 20%-25% of breast cancer patients have a positive family history but only 5%-10% of breast cancer cases demonstrate an autosomal dominant inheritance [52, 53]. Genetic predisposition alleles have been described in terms of clinical significance [51]. High-risk predisposition alleles conferring a 40%-85% lifetime risk of developing breast cancer include BRCA1 and BRCA2 mutations, mutations in TP53 gene resulting in Li-Fraumeni syndrome, PTEN resulting in Cowden syndrome, STK11 causing Peutz-Jegher's syndrome, Neurofibromatosis (NF1) and (CDH-1) E-Cadherin [54].

Half of the breast cancer predisposition syndromes are associated with mutations in BRCA1 and BRCA2. Women with BRCA1 or BRCA2 deleterious mutations have a significantly higher risk of developing breast cancer. Lifetime breast cancer risk ranges from 65% to 81% for BRCA1 mutation carriers and 45% to 85% for BRCA2 carriers [55-57]. Moderate risk genes including homozygous ataxia-telangiectasia (ATM) mutations [58]. Numerous low-risk common alleles have been identified largely through genome-wide association studies, and the clinical application in the presence of these mutations is yet to be determined [51].

2.5.8 Endogenous hormone exposure and reproductive factors:

2.5.8.1 Early menarche:

Early age at menarche is a risk factor among both pre- and postmenopausal women for developing breast cancer. Delay in menarche by two years is associated with corresponding risk reduction of 10% [51].

2.5.8.2 Parity and age at first full term pregnancy:

Nulliparous women are at an increased risk for the development of breast cancer compared to parous women. Young age at first birth has an overall protective effect, whereas relatively advanced age at first birth confers a relative risk of breast cancer greater than that of a nulliparous woman [59].

2.5.8.3 Breast feeding:

Evidence suggests that breast feeding has a protective effect against the development of breast cancer. Breast feeding may delay return of regular ovulatory cycles and decrease endogenous sex hormone levels. It has been estimated that there is a 4.3% reduction for every one-year of breast feeding [51].

2.5.8.4 Testosterone:

High endogenous sex hormone levels increase the risk of breast cancer in both premenopausal and postmenopausal women. High levels of circulating testosterone in postmenopausal women have been linked to increased risk of developing breast cancer [60].

2.5.8.5 Age at menopause:

Later onset of menopause has also been associated with increased breast cancer risk. Every year delay in the onset of menopause confers a 3% increase in risk and every five year delay in the onset of menopause confers a 17% increase in risk of breast cancer [51].

2.5.9 Exogenous hormone exposure:

Evidence suggests a relationship between the use of hormone replacement therapy (HRT) and breast cancer risk. Breast cancers related to HRT use are usually hormone receptor positive. When compared with patients who do not use HRT, breast cancer risk is higher in HRT users [61].

2.5.10 Life style factors:

2.5.10.1 Alcohol consumption:

Alcohol consumption has been associated with increased breast cancer risk. Alcohol intake both earlier and later in adult life was independently associated with risk [62].

2.5.10.2 Smoking:

Smoking tobacco also increases the risk of breast cancer with the greater the amount smoking and the earlier in life smoking begins the higher the risk [63].

2.5.10.3 Obesity:

Obesity, specifically in postmenopausal women, has also been shown to increase a woman's risk of breast cancer [61]. Insulin resistance and hyperinsulinemia have been studied as a risk factor for the comorbidities associated with obesity including cardiovascular disease and diabetes. Insulin has anabolic effects on cellular metabolism and insulin receptor overexpression has been demonstrated in human cancer cells [64]. Hyperinsulinemia has been shown to be an independent risk factor for breast cancer in non-diabetic postmenopausal women and may help to explain the relationship between obesity and breast cancer [65].

2.5.10.4 Radiation:

Radiation exposure from various sources including medical treatment and nuclear explosion increases the risk of breast cancer. Radiation to the chest wall for treatment of childhood cancer increases the risk of breast cancer linearly with chest radiation dose [66].

2.6 Diagnosis of breast cancer:

2.6.1 Mammography:

A mammogram is an X-ray picture of the breast. Digital mammography has replaced conventional (film screen) mammography in some breast screening services. Potential advantages of DM include the use of computer-aided detection, algorithm-based computer programs that alert the radiologist to possible abnormalities on the mammogram and allowing centralized film reading. Mammography frequent use, however, warrants diligent analysis of potential radiation risk. Moreover, false-positive calls lead to additional imaging or histopathological assessment, mainly percutaneous breast biopsy [45].

2.6.2 Magnetic resonance imaging (MRI):

MRI is a powerful imaging tool that produces high-resolution images without requiring the application of harmful radiation. This technique is similar to nuclear magnetic resonance where a proton density image of the tissue is studied to generate an MRI image. MRI of breast depends on the enhancement of lesions after intravenous injection of contrast agent. The neovascularization of the tumor tissues is characterized by high permeability and thus the contrast material extravasates in the tumor tissue [45].

2.6.3 Molecular breast imaging (MBI):

MBI uses a radioactive tracer that lights up cancer tissues of the breast, visualized by a nuclear medicine scanner. MBI has comparable sensitivity to MRI and rather a higher specificity that can detect small breast lesions [45].

2.6.4 Ultrasound:

There are several studies supporting the use of adjunctive screening ultrasound in high risk patients with dense breast tissue, which imparts substantial but accepted number of false positives [51].

2.6.5 Breast biopsy:

The only definitive method for diagnosing breast cancer is with a breast biopsy. There are several different types of breast biopsies. To increase diagnostic accuracy and eliminate as many false negative results as possible, clinical breast examination, breast imaging, and biopsy are performed simultaneously (triple test). Two types of needle biopsies are used to diagnose breast cancer: fine needle aspiration cytology (FNAC) and core needle biopsy (CNB) [45].

2.6.6 Immunohistochemistry (IHC):

IHC is a technique that uses antibodies as a tool to detect protein expression. 40 Monoclonal or polyclonal antibodies complementary to the antigen of interest are labeled with a marker (either visible by light microscopy or fluorescence), allowing detection of the antibodies bound to regions of protein expression in a tissue sample. Diagnostic IHC is widely used, for example, to detect tissue markers associated with specific cancer [45].

2.6.7 Fluorescence in-situ hybridization (FISH):

FISH is a technique used to identify the presence of specific chromosomes or chromosomal regions through hybridization (attachment) of fluorescently labeled DNA probes to denatured chromosomal DNA. Examination under fluorescent lighting detects the presence of the hybridized fluorescent signal (and hence presence of the chromosome material) [45].

2.6.8 Blood-based assay:

Breast biomarkers are CA 15-3, carcinoembryonic antigen (CEA), and CA 27-29. All have low sensitivity and specificity, and thus are not helpful in the early detection of breast cancer [67]. Mammaglobin is a protein found in mammary tissue and can be detected in serum [68]. Circulating endothelial cells (CECs) as well as bone marrow-derived endothelial precursor cells (EPCs) play an important role in neovascularization and tumor growth [69].

Apoptosis and necrosis of the cancer tissue lead to elevated free DNA/RNA in the blood of the patients by 50-folds. Epigenetic analysis of abnormal DNA methylation has been promising in the detection of breast cancer [67]. It has been demonstrated that extracellular circulating mRNA can be detected in the circulation. Circulating microRNAs (miRNAs) are present and differentially expressed in the serum of breast cancer patients [70].

Antibodies may reflect the immune response to the earliest cancer cells or alternatively a robust antitumor defense associated with reduced risk of developing cancer. Genomic studies have produced a number of useful tissue-based gene signatures that can predict prognosis [67].

2.7 Prognosis of breast cancer:

Prognosis of breast cancers is influenced by the following variables, the first three of which are components of the tumor-node-metastasis (TNM) staging classification [36].

2.7.1 Tumor invasion and size:

In situ carcinomas carry an excellent prognosis (5-year survival rate greater than 90%), as do invasive carcinomas less than 2 cm in size (5-year survival rate of 87%) [36].

2.7.2 Extent of lymph node involvement:

With no axillary node involvement, the 5-year survival rate is close to 80%. Survival is inversely related to the number of involved lymph nodes and is less than 50% with 16 or more involved nodes [36].

2.7.3 Distant metastases:

Patients who develop hematogenous spread are rarely curable, although chemotherapy may prolong survival (the 5-year survival rate is approximately 15%) [36].

2.7.4 Histologic grade:

The most common grading system for breast cancer evaluates tubule formation, nuclear grade, and mitotic rate. Well-differentiated carcinomas are associated with a significantly better prognosis than poorly differentiated carcinomas. Moderately differentiated carcinomas initially have a good prognosis, but survival at 20 years approaches that for poorly differentiated carcinomas [36].

2.7.5 The histologic type of carcinoma:

All specialized types of breast carcinoma (tubular, medullary, and mucinous) are associated with a somewhat better prognosis than carcinomas of no special type (ductal carcinomas). A major exception is inflammatory carcinoma, which has a poor prognosis [36].

2.7.6 The presence or absence of estrogen or progesterone receptors:

The presence of hormone receptors confers a slightly better prognosis. However, the practical reason for determining their presence is to predict the response to therapy [36].

2.7.7 Overexpression of HER2/NEU:

Overexpression of this membrane-bound protein is almost always caused by gene amplification and can be determined by immunohistochemistry or by fluorescence in situ hybridization. Overexpression is associated with a poorer prognosis [36].

2.8 Treatment of breast cancer

2.8.1 Surgery:

Breast conservation surgery is the trending approach in the treatment of localized breast cancer [71]. The surgery is preceded by neoadjuvant therapy to shrink tumor bulk. Surgery is usually followed by adjuvant therapy to ensure full recovery and minimize the risk of metastases [20].

2.8.2 Radiotherapy (RT):

Cancer cells that may not be seen during surgery can be killed by radiation to reduce the risk of local recurrence of cancer [20]. RT is a process in which cancer cells are exposed to high levels of radiation directly. RT after surgery shrinks the tumor in combination with CT. But there are some side effects of RT, such as decreased sensation in the breast tissue or under the arm, skin problems in the treated area, for example, soreness, itching, peeling, and/or redness, and at the end of treatment the skin may become moist and weepy [72].

2.8.3 Chemotherapy (19):

Chemotherapy is the standard of care for women with node-positive cancer or with a tumor larger than 1 cm. Hormone receptor–negative disease derives more benefit from chemotherapy than hormone receptor–positive disease. Factors such as age and comorbidities also influence the decision to use chemotherapy [73].

2.8.4 Endocrine therapy (ET):

The purpose of ET is either balancing or blocking hormones. ET is indicated in all patients with detectable ER expression, irrespective of CT and/or targeted therapy. The choice of medication is primarily determined by patient's menopausal status. Other factors include differences in efficacy and side effect profile [20].

2.9 Epithelial mesenchymal transition (EMT):

Epithelial-mesenchymal transition (EMT) is a developmental process in which epithelial cells acquire the motile, migratory properties of mesenchymal cells [21]. The epithelial-mesenchymal transition (EMT) and the reverse process, termed the mesenchymal-epithelial transition (MET), play central roles in embryogenesis. For example, during early embryonic development, the mesoderm generated by EMTs develops into multiple tissue types, and later in development, mesodermal cells generate epithelial organs, such as the kidney and ovary, via METs [74]. The essential features of EMT are associated with disruption of intracellular tight

junctions and loss of cell-cell contact. This results in the loss of epithelial features and the gain of mesenchymal morphology. These cells exhibit an increase in cell self-renewal and an increase in heterogeneous subpopulations. These features enhance cell motility, resulting in the release of cells from the parental epithelial tissue site. These cells gain the ability to reconstitute metastatic colonies at distant sites [75, 76].

2.10 EMT and breast cancer aggressiveness:

Breast cancer originates from epithelial tissue and, hence, originally is characterized by a typical "sheet-like" morphology with apical-basal polarity, with intact tight and adherent junctions, which are separated from the underlying tissues by the basement membrane. Mesenchymal cells are oppositely characterized by loosely associated cells and disorganized cellular layers that lack polarity and tight cell-to-cell adhesion proteins. The morphology of mesenchymal cells is better adapted to cell migration. EMT is typically characterized as loss of epithelial cell adhesion protein E-cadherin and cytokeratins together with the gain of mesenchymal-associated molecules N-cadherin, Vimentin, and fibronectin. The process is described as "cadherin switching", i.e., down-regulation of E-cadherin and up-regulation of N-cadherin [77, 78]. The status of these biomarkers has not been fully evaluated in clinical breast cancer tissues undergoing EMT. However, the expressions of some EMT-associated biomarkers have been detected in a variety of clinical human cancer tissues including breast cancer. Several studies examined EMT-related markers, such as Vimentin, N-cadherin, Snail, Slug, Twist, E-cadherin and cytokeratins expression, in different subtypes of breast cancer tissues by immunohistochemistry (IHC). The data suggest that the EMT-related markers were more likely to be expressed in the basal-like subtype of breast cancer and are related to the aggressiveness of the tumors [79-81].

2.10.1 E-cadherin:

E-Cadherin is a single-span transmembrane glycoprotein that establishes homophilic interactions with adjacent E-cadherin molecules expressed by neighboring cells, thereby forming the core of the epithelial adherens junction. In its cytoplasmic domain, E-cadherin associates with a number of proteins, including three catenins (α, β, and p120), which link E-cadherin to the actin cytoskeleton [82]. Loss or reduction of E-cadherin expression can be caused by somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the *CDH1* promoter [82]. E-cadherin loss ostensibly promotes metastasis by enabling the first step of the metastatic cascade: the disaggregation of cancer cells from one another. However, it has been unclear whether E-cadherin loss also supports the successful completion of additional steps of the invasion-metastasis cascade [83-85].

2.10.2 Vimentin:

Vimentin is an intermediate filament that is used as a marker of mesenchymal cells to distinguish them from epithelial cells [27]. Vimentin's role in cell motility has been implicated in aggressive tumors throughout the body, including the prostate, breast, and lung, and high levels of vimentin expression in cancer cells correlate with accelerated tumor growth and poor prognosis [86]. As a result, Vimentin has been recognized as a well-known marker for the prognosis of EMT [87].

Gamallo, C., *et al.* (1993), reported that the frequency of tumors with reduced E-cadherin expression was significantly higher ($P = 0.0233$) in histological grade 2 and 3 breast carcinomas than in grade 1 tumors. No correlation was observed with nuclear atypia, hormonal receptor levels (estrogen and progesterone), lymph node status, or tumor size [88].

Shiozaki, *et al.* (1991), have reported that nine of 20 cases (45%) displayed reduced E-cadherin expression in infiltrating ductal carcinomas [89].

Jeschke, Udo, *et al.* (2005), found a strong expression of E-cadherin in carcinoma in situ in all cases investigated. A median expression was detected in invasive tumor cells without metastasis in 80% of all cases investigated. Expression of E-cadherin was down-regulated in invasive tumor cells with lymph node metastasis (LNM) in 90% of all cases. Significant differences of E-cadherin staining between carcinoma in situ and invasive carcinoma with LNM (p=0.043), and carcinoma in situ and LNM (p=0.018) [90].

Heatley, M., *et al.* (1993), reported that, associations were identified between vimentin expression and tumor grade, size, number of lymph nodes affected. Only the association with grade was significant ($p = 0.045$) [91].

Hemalatha, *et al.* (2013), reported that, vimentin expression was seen in 18% of cases and its expression correlated with high tumor grade and high growth fraction (P value < 0.01). It did not correlate with lymph node status and tumor size [92].

Domagala, *et al.* (1993), reported that, vimentin was not expressed in all of 26 lobular carcinomas. Vimentin expression in 10% or more of tumor cells was found in 78% of medullary (14 of 18), in 16% of ductal not otherwise specified (NOS) (35 of 214), and in two of four mucinous carcinomas. A further seven tumors showed vimentin expression in less than 1% to 10% of the cells. Vimentin was expressed in tumor cells of 30% (28 of 93) of grade III invasive ductal NOS carcinomas versus 7% (7 of 105) of grade II and 0% of grade I carcinomas (0 of 10) [93].

Chapter Three
Material and Methods

3. Materials and Methods

3.1 Materials:

Archived tissue block previously diagnosed as breast cancer were selected randomly for this study.

3.2 Methods:

3.2.1 Study design:

This is a descriptive retrospective laboratory based study, aimed to detect the expression of E-cadherin and Vimentin among Sudanese breast cancer patients using immunohistochemistry.

3.2.2 Study sampling and sample size:

65 paraffin blocks previously diagnosed as breast cancer were selected according to the availability of samples from El-rahama medical center. Patient's identification information (age, tumor grade and histological type) were obtained from patients records.

3.2.3 Study area:

This study was conducted at El-rahama medical center in Khartoum state during the period from March to July 2018.

3.2.4 Sample processing:

TMA samples were selected from 56 formalin-fixed paraffin embedded tissue blocks, processed, cut at thickness of (3µm), and mounted on positively charged glass slides (Thermo) and baked at 60 o C for 30 minutes.

3.2.5 Immunohistochemical staining:

The immunohistochemical procedure was done as follows: Following deparaffinization in xylene, TMA slides were rehydrated through a graded series of alcohol and were placed in distilled water. TMAs were steamed for antigen retrieval for E-cadherin and Vimentin using high PH (9) by water bath at 95C for 40 min. After washing with PBS for 3 min Endogenous peroxides activity were

blocked with 3% hydrogen peroxide and methanol for 10 min, and after washing with PBS for 3 min, then each TMA slide were treated separately with (100 µ L) of (mouse monoclonal antibody against E-cadherin, Dako), and (100 µ L) of (mouse monoclonal antibody against Vimentin, Dako) for 30 min at room temperature in a moisture chamber. After washing with PBS for 3 min, binding of antibodies will be detected by incubating for 20 min with dextran labeled polymer (Dako). Finally, the sections washed in three changes of PBS, followed by adding 3, 3 diaminobenzidinetetrahydrochloride (14) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for up to 5 min. After washing with distal water for 3 min Slides were counterstained with haematoxylin (Mayer's) for one minute and washed in running tap water for several minutes 7-10 (bluing), then dehydrated, cleaned, and mounted in DBX.

3.2.6 Data analysis:

Data analysis was done using SPSS 22.0 computer program. Frequencies, mean and Chi-square test values were calculated. Pearson's correlation method was used to evaluate the correlation between the protein expressions in the studied population. $p < 0.05$ was considered as statistically significant.

3.2.7 Result interpretation:

Vimentin expression was evaluated by counting the cells with positive cytoplasmic staining in the region of hotspots. Similarly, E-cadherin expression was evaluated by enumeration of cells exhibiting positive reaction in the membrane and cytoplasmic region as the protein is present in major concentration on the membrane. Staining intensity in both the cases were assigned a scores such as unstained (0), weak (1+), moderate (2+), and strong (3+) stained cells. Immunoscore was calculated with the help of percentage of cells and staining intensity in each of the cases [94].

3.2.8 Ethical consideration:

The samples were collected after permission according to the laboratory guidelines and regulations.

Chapter Four

Result

4. Results

The study includes fifty six samples, previously diagnosed as breast cancer. The age of patients ranges between 30 to 80 years with mean age of 50.1 years and standard deviation 10.1, as indicated in table (4.1).

Majority of the patients were in the age group of 50-59 representing 19/56 (33.9%), followed by 40-49 representing 16/56 (28.6%), 60-69 representing 10/56 (17.9%), 30-39 representing 9/56 (16.1%) and 70-79 representing 2/56 (3.6%) as indicated in table (4.2).

The majority of diagnosed samples were invasive ductal carcinoma (IDC) in 40/56 (71.4%), invasive medullary carcinoma (MC) in 14/56 (25%) and mucinous carcinoma (CC) in 2/56 (3.6%) as indicated in table (4.3).

The histological grade of the study samples includes grade I in 6/56 (10.7%), grade II in 20/56 (35.7%) and grade III in 30/56 (53.6%) as indicated in table (4.4).

E-cadherin expression was positive in 49/56 (87.5%) and negative in 7/56 (12.5%). Positive expression was weakly expressed in 18/49 (36.7%), moderately expressed in 27/49 (55.1%) and strongly expressed in 4/49 (8.2%), as indicated in table (4.5) and (4.6) respectively.

Vimentin expression was positive in 55/56 (98.2%) and negative in 1/56 (1.8%). Positive expression was weakly expressed in 29/55 (52.7%), moderately expressed in 26/55 (47.3%) and strongly expressed in 0/55 (0%) as indicated in table (4.7) and (4.8) respectively.

The correlation between age and expression of E-cadherin revealed a significant correlation (p-value = 0.028), as indicated in table (4.9).

The correlation between age and expression of Vimentin revealed a significant correlation (p-value = 0.016), as indicated in table (4.10).

The correlation between the histological type of tumors and expression of E-cadherin revealed no significant correlation (p-value = 0.126), as indicated in table (4.11).

The correlation between the histological type of tumors and expression of Vimentin revealed a significant correlation (p-value = 0.004), as indicated in table (4.12).

The correlation between the histological grade and expression of E-cadherin revealed a significant correlation (p-value = 0.027), as indicated in table (4.13).

 The correlation between the histological grade and Vimentin expression revealed no significant correlation (p-value = 0.051) as indicated in table (4.14).

Analysis of correlation between E-cadherin and Vimentin expression revealed an inverse correlation with (r value = -0.389) and strong significant correlation (p-value = 0.002) as indicated in table (4.15).

Table (4.1): Mean and standard deviation of age distribution among patients:

No. of patients	Mean	Standard deviation
56	50.1	10.1

Table (4.2): Distribution of age groups among the Patients:

Age group	Frequency	Percentages
30-39	9	16.1
40-49	16	28.6
50-59	19	33.9
60-69	10	17.9
70-79	2	3.6
Total	56	100

Table (4.3): Distribution of histological type among the patient's sample:

Tumor type	Frequency	Percent
IDC	40	71.4
MC	14	25.0
CC	2	3.6
Total	56	100

Table (4.4): Distribution of the histological tumor grade among the study cases**:**

Histological Tumor grade	Frequency	Percent
Grade I	6	10.7
Grade II	20	35.7
Grade III	30	53.6
Total	56	100

Table (4.5): Distribution of E-cadherin expression among the study cases:

E-cadherin expression	Frequency	Percent
Positive	49	87.5
Negative	7	12.5
Total	56	100

Table (4.6): Distribution of E-cadherin positive expression:

Positive Expression	Frequency	Percent
Weak	18	36.7
Moderate	27	55.1
Strong	4	8.2
Total	49	100

Table (4.7): Distribution of Vimentin expression among the study cases:

Vimentin expression	Frequency	Percent
Positive	55	98.2
Negative	1	1.8
Total	56	100

Table (4.8): Distribution of Vimentin positive expression:

Positive Expression	Frequency	Percent
Weak	29	52.7
Moderate	26	47.3
Strong	0	0
Total	55	100

Table (4.9): The correlation between age and expression of E-cadherin:

Expression			Age					Total	P-value
			30-39	40-49	50-59	60-69	70-79		
E-cadherin	Negative		3	3	1	0	0	7	0.028
	Positive	Weak	4	4	7	3	0	18	
		Moderate	1	7	11	6	2	27	
		Strong	1	2	0	1	0	4	
Total			9	16	19	10	2	56	

Table (4.10): The correlation between age and expression of Vimentin:

Expression			Age					Total	P-value
			30-39	40-49	50-59	60-69	70-79		
Vimentin	Negative		0	0	0	1	0	1	0.016
	Positive	Weak	1	10	10	7	1	29	
		Moderate	8	6	9	2	1	26	
		Strong	0	0	0	0	0	0	
Total			9	16	19	10	2	56	

Table (4.11): The correlation between histological type and expression of E-cadherin:

Expression			Histological Type			Total	P-value
			IDC	MC	CC		
E-cadherin	Negative		5	1	1	7	
	Positive	Weak	10	7	1	18	
		Moderate	22	5	0	27	0.126
		Strong	3	1	0	4	
Total			40	14	2	56	

Table (4.12): The correlation between the histological type and expression of Vimentin:

Expression			Histological Type			Total	P-value
			IDC	MC	CC		
Vimentin	Negative		1	0	0	1	
	Positive	Weak	14	14	1	29	
		Moderate	25	0	1	26	0.004
		Strong	0	0	0	0	
Total			40	14	2	56	

Table (4.13): The correlation between histological grade and expression of E-cadherin:

Expression			Histological grade			Total	P-value
			Grade I	Grade II	Grade III		
E-cadherin	Negative		2	1	4	7	
	Positive	Weak	3	10	5	18	
		Moderate	1	8	18	27	0.027
		Strong	0	1	3	4	
Total			6	20	30	56	

Table (4.14): The correlation between histological grade and expression of Vimentin:

Expression			Histological Type			Total	P-value
			Grade I	Grade II	Grade III		
Vimentin	Negative		0	0	1	1	
	Positive	Weak	5	13	11	29	
		Moderate	1	7	18	26	0.051
		Strong	0	0	0	0	
Total			6	20	30	56	

Table (4.15): The correlation between E-cadherin and Vimentin expression:

		E-cadherin	Vimentin
E-cadherin	Pearson Correlation	1	-.398[**]
	Sig. (2-tailed)		.002
	N	56	56
Vimentin	Pearson Correlation	-.398[**]	1
	Sig. (2-tailed)	.002	
	N	56	56

* There is an inverse correlation between E-cadherin and Vimentin expression, ($r =$ -398). This reverse correlation is statistically significant (p-value = 0.002).

Chapter Five

Discussion

5. Discussion

The present study focused on detection of expression of E-cadherin and Vimentin, and correlating their expression with various diagnostic parameters of breast cancer. It involved 56 cases of women with breast cancer stained by immunohistochemistry for EMT markers (E-cadherin & Vimentin).

Regarding the age group of the study population, the study revealed that majority of patients was more than 40 years 45/56 (80%), indicating that older women are more susceptible to breast cancer than younger women. This result agrees with Hemalatha *et al.* (2013), they reported that age is an important factor in occurrence of carcinoma, breast with carcinoma rarely occurring in young. Also compatible with Bakhet *et al.* (2016), they reported that risk of developing breast cancer increases with age.

Regarding the histopathological diagnosis of the study cases, the study revealed that the majority of diagnosed samples were invasive ductal carcinoma 40/56 (71.4%), and this finding agrees with Domagala *et al.* (1990), they reported that 214/262 (81.7%) cases of breast cancer is invasive ductal carcinoma, also agrees with Bakhet *et al.* (2016), they reported that most frequent type is invasive ductal carcinoma.

Regarding the histological grade of the study cases, the study revealed that, most frequent grade is grade III, indicating that delay in diagnosis lead to delay in the treatment. This result is compatible with Bakhet *et al.* (2016), they reported that grade III were more frequent malignant tumor grade, and this associates with poor prognosis. But it's not compatible with Hemalatha *et al.* (2013), they reported that (22/50) cases were of grade I.

The present study found that, the expression of E-cadherin was negative in (7/56) cases, weakly expressed in (18/56) cases, moderately expressed in (27/56) cases and strongly expressed in (4/56) cases, indicating that down-regulation of E-

cadherin act as a major role in EMT and tumor cells metastasis. This result agrees with Shiozaki *et al*. (1991), they reported that reduced expression of E-cadherin may be a characteristic acquired during malignant transformation. Also agrees with Gamallo, C., *et al.* (1993), they reported that a correlation has been suggested between a loss of E-cadherin and increased invasiveness of neoplastic cells.

Regarding Vimentin expression, the study found that it was negative in (1/56) cases, weakly expressed in (29/56) cases, moderately expressed in (26/56) cases and strongly expressed in (0/56) cases, indicating that Vimentin expression works as a prognostic marker for EMT and plays a major role in prognosis of breast cancer. This result agrees with Heatley, M., *et al.* (1993), they reported that Vimentin expression in breast tumors is an indicator of prognosis, and also agrees with Hemalatha *et al.* (2013), they reported that Vimentin-positive cells are associated with increased tumor proliferation.

The correlation between age and expression of E-cadherin and Vimentin in this study is significant (p-value = 0.028 / 0.016) respectively, indicating an association between age and the EMT markers under investigation.

There is no significant correlation between the histological type of the study cases and E-cadherin expression (p-value = 0.126), this result may be due to the limited number and types of the study cases, suggesting that use of all types of breast cancer with reasonably large sample size may reveal the correlation. The result of current study disagrees with Gamallo, C., *et al.* (1993) and Qureshi et al. (2006), they reported that E-cadherin expression correlates with histological type in breast carcinomas. On the other hand, there is a significant correlation between the histological type of the study cases and Vimentin expression (p-value = 0.004) and this result agrees with Domagala *et al.* (1990), they reported that Vimentin expression is unevenly distributed among the various histologic types of breast cancers and seems to be associated with ductal carcinomas.

There is a significant correlation between the histological grade of the study cases and E-cadherin expression (p-value = 0.027), this result agrees with Gamallo, C., *et al.* (1993) and Shiozaki *et al.* (1991), they reported that an association observed between E-cadherin expression and histological grade in breast cancer. On the other hand, there is no significant correlation between the histological grade and Vimentin expression (p-value = 0.051), and this result disagrees with Domagala *et al.* (1990), they reported that there is a correlation between vimentin expression and histological grade of ductal breast carcinoma. Also disagrees with Hemalatha *et al.* (2013), they reported that a significant correlation is present between vimentin expression and tumor grade.

The present study found that, there is an inverse correlation between E-cadherin and Vimentin expression (r = -0.389; p-value = 0.002), indicating that loss of E-cadherin expression and increase in Vimentin expression is associated with the increase in aggressiveness of tumor cells. The detection of E-cadherin and Vimentin as prognostic markers will help in evaluation of EMT, and thus help in prognosis and treatment of breast cancer.

Chapter Six

Conclusion and Recommendations

6. Conclusion and recommendations

6.1 Conclusion:

From this study we conclude that:

- Decreased expression of E-cadherin and increased expression of Vimentin were associated with epithelial-mesenchymal transition and tumor aggressiveness.

- There is a significant correlation between E-cadherin expression with age and histological grade, and no correlation with histological type.

- There is a significant correlation between Vimentin expression with age and histological type, and no correlation with histological grade.

- There is an inverse correlation and strong significant association between E-cadherin and Vimentin expression.

6.2 Recommendations:

- E-cadherin and Vimentin recommended to be used as prognostic markers for EMT and tumor aggressiveness.

- E-cadherin and Vimentin recommended to be used in prognostic panels of breast cancer.

- Studies should be done including large sample size, all breast cancer types with addition of other diagnostic, prognostic parameters and advance techniques.

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Appendices

Appendix 1:

Materials and instrument used for processing and staining of the specimens include:

- Disposable gloves
- Microtome knife
- Positively charged slides (Thermo)
- Cover glass
- Dry oven
- Water bath
- Embedding center
- Coplin jar
- Humidity chamber
- Ethanol (100%, 90%, 70%, 50%)
- Mayer's haematoxylin
  (haematoxylin , D.W, K or ammonium alum ,sodium iodated ,citric acid, chloral hydrate)
- Citrate buffer (pH 6.8)
- Primary antibody (E-cadherin - Vimentin)
- Secondary antibody
  (Dextran polymer conjugated secondary antibody – HRP)
- Tris EDTA buffer (pH 9)
- Phosphate buffer saline (pH 7.4)
- Peroxides blocker(3% hydrogen peroxide in methanol)

- DAB (3,3 diaminobenzidinetetrahydrochloride) substrate chromogen
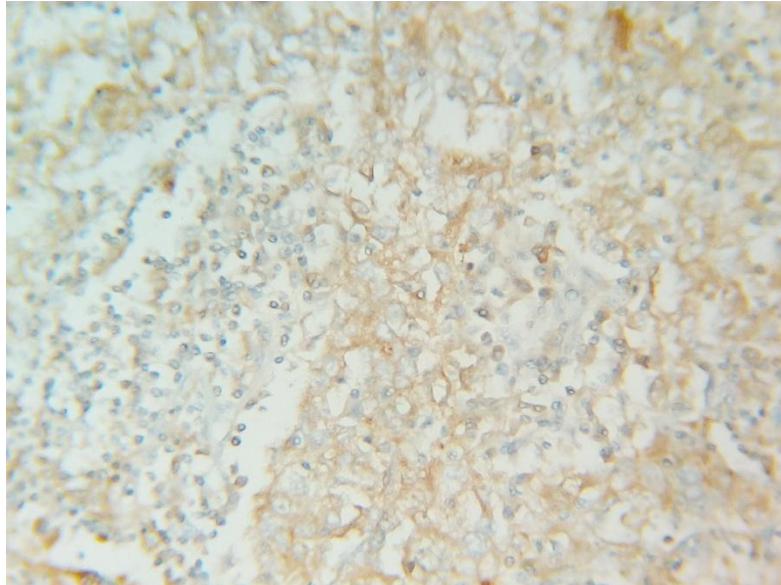- Bluing Reagent
- Xylene
- DPX

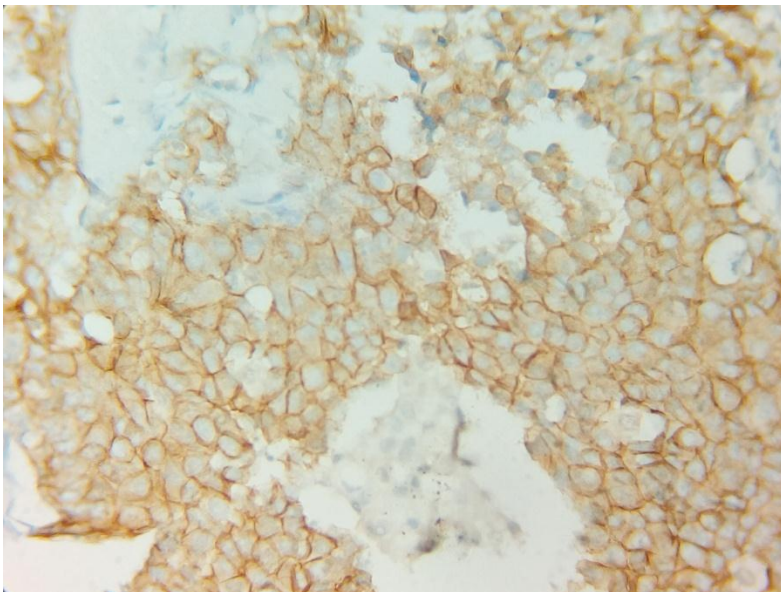**Microphotograph (4.1):** Tumor shows negative expression of E-cadherin
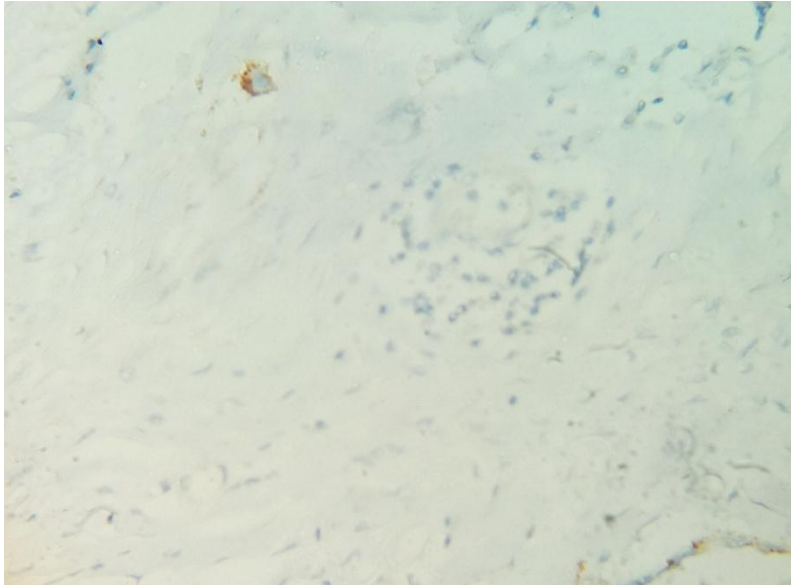
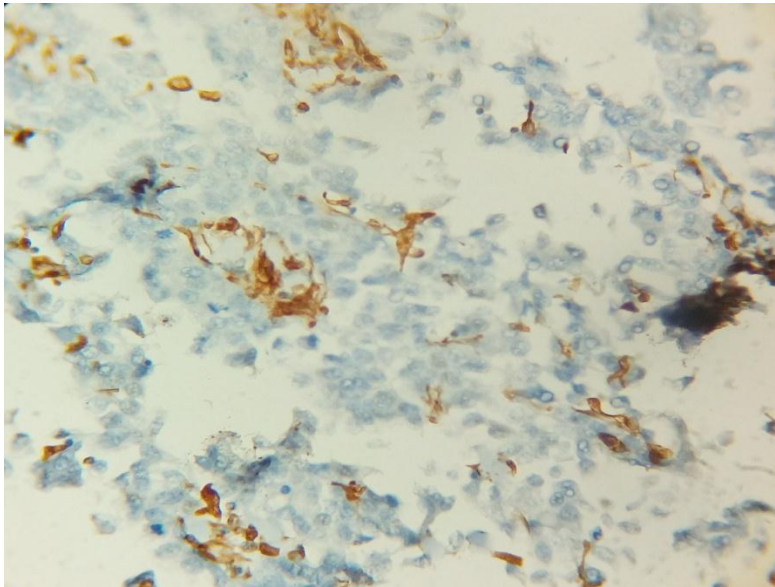**Microphotograph (4.2):** Tumor shows weak expression of E-cadherin
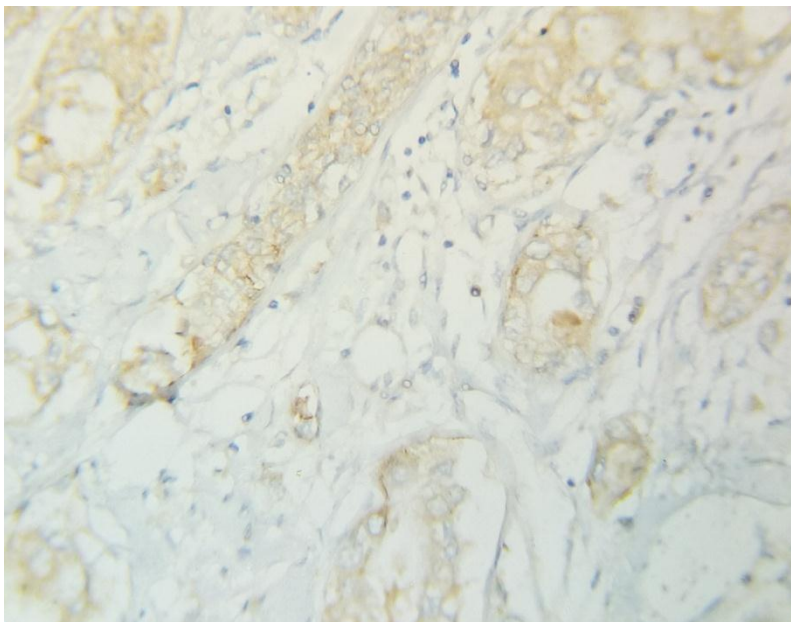
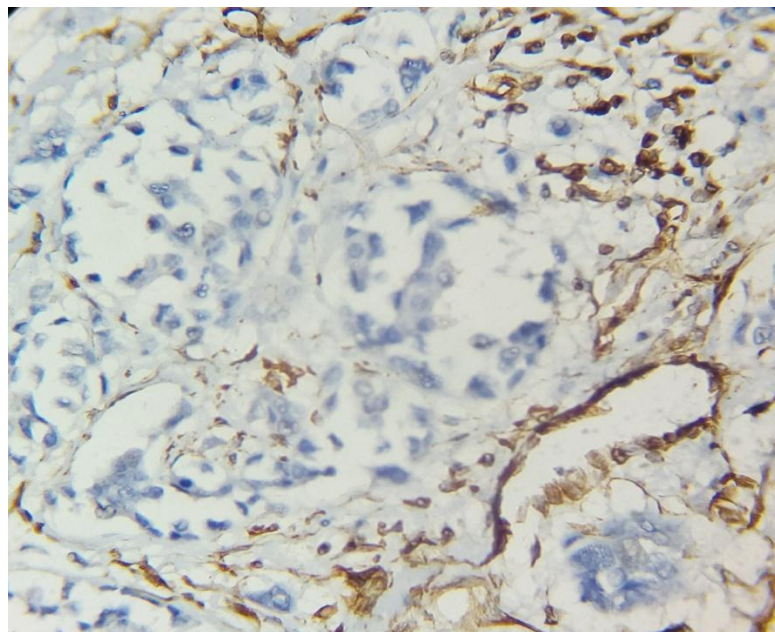**Microphotograph (4.3):** Tumor shows moderate expression of E-cadherin

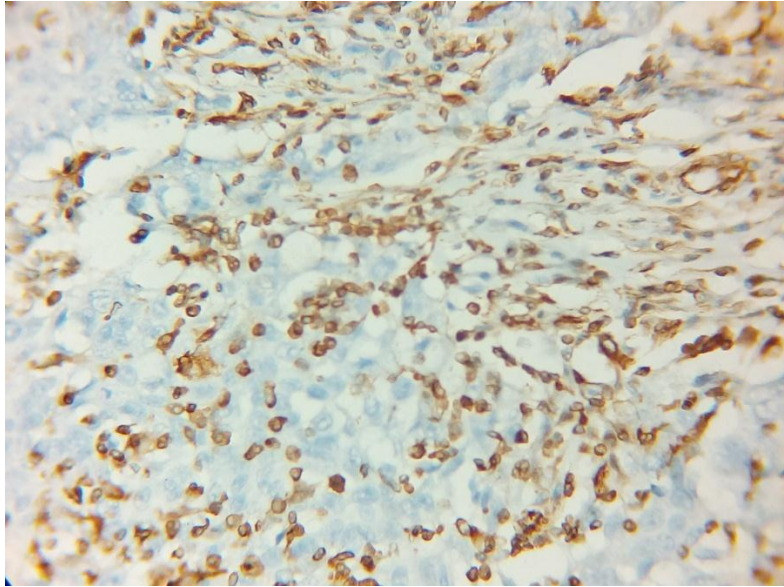**Microphotograph (4.4):** Tumor shows strong expression of E-cadherin

Microphotograph (4.5): Tumor shows negative expression of Vimentin

Microphotograph (4.6): Tumor shows weak expression of Vimentin

Microphotograph (4.7): Tumor shows moderate expression of Vimentin

Dako

**Monoclonal Mouse
Anti-Human
E-cadherin
Clone NCH-38**

**English
Code M3612**

Intended use
For in vitro diagnostic use.

Monoclonal Mouse Anti-Human E-cadherin, Clone NCH-38 is intended for use in immunohistochemistry (IHC). This antibody is useful for the identification of E-cadherin-expressing cells in normal and neoplastic tissues.[1-3,5-6,10,11] Results aid in the classification of ductal breast carcinoma. Differential classification is aided by the results from a panel of antibodies. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist. This antibody is intended to be used after the primary diagnosis of tumor has been made by conventional histopathology using nonimmunologic histochemical stains.

Synonyms
E-CD, uvomorulin, L-CAM, Arc-1, or cell-CAM 120/180[1-3]

Summary and explanation
E-cadherin is a 120 kDa transmembrane cell adhesion molecule. The gene has been localized on chromosome 16q22.1. In its extracellular domain, E-cadherin is involved in cell-cell adhesion through calcium-regulated homophilic interactions, whereas in its intracellular domain, E-cadherin connects to the actin cytoskeleton via catenins. E-cadherin has a significant function in intercellular adhesion of epithelial cells, the establishment of epithelial polarization, glandular differentiation, and stratification. It is localized mainly in the adherens junctions and concentrates the urokinase plasminogen and the epidermal growth factor receptor to cell contact sites.[5,6]

Refer to *Dako General Instructions for Immunohistochemical Staining* or the detection system instructions of IHC procedures for: Principle of Procedure, Materials Required, Not Supplied, Storage, Specimen Preparation, Staining Procedure, Quality Control, Troubleshooting, Interpretation of Staining, General Limitations.

Reagent provided
Monoclonal mouse antibody provided in liquid form as tissue culture supernatant in 0.05 mol/L Tris-HCl, pH 7.2 and 0.015 mol/L sodium azide. This product contains stabilizing protein.

Clone: NCH-38[4]   Isotype: IgG1, kappa

Mouse IgG concentration: see label on vial.

The protein concentration between lots may vary without influencing the optimal dilution. The titer of each individual lot is compared and adjusted to a reference lot to ensure a consistent immunohistochemical staining performance from lot-to-lot.

Immunogen
E-cadherin (uvomorulin) and GST recombinant protein[4]

Specificity
Anti-E-cadherin, NCH-38 recognizes the 120 kDa mature form and 82 kDa fragment of E-cadherin in Western blots of A431 cells lysates.[4]

Materials required, but not supplied
Refer to *Dako General Instructions for Immunohistochemical Staining* and/or the Detection System instructions. Suggested diluent for IHC procedures: Antibody Diluent (Code S0809)
The following negative control is recommended for IHC procedures: Mouse IgG1 (Code X0931)

Precautions
1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN₃), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN₃ may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
4. As with any product derived from biological sources, proper handling procedures should be used.
5. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
6. Unused reagents should be disposed of according to local, State, and Federal regulations.

Storage
Store at 2–8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user. There are no obvious signs to indicate instability of this product. Therefore, positive and negative

![Dako logo]

**Monoclonal Mouse
Anti-Vimentin
Clone Vim 3B4
Code M7020**

CE

ENGLISH

Intended use

For in vitro diagnostic use.

Monoclonal Mouse Anti-Vimentin, Clone Vim 3B4, is intended for use in immunohistochemistry (IHC). The antibody labels primarily cells of mesenchymal origin in normal and neoplastic tissue, and is a useful aid for classification of tumors of mesenchymal origin (1). Differential classification is aided by the results from a panel of antibodies. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist. This antibody is intended to be used after the primary diagnosis of tumor has been made by conventional histopathology using nonimmunologic histochemical stains.

Summary and explanation

Vimentin is a 55 kDa intermediate filament (IF) protein, which form part of the cytoskeleton of vertebrate cells. Among the IFs, vimentin belongs to class III and is expressed in the cytoplasm of cells of mesenchymal origin (2). Being the predominant IF protein in mesenchymal cells vimentin was initially believed to be a useful marker for classifying non-epithelial neoplasms. The co-expression of intermediate filaments, particularly vimentin and cytokeratin, has been demonstrated in a variety of normal cells/tissues and in neoplastic lesions, necessitating the use of a panel of antibodies in tumor classification (1).

Refer to *Dako General Instructions for Immunohistochemical Staining* or the detection system instructions of IHC procedures for: Principle of Procedure; Materials Required, Not Supplied; Storage; Specimen Preparation; Staining Procedure; Quality Control; Troubleshooting; Interpretation of Staining; General Limitations.

Reagent provided

Monoclonal mouse antibody provided in liquid form purified from cell culture supernatant. In 0.05 mol/L Tris-HCl, 1% bovine serum albumin (BSA) and 15 mmol/L NaN₃, pH 7.2.

Clone: Vim 3B4. Isotype: IgG2a, kappa.

Mouse IgG concentration: See label on vial.

The protein concentration between lots may vary without influencing the optimal dilution. The titer of each individual lot is compared and adjusted to a reference lot to ensure a consistent immunohistochemical staining performance from lot-to-lot.

Immunogen

Vimentin isolated from bovine eye lens (4).

Specificity

SDS-PAGE analysis of immunoprecipitates formed between the antibody and ³⁵S-labeled proteins from metabolically labeled human osteogenic sarcoma 4-996 cells shows reaction with a ~60 kDa polypeptide, corresponding to vimentin. In addition, a few bands of lower molecular weight were observed and may represent degradation products of vimentin (4).

The antibody reacts with an epitope that has been localized to the coil 2 part of the vimentin rod domain (4).

As demonstrated by immunocytochemistry on frozen as well as formalin-fixed tissue, the antibody cross-reacts with the vimentin-equivalent protein in man (5-9).

Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN₃), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
4. As with any product derived from biological sources, proper handling procedures should be used.
5. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
6. Unused solution should be disposed of according to local, State and Federal regulations.

Storage

Store at 2–8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the user must verify the conditions. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact Dako Technical Support.

Specimen preparation

Paraffin sections: The antibody can be used for labeling paraffin-embedded tissue sections fixed in formalin. Pre-treatment of deparaffinized tissue with proteinase K or heat-induced epitope retrieval is required. For heat-induced epitope retrieval, optimal results are obtained with Dako Target Retrieval Solution, Code S1700, 10 mmol/L citrate buffer, pH 6.0, or 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. The tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure.

Frozen sections and cell preparations: The antibody can be used for labeling acetone fixed, frozen sections (3, 6, 7). The user must validate the staining procedure.

Staining procedure

These are guidelines only. Optimal conditions may vary depending on specimen type and preparation method, and should be validated individually by each laboratory. The performance of this antibody should be established by the user when utilized with other manual staining systems or automated platforms.

Dilution: Monoclonal Mouse Anti-Vimentin, Code M7020, may be used at a dilution range of 1:100-1:200 when applied on formalin-fixed, paraffin-embedded sections of malignant melanoma and using 20 minutes heat-induced epitope retrieval in Dako Target Retrieval Solution, Code S1700, and 30 minutes incubation at room temperature with the primary antibody. The recommended negative control is Dako Mouse IgG2a, Code X0943, diluted to the same mouse IgG concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in Dako Antibody Diluent, Code S0809.

Visualization: Dako EnVision+/HRP kits, e.g. Code K4005, are recommended. Follow the procedure enclosed with the selected visualization kit.

Quality control: Positive and negative control tissues as well as negative control reagent should be run simultaneously using the same protocol as the patient specimen.