

Sudan University of Science and Technology
College of Graduate Studies

**Role Of Cytokeratin 5/6 in Differentiation Between Benign and
Malignant Breast Tumors**

دور السايٲوكيراتين 6/5 في التفريق بين الأورام الحميدة والخبيثة للثدي

A Dissertation Submitted for Partial Fulfillment for Requirement of M.Sc.
Degree in Medical Laboratory Science (Histopathology and Cytology)

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الآية

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قال تعالى:

(وَالَّذِينَ يَقُولُونَ رَبَّنَا هَبْ لَنَا مِنْ أَزْوَاجِنَا وَذُرِّيَّاتِنَا قُرَّةَ أَعْيُنٍ وَاجْعَلْنَا لِلْمُتَّقِينَ إِمَامًا)

صدق الله العظيم

سورة الفرقان الآية (74)

Dedication

To you, beloved mother

There are no enough words I can say to describe you.

Love you forever

To you, my dear father

You stood by me and I stood tall

I am grateful for each day you gave to me

I will be forever thankful

To dear Mohammed

For all things you gave to me.

For all those times you stood by me

You was my strength when I was weak

To my lovely daughters

Shahd and Jud

You mean the world to me

To my dear sisters

Manal, Marwa and Manar

My world is a better place because of you

Acknowledgment

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Abstract

This is a hospital based analytical retrospective case control study conducted at Radiation and Isotope Center (RICK) and Ibrahim Malik hospital (Khartoum State), and Sudan university of science and technology - college of medical laboratory science, during the period from April to August 2016. The study aimed to investigate the role of CK 5/6 in differentiation between benign and malignant breast tumors, using immunohistochemistry.

Forty paraffin blocks were collected from patients previously diagnosed as breast tumor, 30 (75%) of them were malignant samples and 10 (25%) of them were benign samples.

The paraffin blocks were cut by rotary microtome, then stained by immunohistochemistry method (Avidin biotin technique). The data obtained was analyzed using SPSS program version 20. Frequencies mean and Chi-square test values were calculated.

The age of patients ranged between 18 to 80 years with mean age of 46 years. The study revealed that most patients were more than 40 years representing 27 (76.5%) and the remaining 13 (32.5%) were less than 40 years.

The histopathological diagnosis of patients revealed invasive ductal carcinoma in 26 (65%) samples, medullary carcinoma in 1 (2.5%) sample, invasive lobular carcinoma in 1 (2.5%) sample, invasive medullary carcinoma in 1 (2.5%) sample, invasive micropapillary carcinoma in 1 (2.5%) sample, fibroadenoma in 7 (17.5%) samples, fibrocytic changes in 2 (5%) samples, mastitis in 1 (2.5%) sample.

The grade of tumors showed grade I in 1 (2.5%) sample, grade II in 7 (17.5%) samples, grade III in 16 (40%) samples, and not graded in 6 (15%) samples.

Positive expression of CK 5/6 was found in (9/30) in malignant samples and (21/30) samples showed negative expression, while all benign samples (10/10) showed positive expression of CK 5/6, this result showed significant association of CK 5/6 expression and benign breast tumors (P. value = 0.000).

The study concluded that positive expression of CK 5/6 was associated with benign tumors of breast.

الخلاصة

أجريت هذه الدراسة المستشفوية التحليلية الإسترجاعية حالة وحالة ضابطة في المركز القومي للعلاج بالأشعة والطب النووي ومستشفى إبراهيم مالك (ولاية الخرطوم) وجامعة السودان للعلوم والتكنولوجيا، كلية علوم المختبرات الطبية في الفترة من أبريل إلى أغسطس 2016م، لدراسة دور السايبتوكيراتين 6/5 في التفريق بين الأورام الحميدة والخبيثة للثدي باستخدام كيمياء الأنسجة المناعية.

جُمع أربعون قالب شمعي من عينات مرضى كانوا مشخصين مسبقاً على أنهم مصابين بأورام الثدي، 30 (75%) منهم كانوا مشخصين أورام ثدي خبيثة، و10 (25%) منهم كانوا مشخصين أورام ثدي حميدة. قُطعت القوالب باستخدام المشراح الدوار وصُبغت بواسطة طريقة كيمياء الأنسجة المناعية (طريقة البايوتين أفيدين) واستخدم برنامج الحزم الإحصائية للعلوم الإجتماعية، النسخة 20 لتحليل البيانات. حسب متوسط التكرار وقيم إختبار مربع كاي.

تراوحت أعمار المرضى بين 18-80 عام بمتوسط عمر 46 سنة. أظهرت الدراسة أن معظم المرضى كانت أعمارهم أكثر من 40 سنة، وكان عددهم 27 مريض بنسبة (67.5%) و13 مريضا بنسبة (32.5%) كانت أعمارهم أقل من 40 سنة.

كان تشخيص حالات الورم الخبيث كالاتي: سرطان الأفتية الغازي 26 (65%) عينة، وسرطان النخاع 1 (2.5%) عينة، وسرطان سرطان مفصص الغازي 1 (2.5%) عينة، وسرطان النخاع الغازي 1 (2.5%) عينة، وسرطان مايكروباييلاري الغازي 1 (2.5%) عينة، ورم غدي ليفي 7 (17.5%) عينة، تغييرات فيبروسيستيك 2 (5%) عينة، إلتهاب الثدي 1 (2.5%) عينة.

كان تمايز الأورام يشتمل على النوع الأول 1 (2.5%)، والنوع الثاني 7 (17.5%)، والنوع الثالث 16 (40%)، وليست مصنفة 6 (15%).

اظهرت الدراسة أن السايبتوكيراتين 6/5 موجب الظهور في (30/9) عينة في أورام الثدي الخبيثة، وسالب الظهور في (30/21) عينة، بينما كل عينات أورام الثدي الحميدة موجبة الظهور (10/10)، أظهرت هذه النتيجة علاقة ذات دلالة احصائية بين السايبتوكيراتين 6/5 وأورام الثدي الحميدة (قيمة P = 0.000).

خلصت الدراسة إلى أن إيجابية الظهور للسايبتوكيراتين 6/5 ترتبط بالأورام الحميدة للثدي.

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Chapter One

Introduction

1.1 Introduction

Breast cancer is a type of cancer that originate from tissue of the breast either from inner lining of milk ducts or the lobules that supply the ducts with milk. Two main types of breast cancer usually exists lobular carcinoma and ductal carcinoma. Breast cancer may also start in other areas of the breast, but that is rare. Therefore, sometimes it may also classified as invasive or non-invasive. Non-invasive breast cancer is called carcinoma in situ (Carlson, *et al.* 2009).

Worldwide, breast cancer is the commonest form of malignancy in women (Abdoelrahman, *et al.* 2015).

In Sudanese population cases comprised 1255 women from central Sudan diagnosed with breast cancer and referred to and treated of Institute of Nuclear Medicine (Abdoelrahman, *et al.* 2015).

Risk factors which increases the incidence of getting breast cancer include age, socio-economic status, reproductive factors, age at first child birth, breastfeeding, endogenous hormones, oral contraceptive pills, breast density, family history, overweight and physical activity, diet and vitamin intake, alcohol consumption, sleep routine and night shift, health related issues and use of medicines and smoking (Rabia, *et al.* 2013).

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 (Mohamed, *et al.* 2015).

The main types of treatment for breast cancer are surgery, radiation therapy (RT), chemotherapy (CT), endocrine hormone therapy (ET) and targeted therapy (Mohamed, *et al.* 2015).

Cytokeratin 5/6 are intermediate sized basic keratins. In normal tissue, Ck 5/6 is mainly expressed in keratinizing (epidermis) and non-keratinizing (mucosa) squamous epithelium, as well as in basal myoepithelial cell layer of prostate, breast, and salivary gland. Ck 5/6 are also seen in benign and malignant tumors of epidermal, squamous mucosal and myeoepithelial origins (Peiguo and Lawrence, 2002).

Ck 5/6 applied on cell block sections of benign and malignant breast lesions. All benign lesions showed positive imuunoreaction. The malignant lesions comprised ductal carcinoma insitu (DCIS) and infiltrating ductal carcinoma (IDC). None of the DCIS showed positive immunoreaction. Among IDC cases of grade III breast carcinoma exhibited a positive immunohistochemical reaction (Amarpreet, *et al.* 2010).

1.2 Objectives:

1.2.1 General objective:

To study the role of CK 5/6 in differential diagnosis of breast tumors.

1.2.2 Specific objective:

Detection of CK 5/6 in breast tumors tissues by immunohistochemical method to correlate between CK 5/6 expression with histological diagnosis.

Chapter Two

Literature Review

2.1 Scientific background:

Breast cancer represents a significant health problem, because of number of individuals affected by this disease. 30% of all cancers in women occur in the breast, making it the most commonly diagnosed female cancer. Prevalence data of breast cancer indicate that one million new cases are reported each year in the world (Rabia, *et al.* 2013).

2.2 Structure of the breast:

Histologically, breast consists of glandular tissue and fatty tissue. The glandular tissue is arranged predominantly on the upper outer quadrant of the breast. The glandular tissue consists of fifteen to twenty lobes where each lobe divided into numerous lobules. Each lobule then consists of alveoli, which are sites of milk secretion. Around the areola, the lactiferous ducts have dilated areas called lactiferous sinuses where the accumulation of milk occurs during lactation. The lactiferous ducts all open at the nipple. The fibrous connective tissue of the breast separates the lobes from one another and also forms the suspensory (Cooper's) ligaments which extend from the underlying muscle to the skin and provide natural support for the breast. In advanced stages of carcinoma of the breast, these ligaments contract and dimple the skin like and orange peel. The fatty tissue of the breast covers the glandular surface and lies between the lobes. Most of breast size due to fat deposits, whereas, the superficial fat gives the breast its shape (Marieb, 1989).

2.3 Disorder of the breast:

2.3.1 Benign disorder:

2.3.1.1 Inflammatory breast lesions:

Mastitis or inflammation of the breast is uncommon. Mastitis can be divided into acute and chronic, and acute mastitis may be associated with abscess formation. 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 suckling baby's oral cavity. Chronic mastitis due to specific microorganisms is rare, and among these, granulomatous mastitis due to mycobacterium tuberculosis is probably the most common (Bakaris, *et al.* 2006).

2.3.1.2 Fibrocystic changes:

It represents the most common lesions of the breast. The clinical presentation is variable, ranging from asymptomatic to mastalgia that is related to menstrual cycle. A wide range of lesions are seen within fibrocystic changes, including epithelial metaplasia, hyperplasia of benign or usual type, and adenosis, cyst formation (Tse, *et al.* 2002).

2.3.1.3 Fibroadenomas:

It is probably most common benign breast tumor, presenting as solitary painless, mobile and well defined nodules. Multiple lesions are less frequent. The use of immunosuppressant cyclosporine in transplant patients has resulted in an increased risk of fibroadenoma development (Tse, *et al.* 2002).

2.3.1.4 Phyllodes tumor:

Uncommon fibroepithelial neoplasm that resembles fibroadenoma grossly. Patients with phyllodes tumor usually are older than patients with fibroadenomas, and there may be a history of rapidly growing mass (Tse and Tan, 2005).

2.3.1.5 Papilloma:

It is 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. Microscopically papillomas are characterized by an arborescent growth derived from the wall of dilated duct, and these stromal tissue fibrovascular cores are lined by epithelial cells, together with an intervening layer of myoepithelial cells (Mulligan and O'Malley, 2007).

2.3.1.6 Sclerosing adenosis:

It is a variant of breast proliferation. Clinically it is characterized by an irregular hard mass that is fixed to the adjacent structures, and by imaging, it also shows significant architectural distortion, rendering this indistinguishable from carcinoma (Tse, *et al.* 2002).

2.3.1.7 Hamartoma:

It may present as a soft palpable mass or as breast asymmetry, and is usually round to oval and lobulated (Tse, *et al.* 2002).

2.3.2 Malignant disorders:

2.3.2.1 Carcinoma in situ:

Ductal carcinoma in situ (DCIS) is increasingly diagnosed as non-palpable lesions. Newer classification/grading always use nuclear grade as one of the defining features of DCIS. Other histologic features being

used are necrosis and the presence of tumor cell polarization (Silverstein, *et al.* 1995).

High grade DCIS is easily differentiated from benign lesions, with the highly pleomorphic tumor cells present within the enlarged ducts associated central comedo necrosis. In lobular carcinoma in situ neoplastic cells are small and uniform, smaller than ductal lesions, with higher nuclear cytoplasmic ratio, mild nuclear pleomorphic, rare mitoses and occasional cytoplasmic vacuoles (Page, *et al.* 1996).

2.3.2.2 Papillary carcinoma:

Papillary carcinomas are uncommon malignant lesions, representing several different morphological entities, all possessing a common papillary architecture, characterized by epithelial proliferation overlying elaborate fibrovascular cores (Page, *et al.* 1996).

2.3.2.3 Invasive carcinoma:

Clinically, it presents as an ill-defined mass, sometimes adhered to the skin or underlying muscle. Among the tumor types, the most common is invasive ductal carcinoma (Rokha, *et al.* 2008).

2.4 Epidemiology of breast cancer:

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed and 521,900 death in 2012 (second most common cancer overall). This represents about 12% of all new cancer cases (14.1 million). Breast cancer alone account for 25% of all cancer cases and 15% of all cancer deaths among females (Mohamed, *et al.* 2015).

Female breast cancer is by far the leading cancer in Sudan. It accounts to 16.5% of all cancers (30.24% of all female cancers in 2009). A vast majority of these cancers were from Khartoum, with 41.7% in 2009 followed by El-Gezira State with 10.4% in that year, as reported by the

Radio and Isotope Center Khartoum (RICK). This is the prime center that has promoted these services in Sudan. In 2009 about 948 patients of breast cancer were presented to the center with a majority of patients aged 35 to 54 year (Abdulla, *et al.* 2015).

2.5 Risk factors:

2.5.1 Age:

Age is proportional to risk i.e. the older the woman, the higher the risk throughout her life, but chances increase markedly in post-menopausal years (Rabia, *et al.* 2013).

2.5.2 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 onset of menopause confers a 17% increase in risk of breast cancer (Rupen, *et al.* 2014).

2.5.3 Age at first child birth:

Childbearing age is inversely related to the risk of breast cancer i.e. the younger the woman is when she begins childbearing, the lower the risk to develop breast cancer. It is estimated that a 3% increase in risk occurs for each year delay. It is also an established fact that childbearing not only reduces the risk, but the higher number of full-term pregnancies ensures higher protection for females (Rabia, *et al.* 2013).

2.5.4 Family history:

A woman's risk of breast cancer is increased if she has a family history of the disease. The highest risk is associated with increasing number of first degree relatives diagnosed with breast cancer at a young age (under age 50) (Rupen, *et al.* 2014).

2.5.5 Breast feeding:

Evidence suggested 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 (Rupen, *et al.* 2014).

2.5.6 Breast pathology:

Proliferative breast disease is associated with an increased risk of breast cancer. Proliferative breast lesions without a typia, including usual ductal hyperplasia, intraductal papillomas, sclerosing adenosis and fibroadenomas cancer only, approximately 1.5 – 2 times that of general population (Rupen, *et al.* 2014).

2.5.7 Endogenous hormones:

It has been hypothesized that higher level of endogenous hormones increase the risk of breast cancer. Post-menopausal women with highest levels of testosterone and oestrogen have 2-3 times the risk compared to women with lowest levels (Rabia, *et al.* 2013).

2.5.8 Obesity:

Obesity, especially in postmenopausal women, has also shown to increase a woman's risk of breast cancer (Rupen, *et al.* 2014).

2.5.9 Alcohol consumption:

Alcohol consumption is also associated with an increased risk of breast cancer. There is a higher level of sex hormones in blood stream in alcohol consumers compared to non-consumers (Rabia, *et al.* 2013).

2.5.10 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 (Abdullah, *et al.* 2015).

2.5.11 Health related issues and use of medicines:

Use of anti-hypertensive drugs for 5 years or more, oestrogen use during pregnancy, Grave's disease and longer exposure to ionizing radiation increase the risk factor of breast cancer (Rabia, *et al.* 2013).

2.5.12 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. Genetic predisposition alleles has been described in terms of clinical significance (Rupen, *et al.* 2014)

2.6 Diagnosis of breast cancer:

2.6.1 Mammography:

Mammographic screening for breast cancer uses X-rays to examine breast for any uncharacteristic masses or lumps. The cochrane collaboration in 2009 concluded the mammograms reduce mortality from breast cancer by 15% but also result in unnecessary surgery and anxiety, resulting in their view that it is not clear whether mammography screening does more good of harm. In women at high risk an earlier age and additional testing may include genetic screening that test for the BRCA genes and magnetic resonance imaging (Abdullah, *et al.* 2015).

2.6.2 Magnetic resonance imaging (MRI):

Magnetic resonance imaging (MRI) has become an important modality in the detection, assessment, staging and management of breast cancer in selected patients. MRI is more sensitive, but less specific for the detection of cancer in high risk women (Rupen, *et al.* 2014).

2.6.3 Molecular breast imaging (MBI):

MBI uses radioactive tracer that lights up cancer tissues of the breast, visualized by a nuclear medicine scanner. This technique is also called Miraluma test, sestamibi test, scintimammography, or special gamma imaging. MBI has comparable sensitivity to MRI and rather a higher specificity that can detect small breast lesions (Mohamed, *et al.* 2015).

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 (Rupen, *et al.* 2014).

2.6.5 Breast biopsy:

The only definitive method for diagnosing breast cancer is with 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). In FNAC a thin, hollow needle is inserted into the breast to withdraw cells from the suspicious lesion; the cells are submitted to a laboratory for analysis. CNB uses a larger needle than FNAC, and instead of cells, CNB removes a small cylinder of tissue (a core) about the size of a grain of rice (Mohamed, *et al.* 2015).

2.6.6 Immunohistochemistry (IHC):

IHC is a technique that uses antibodies as a tool to detect protein expression. 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 (Mohamed, *et al.* 2015).

2.7 Treatment of breast cancer:

2.7.1 Surgery:

Breast conservation surgery is the tending approach the treatment of localized breast cancer. Surgery is usually followed by adjuvant therapy to ensure full recovery and minimize the risk of metastases. Cancer cells that may not be seen during surgery can be killed by radiation to reduce the risk of local recurrence of cancer (Mohamed, *et al.* 2015).

2.7.2 Radiation therapy (RT):

Radiation therapy is one of the most affect method used to kill breast tumor cell which aims to deliver maximum dose of radiation to the tumor while minimizing dose to the critical structure (Abdoelrahman, *et al.* 2015).

2.7.3 Endocrine therapy (ET):

The purpose of ET is either balancing, or blocking hormones. The choice of medication is primarily determined by patients' menopausal status. Other factors include differences in efficacy and side effect profile (Mohamed, *et al.* 2015).

2.7.4 Chemotherapy:

Chemotherapy is the standard of care for women with node – positive cancer or with tumor larger than 1 cm. Factors such as age and comorbidities also influence the decision to use chemotherapy (Karen, *et al.* 2010)

2.8 Cytokeratin 5/6 and its relation with breast cancer:

Cytokeratin (CK) an intermediate filament protein, reflects the epithelial cell type, state of tissue growth, differentiation, functional status and used for the fingerprinting of various carcinomas (Amarpreet, *et al.* 2010).

Cytokeratin 5/6 is normally expressed in both keratinizing and non-keratinizing squamous epithelia, in addition to basal or myoepithelial cells in the breast, prostate and salivary glands. CK 5/6 is considered to be marker of basal and squamous cells differentiation in several normal epithelia and human tumors (Ingunn, *et al.* 2006).

Ding and Ruan reported that positive rate of CK 5/6 expression in benign breast lesions was 100%. In cases of a typical ductal hyperplasia, there were few positive cells in the ducts. No CK 5/6 was detectable in cases of invasive ductal carcinoma. CK 5/6 has value in differentiating ductal proliferation of varying degree, especially in the differentiation between cancerous and non-cancerous changes (Ding and Ruan, 2006).

Kritins *et al.* reported that cytokeratin 5/6 was expressed in all normal breast tissues, and in 14% of invasive breast cancers (Kristin, *et al.* 2008).

Amarpreet *et al.* reported that all benign lesions showed positive immunoreaction with the staining index varying from 6-9. (22/25) cases of infiltrating ductal carcinoma (IDC) showed negative expression of CK 5/6. CK 5/6 can be used as component of panels to differentiate between benign and malignant breast lesions (Amarpreet, *et al.* 2010).

Kafil *et al.* reported that all benign lesions of breast showed positive cytokeratin 5/6 expression with variable staining score, all cases of ductal carcinoma in situ showed positive immunostaining for CK 5/6 and (16/22) cases of invasive ductal carcinoma showed negative expression of CK 5/6. CK 5/6 is efficient in differentiating the usual ductal hyperplasia (UDH), ruling out micro-invasion, and distinguishing invasive carcinoma from pseudo-invasive lesions (Kafil, *et al.* 2015).

Chapter Three

Material and Methods

3.1 Materials:

Archived tissue blocks of breast tumors were selected for this study.

3.2 Methods:

3.2.1 Study design:

This is hospital based analytical retrospective case control study aimed to study role of CK 5/6 in differentiation between benign and malignant breast tumor.

3.2.2 Study population:

Tissue blocks obtained from thirty samples were previously diagnosed as malignant breast tissues and ten samples were diagnosed as benign tumor. Patients' identification information (age, histopathological diagnosis, malignant tumor grade) were obtained from patients files.

3.2.3 Study area:

This study held in Ibrahim Malik Hospital, and Radiation and Isotope Centre (Khartoum State), and Sudan university of science and technology - college of Medical laboratory science during the period from April to August 2016.

3.2.4 Sample processing:

Section to be stained were cut at (3 μ m) thickness by rotary microtome, mounted in positively charged glass slides and baked at 60°C for 30 minutes (Kristin, *et al.* 2008).

3.2.5 Immunohistochemical staining:

Immunohistochemical staining was carried out using indirect streptoavidin – biotin immune peroxidase technique. Tissue sections (3 μ m) were deparaffinized in xylene and rehydrated in grade of a sending

alcohol (100%, 90%, 70%, 50%). Sections were incubated for 10 minutes in 0.3% hydrogen peroxide, in absolute methanol to block endogenous peroxidase activity. Antigen retrieval was performed by using Dako water path with citrate buffer (PH 6.8) for 30 minutes at 95°C. The sections then treated with anti CK 5/6 primary antibody for 30 minutes. Then sections were incubated in biotinylated secondary antibody for 15 minutes, then washed in phosphate buffer saline (PH 7.4), incubated in streptavidin – HRP (horseradish peroxidase) for 15 minutes, washed in phosphate buffer saline (PH 7.4), incubated in DAB substrate solution for 5 minutes, washed in running water, then counterstained in Mayer's hematoxylin stain for one minute. Dehydrated, cleared and mounted in DPX mounting media (Bancroft, *et al.* 2013).

3.2.6 Data analysis:

Data analysis was done using SPSS 20 computer program. Frequencies mean and Chi-square test values were calculated.

3.2.7 Result interpretation:

All quality control measures were adopted, positive and negative control sections were used during immunohistochemical staining. Detection of more than 5 cells with cytoplasmic reaction per one field considered as positive result.

3.2.8 Ethical consideration:

Samples were collected after taking ethical acceptance from hospital administration.

Chapter Four

4. Results

The study includes forty samples, 30 (75%) samples of them were malignant and 10 (25%) samples of them were benign.

The age of study population range between 18 and 80 with mean age of 46 years, and standard deviation was 16.1.

Most patients were more than 40 years representing 27 (67.5%) and the remaining 13 (32.5%) were less than 40 years as indicated in table (4.1).

The diagnosis of study population includes invasive ductal carcinoma in 26 (65%) samples, medullary carcinoma in 1 (2.5%) sample, invasive lobular carcinoma in 1 (2.5%) sample, invasive medullary carcinoma in 1 (2.5%) sample, invasive micropapillary carcinoma in 1 (2.5%) sample, fibroadenoma in 7 (17.5%) samples, fibrocytic changes in 2 (5%) samples, mastitis in 1 (2.5%) sample as indicated in table (4.2).

The grade of study population includes grade I in 1 (2.5%) sample, grade II in 7 (17.5%) samples, grade III in 16 (40%) samples, and not graded in 6 (15%) samples as indicated in table (4.3).

Cytokeratin 5/6 positive expression was found (9/30) in malignant samples, and (21/30) samples showed negative expression, while all benign samples (10/10) showed positive expression for CK 5/6. This result showed significant association (P. value = 0.000) as indicated in table (4.4).

Table (4.1): Distribution of age group among the study population

Age group	Frequency	Percentage
Less than 40 years	13	32.5%
More than 40 years	27	67.5%
Total	40	100%

Table (4.2): Distribution of histopathological diagnosis among the study population

Histological diagnosis	Type	Frequency	Percentage
Benign	Fibroadenoma	7	17.5%
	Fibrocytic changes	2	5%
	Mastitis	1	2.5%
Malignant	Invasive ductal carcinoma	26	65%
	Invasive lobular carcinoma	1	2.5%
	Invasive medullary carcinoma	1	2.5%
	Invasive micropapillary carcinoma	1	2.5%
	Medullary carcinoma	1	2.5%
Total		40	100%

Table (4.3): Distribution of malignant tumor grade

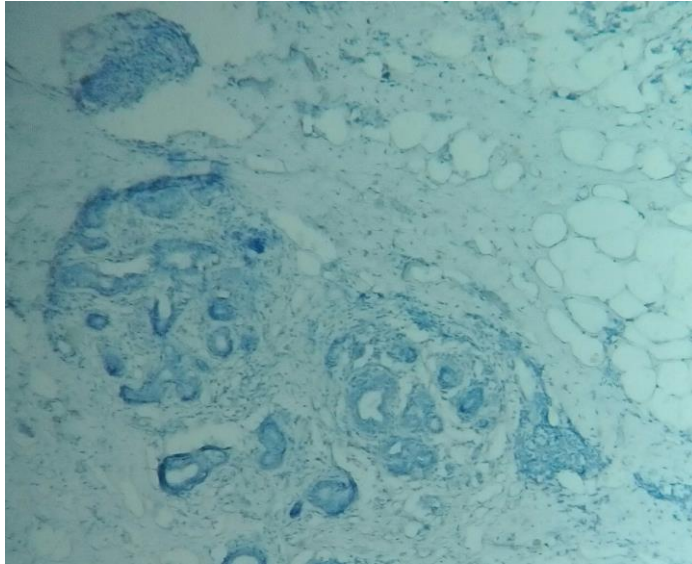
Grade	Frequency	Percentage
Grade I	1	3.3%
Grade II	7	23.3%
Grade III	16	53.4%
Not graded	6	20%
Total	30	100%

Table (4.4): Relation between histopathological diagnosis and CK 5/6 expression

Histological diagnosis	CK5/6 Expression		P. value
	Positive	Negative	
Benign	10 (25%)	0 (0.0%)	0.000
Malignant	9 (22.5%)	21 (52.5%)	
Total	19 (47.5%)	21 (52.5%)	



Microphotograph(4.1): Fibrocytic change showed positive cytoplasmic expression of CK 5/6 (40X)



Microphotograph(4.2): Invasive ductal carcinoma showed negative expression of CK 5/6 (40X)

Chapter Five

5. Discussion

The present study involves 40 cases of breast lesions for immunohistochemical staining by cytokeratin 5/6. Regarding the age group of the study population, the study revealed that most of patients were more than 40 years indicating that older women are more susceptible to breast cancer due to endogenous hormones level, genetic factors, and family history. This result is compatible with Rupen *et al.* (2014), they reported that risk of developing breast cancer increases with age. Also agree with Abdullah *et al.* (2015), they reported that number of the patients was increased with age and reached its maximum higher in age group 40-45. This result also agrees with Rabia *et al.* (2013), they reported that the older women, the higher risk throughout her life.

The histopathological diagnosis of the study population revealed that more frequent type is invasive ductal carcinoma due to delay of the treatment, patient was not respond to the treatment and that was lead to cancer invaded or spread to the surrounding breast tissues. This result is compatible with Kafil *et al.* (2015), they reported that (16/22) cases of malignant tumors were diagnosed as invasive ductal carcinoma. Also agree with Amarpreet *et al.* (2010), they reported that (22/25) cases of malignant lesions were diagnosed as invasive ductal carcinoma.

The malignant tumor grade revealed that more frequent grade is grade III due to late diagnosis of cancer, that lead to delay in the treatment of cancer. This result is compatible with Fereshteh *et al.* (2009), they reported that grade III were more frequent malignant tumor grade, and this associates with poor prognosis. Also agree with Amarpreet *et al.* (2010), they reported that most malignant tumors were classified as grade

III, if positive for CK 5/6 imply a “basal like” molecular phenotype and signify poor prognosis.

The expression of CK 5/6 revealed that (9/30) of malignant lesions showed positive expression, and (21/30) showed negative expression, and all benign cases of breast lesions showed positive expression for CK 5/6 because CK 5/6 normally present in the basal layer of the breast tissues. This result is compatible with Kafil *et al.* (2015), they reported that (16/22) of malignant lesions showed negative expression, and all benign lesions of breast showed positive CK 5/6 expression with variable staining score. Also agree with Ding and Ruan (2006), they reported that all malignant lesions showed negative expression, and positive rate of CK 5/6 in benign breast lesions was 100%. Also agree with Amarpreet *et al.* (2010), they reported that (22/25) of malignant lesions showed negative expression, and all benign lesions of breast showed positive expression of CK5/6. Also this result agrees with Kristin *et al.* (2008), they reported that 14% of invasive ductal carcinomas showed positive expression, and CK5/6 was expressed in all normal breast tissues.

Chapter Six

Conclusion and Recommendations

6.1 Conclusion:

From this study we conclude that:

- The age of the breast cancer patients in Sudan is commonly more than 40 years.
- Most histological type of breast cancer is invasive ductal carcinoma.
- Grade of breast cancer found mostly in grade III.
- CK 5/6 expression is associated with benign breast tumors.

6.2 Recommendations:

From this study we recommended that:

- CK 5/6 should be used to help in differentiation between benign and malignant breast tumors.
- CK 5/6 should be used in prognostic panels of breast cancer.

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Appendices

Appendix 1:

Materials and instruments used for processing and staining of the specimens.

Include:

Disposable gloves.

Rotary microtome.

Microtome knives.

Positively charged slides (Thermo).

Cover glasses.

Dry oven.

Water path (Dako water path).

Coplin jars.

Humidity chamber.

Ethanol (100%, 90%, 70%, 50%).

Xylene.

Mayer's haematoxylin

(Haematoxylin, DW, K or ammonium alum, sodium iodate, citric acid, chloral hydrate).

Citrate buffer (PH 6.8).

Phosphate buffer (PH 7.4).

0.3 Hydrogen peroxidase.

Primary antibody (CK 5/6).

Secondary antibody (biotinylated secondary antibody).

Streptavidin – HRP

Substrate Chromogen (DAB).

DPX

Appendix 2:

BIOCARE
MEDICAL

continually researching the most cost-effective and quality-aware diagnostic solutions



Cytokeratin 5/6 (CK 5/6)

Prediluted Monoclonal Antibody

Control Number: 901-105VP-073109

ISO
9001:2000
CERTIFIED

Catalog Number: VP 105 G5
Description: 5.0 ml, prediluted
Dilution: Ready-to-use

Intended Use:
For In Vitro Diagnostic Use

Summary and Explanation:

Cytokeratin 5/6 reacts with human epidermis and non-keratinizing epithelium as determined by immunoblotting. It also reacts with cytokeratin No. 6, and weakly reacts with cytokeratin No. 4. It does not react with cytokeratins 1, 7, 8, 10, 13, 14, 18 and 19. CK5/6 has been shown to be a reliable marker for mesothelioma and squamous cell carcinoma of the lung. It does not react with pulmonary adenocarcinoma.

Principle of Procedure:

Antigen detection, in tissues and cells, is a multi-step immunohistochemical process. The initial step binds the primary antibody to its specific epitope. After labeling the antigen with a primary antibody, a universal, affinity-purified, secondary antibody is added to bind to the primary antibody. An enzyme label is then added to bind to the secondary antibody; this detection of the bound antibody is evidenced by a colorimetric reaction.

Source: Mouse monoclonal

Species Reactivity: Human, others not tested.

Clone: CK5/6.007

IsoType: IgG₁

Total Protein Concentration: ~10 mg/ml. Call for lot specific Ig Concentration.

Epitope/Antigen: CK5/6

Cellular Localization: Cytoplasmic

Positive Control: Prostate or skin

Normal Tissue: skin, breast and prostate

Abnormal Tissue: Mesothelioma, squamous cell carcinoma

Known Applications:

Immunohistochemistry (formalin-fixed paraffin-embedded tissues)

Supplied As: Buffer with protein carrier and preservative.

Storage and Stability:

Store at 2°C to 8°C. Do not use after expiration date printed on vial. If reagents are stored under conditions other than those specified in the package insert, they must be verified by the user. Diluted reagents should be used promptly; any remaining reagent should be stored at 2°C to 8°C.

Protocol Recommendations

Pretreatment Solution (recommended): CC1

Pretreatment Protocol: Standard

Primary Antibody: Incubate for 32 minutes at 37°C.

Counterstain: Use BIOCARE'S CAT Hematoxylin for 30 seconds. Do not overstain with hematoxylin.

Technical Note:

BIOCARE's VP-Echelon Series of predilutes have been optimized with rVIEW™ detection system using the BENCHMARK® XT Automated slide stainer. If staining is not optimal enhance signal with Ventana's Amplification Kit. Use buffer for washing steps as suggested in Ventana protocol.

Performance Characteristics:

The optimum antibody dilution and protocols for a specific application can vary. These include, but are not limited to: fixation, heat-retrieval method, incubation times, tissue section thickness and detection kit used. Due to the superior sensitivity of these unique reagents, the recommended incubation times and titers listed are not applicable to other detection systems, as results may vary. Ultimately, it is the responsibility of the investigator to determine optimal conditions. These products are tools that can be used for interpretation of morphological findings in conjunction with other diagnostic tests and pertinent clinical data by a qualified pathologist.

Quality Control:

Refer to NCCLS Quality Assurance for Immunocytochemistry approved guidelines, December 1999 MM4-A Vol.19 No.26 for more information about Tissue Controls.

Precautions:

This antibody contains less than 0.1% sodium azide. Concentrations less than 0.1% are not reportable hazardous materials according to U.S. 29 CFR 1910.1200, OSHA Hazard communication and EC Directive 91/155/EC.

Sodium azide (NaN₃) used as a preservative is toxic if ingested. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing. (Center for disease control, 1976, National Institute of Occupational Safety and Health, 1976)

Specimens, before and after fixation and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions. Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents or specimens come in contact with sensitive areas, wash with copious amounts of water.

Microbial contamination of reagents may result in an increase in nonspecific staining. Incubation times or temperatures other than those specified may give erroneous results. The user must validate any such change. The MSDS is available upon request.

Troubleshooting:

Follow the antibody specific protocol recommendations according to data sheet provided. If atypical results occur, contact BIOCARE's Technical Support at 1-800-542-2002.

Limitations and Warranty:

There are no warranties, expressed or implied, which extend beyond this description. BIOCARE is not liable for property damage, personal injury, or economic loss caused by this product.

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