Sudan University of Science and Technology
College of Graduate Studies

Immunohistochemical Detection of Cytokeratin 5/6 and Smooth Muscle Actin among Triple Negative Breast Carcinoma in Sudanese Patients

الكشف النسيجي الكيميائي المناعي عن السايتوكيراتين(5/6)وأكتين العضلات الناعمة لدى المرضى السودانيات المصابات بسرطان الثدي السالب الثلاثي

A dissertation Submitted in Partial Fulfillment for the Requirement of Master Degree in Medical Laboratory Science (Histopathology and Cytology).

BY:

Ashraf Kamal Ibrahim Mohammed

B.Sc (Honor) in Medical Laboratory Sciences (Histopathology and Cytology)

National Ribat University 2012

Supervisor:

Dr. Abu Elgasim Abass Awad Elkareem

2021

الآية

قَالَ اللهُ تَعَالَى :

" وَلِمَنْ خَافَ مَقَامَ رَبِّهِ جَنَّتَانِ"

(سورة الرحمن الآية-46)

صدق الله العظيم

Dedication

To my mother and father who helped me through out of my whole life to be better person. .

And to my friends and colleagues who helped me to complete this research..

I dedicate this work..

Acknowledgement

I would like to thank my supervisor: Dr: Abu Elgasim Abass Awad Elkareem who helped and supported me patiently to complete this research.

Abstract

This is a hospital based descriptive retrospective study conducted in Radiation and Isotopes Center-Khartoum (RICK) hospital, during the period from March to June 2019. The study aimed to detect the expression of cytokeratin 5/6 and smooth muscle actin in triple negative invasive ductal carcinoma tissues using immunohistochemical method. Thirty formalin fixed paraffin embedded blocks from patients samples previously diagnosed as invasive ductal breast carcinoma with triple negative phenotype were collected. A tissue microarray block made and section of 4 micron thickness was cut from the microarray block by rotary microtome and stained by immunohistochemical method for detection of cytokeratin 5/6 and the same done for detection of smooth muscle actin. Data collected from patient's files and obtained results were analyzed using SPSS computer program.

All thirty samples were invasive ductal carcinoma with triple negative phenotype. The patients age range between 20 to 70 years with mean age of 45 years. Based on the expression of cytokeratin 5/6 which differentiate between the basal and non-basal phenotype among the triple negative breast carcinoma, the Basal-like phenotype indicated by positive expression of cytokeratin 5/6 was in 25/30 (83.3 %) and the Non-basal-like phenotype indicated by the negativity of cytokeratin 5/6 was in 5/30 (16.7%) , while only 6/30 (20%) showed positive expression of smooth muscle actin among Triple Negative breast Carcinoma (TNBC).

The study concluded that the expression of CK 5/6 is high (83.3%) among TNBC and the expression of SMA is low (20%) among TNBC. Also the lack of the expression of SMA in positive CK 5/6 expressed tissues is high (84%) among TNBC.

المستخلص

أجريت دراسة الحالة الوصفية الإسترجاعية في مستشفى الخرطوم لعلاج الأورام خلال الفترة من مارس الى يونيو2019 وقد هدفت الدراسة للكشف عن تعبير السايتوكراتين (5/6) وأكتين العضلات الناعمة في انسجة سرطان القنوات المتسلل نوع السالب الثلاثي بالثدي باستخدام كيمياء الأنسجة المناعية . جمعت ثلاثون قالب محفوظ في الفورمالين ومطمور بشمع البارفين من عينات مرضى تم تشخيصهم مسبقا بسرطان القنوات المتسلل نوع السالب الثلاثي بالثدي.

وتم عمل قالب ثانوي باستخدام تقنية مايكروأري الانسجة مكونا من الثلاثين عينة .تم قطع مقطع من القالب الثانوي بسمك 4 ميكرون بواسطة جهاز المشراح وتم صبغ العينات بواسطة كيمياء الانسجة المناعية للكشف عنالسايتوكراتين (5/6) وأكتين العضلات الناعمة .جمعت البيانات من ملفات المرضى وحللت النتائج باستخدام برنامج الحزمة الاحصائية للعلوم الاجتماعية.

جميع العينات التي تم جمعها تنتمي لمرضى تم تشخيصهم مسبقا بسرطان القنوات المتسلل نوع السالب الثلاثي بالثدي وتراوحت أعمار المرضى بين 20 الى 70 سنة بمتوسط عمر 45 سنة.

بالإعتماد على التعبير الكيميائي النسيجي للسايتوكراتين (5/6) والذي بدوره يفرق بين النمط الظاهري لشبيه القاعدية و النمط الظاهري غير شبيه القاعدية ضمن سرطان القنوات المتسلل نوع السالب الثلاثي، فقد كانت نسبة النمط الظاهري بشبيه القاعدية (83.3 %) والتي تمثل التعبير الإيجابي للسايتوكراتين (5/6) ، اما نسبة النمط الظاهري لغيرشبيه القاعدية فقد كانت (16.7%) والتي تمثل التعبير السلبي للسايتوكراتين (5/6) .اما نسبه التعبير الكيميائي النسيجي أكتين العضلات الناعمة فقد كانت بنسبة (20%) ضمن أنسجة سرطان القنوات المتسلل نوع السالب الثلاثي.

استخلصت هذه الدراسة إلى أن ظهور تعبير السايتوكراتين (5/6) عالي بنسبة (83.3%) في أنسجة سرطان القنوات المتسلل نوع السالب الثلاثي.اما ظهور تعبير أكتين العضلات الناعمة منخفض بنسبة (20%) في أنسجة سرطان القنوات المتسلل نوع السالب الثلاثي. وايضا التعبير السلبي لأأكتين العضلات الناعمة بنسبة عاليه (84%) في الأنسجه ذات التعبير الإيجابي للسايتوكراتين (5/6) ضمن أنسجة سرطان القنوات المتسلل نوع السالب الثلاثي.

List of contents

List of tables

List of Photographs

Chapter One

Introduction

Chapter One

Introduction

1.1 Introduction:

Triple negative breast cancer (TNBC) is a subtype of breast cancer that based on immunohistochemistry (IHC) and defined by the lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2 neu) expressions, it's generally larger in size, higher in grade, have lymph node involvement at diagnosis, and biologically more aggressive than other types of breast cancers (Morris *et al.*,2007).

It constitutes about 10%–20% of all breast cancers; more frequently affects younger patients, and is more prevalent in African-American women who have a family history of breast cancer, and have a mutation in the Breast Cancer gene 1 (BRCA1) (Pal *et al.*,2011).

This clinically defined subtype of breast cancer also comprise basal like molecular subtype, however triple negative and basal breast cancers are not synonymous and there is substantial overlap and heterogeneity among these two groups. For example in a study; 71% of TNBC were found to be of basal subtype and conversely 77% basal tumors were triple negative by IHC analysis (Shah et *al.*,2012).

Triple negative breast cancers (TNBC) are usually high-grade, invasive ductal carcinomas. It can be divided into two subtypes by IHC: basal-like and non-basal-like tumors (Foulkes *et al.*,2010).The basal-like TNBCs are defined by CK5 or CK5/6 positivity and have a worse prognosis than non-basal-like TNBCs which have negative expression of CK5 or CK5/6 (Sutton *et al*.,2010).

The term basal has been applied to the well-defined myoepithelial (ME) cell (exhibits features of both epithelial and smooth muscle cells.) and to a specific subpopulation of basal CK-expressing cells that may be found in either a luminal or basal location regardless of smooth muscle marker expression (Rakha *et al.*,2009).

Loss of the myoepithelial/basal layer is the gold standard for the diagnosis of invasive breast carcinomas. Using smooth muscle actin (SMA) directed against muscle and myoepithelial cell layer related antigens to distinguish the myoepithelial cell layer from the epithelial layer and surrounding fibroblasts (Kalof *et al.*,2004).

Triple negative breast cancer with basal-like features (TN-BCBL) is apathological subtype, defined by molecular profiling similar to that of the basal/myoepithelial cells of the breast and

has an aggressive behavior and poor prognosis. Basal-like phenotype expresses high molecular weight cytokeratins (CK5/6, CK14 and CK17) and/or human epidermal growth factor receptor (EGFR) ,Therefore, screening for basal markers (CK5/6 and/or EGFR) is important in predicting prognosis and therapeutic strategies in TNBC (Aziza *et al.*,2017).

The lack of targeted growth driver (ER, PR, and Her2) require another therapeutic strategy other than chemotherapy because more than one-half of TNBCs do not respond to chemotherapy. These strategies include EGFR targeted agents, androgen receptor targeted agents, anti-antigenic agents (Oakman*etal.*,2011).

1.2 Objectives:

1.2.1. General objective:

To study cytokeratin 5/6 and SMA expressions in TNBC tumors among Sudanese women.

1.2.2. Specific objectives:

1. To detect SMA and cytokertain 5/6 in triple negative breast tissues using immunohistochemistry.
2. To classify TNBCs into basal-like and non basal-like according to cytokertain 5/6 expression.
3. To correlate between the expression of SMA and cytokeratin 5/6 in TNBCs.

Chapter Two

Literature Review

Chapter Two

Literature Review

2.1 Scientific background:

Breast carcinomas are classified as ER (luminal A and luminal B), HER2þ, or triple negative, and these different types demonstrate different tumor biology, prognoses, and therapy responses based on their gene expression profiles. Triple negative breast carcinomas (TNBCs) represent approximately 15% of all breast carcinomas and have the worst 5-year survival rate of any type of breast cancer (Foulkes *et al.*,2010).

2.2Basal and myoepithelial:

The term ‘‘basal’’ was first introduced to refer to the cells in normal multi-layered epithelia that are juxtaposed next to the stroma and/or the basement membrane. It has also been used to refer to cells that are similarly positioned in a benign or malignant lesion. In the mammary glands of adult mice and humans, most of the basal cells have features of smooth muscle cells. These include the presence of contractile proteins (such as myosin and smooth muscle actin) that enable the gland to express the milk produced during lactation down the ducts and out the nipple, hence the alternate description of basal mammary cells as myoepithelial cells (Linzell.,1952).

The normal breast is composed of two cell layers, an inner luminal cell population and a distinct outer cell layer juxtaposed to the basement membrane, termed the basal layer (Anbazhagan *et al.*,1998).

The luminal and basal layers have different immunoprofile. Cytokeratins are intermediate filament forming proteins which are expressed in different combinations in these distinct epithelial cell types. Basal cells typically express CK5/6 and CK 17, while luminal cells typically express cytokeratins 8 and 18 (Putti *et al.*,2005).

2.3Triple negative and basal-like:

Triple-negative is a term based on clinical assays for ER, PR, and HER2, whereas basal-like is a molecular phenotype initially defined using cDNA microarrays (Sorlie *et al.*,2001).

Although most triple-negative breast tumors do cluster within the basal-like subgroup, these terms are not synonymous; there is up to 30% discordance between the two groups (Bertucci *et al.*,2008).

2.4 Tumor heterogeneity of TNBC:

Molecular analysis have demonstrated that TNBCs are a heterogeneous group of tumors. Six TNBCs were identified using gene expression profiling: basal-like carcinoma (BL1 and BL2), which is the major subtype; immunomodulatory; mesenchymal; mesenchymal stem-like; luminal androgen receptor; and unstable subtypes, which has allowed for specific targeting of the unique biologic behavior of each subtype (Lehmann*et al.*,2011).

2.5 Epidemiology of TNBC:

Triple-negative breast cancers have been characterized by several aggressive clinicopathologic features including onset at a younger age, higher mean tumor size, higher-grade tumors, and, in some cases, a higher rate of node positivity (Carey *et al.*,2006).

Among approximately 500 women evaluated in the Carolina Breast Cancer Study, those with basal-like tumors (defined as ER-negative, PR-negative, HER2-negative, CK 5/6-positive, and/or HER1-positive) were more likely to be African-American (prevalence of 26% vs 16% in non–African-Americans) and premenopausal (24% vs 15% postmenopausal), in which they observed a particularly high prevalence of basal-like tumors among premenopausal, African-American women compared to postmenopausal African-American women and non–African-American women of any age (39% vs 14% and 16%, respectively; $P < .001$) (Carey *et al.*,2006).

As compared with patients who have non-TNBCs, patients with TNBCs are younger at disease onset (50 years) with a higher tumor grade at diagnosis and a higher rate of developing metastatic disease. These aggressive tumors also have different metastatic patterns compared with non-TNBC tumors. Distant metastases typically occur early in the disease course, with a propensity for visceral metastasis to the lung and brain, rather than the lymph nodes, bone, or liver as seen in non-TNBCs (Foulkes*et al.*,2010).

2.6 Risk factors of TNBC:

2.6.1 Age:

Triple-negative breast cancer is more likely to be diagnosed in people younger than age 50. Other types of breast cancer are more commonly diagnosed in people age 60 or older (Partridge *et al.*,2016).

2.6.2 Race:

Triple negative breast cancer is more likely to be diagnosed in Black women and Hispanic women. Asian women and non- Hispanic white women are less likely to be diagnosed with this type of cancer (Kohler *et al.*,2015).

2.6.3 Genetics:

About 70% of breast cancers diagnosed in people with an inherited BRCA mutation, particularly BRCA1, are triple-negative (Atchley *et al.*,2008).

2.6.4 Oral contraceptives:

The TNBC risk was 2.5 times increased in women who had used oral contraceptives for more than 1 year compared to women who had used oral contraceptives for less than 1 year or never (Dolle *et al.*,2009).

2.7 Diagnosis of TNBC:

Once a breast cancer diagnosis has been made using history, physical examination, imaging tests and a biopsy, then cancer cells will be checked immunohistochemically for definitive diagnosis of TNBC.

2.7.1 Immaging techniques:

Different screening tests can be used to look for breast cancer.

2.7.1.1 Mammography:

Is the process of using low-energy X-rays (usually around 30 kVp) to examine the human breast for diagnosis and screening. The goal of mammography is the early detection of breast cancer, typically through detection of characteristic masses or microcalcifications. A mammogram is an X-ray picture of the breast (Kerlikowskeet *et* al.,2011).

2.7.1.2 Magnetic resonance imaging (MRI):

MRI is a powerful imaging tool that produces high-resolution images without requiring the application of harmful radiation (Van Goethem *et al.*,2006).

2.7.1.3 Molecular breast imaging (MBI):

MBI uses a radioactive tracer that lights up cancer tissues of the breast, visualized by a nuclear medicine scanner (O'Connor *et al.*,2009).

2.7.2 Laboratory tests:

2.7.2.1 Blood-based assay:

Serum Breast biomarkers are Serum tumor markers are soluble molecules released into the blood stream by cancer cells or other cell types belonging to tumor microenvironment such as CA 15-3, carcinoembryonic antigen (CEA), and CA 27-29 (Bast *et* al., 2001).

2.7.2.2 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) (Palmer *et al.*,1993).

A histological study of basal-like tumors, of which all were ER/HER2–negative, illustrated marked increases in mitotic count, geographic necrosis, pushing borders of invasion, and stromal lymphocytic response (Livasy *et al.*,2006).

2.7.2.3 Immunohistochemistry (IHC):

This is a technique for identifying cellular or tissue constituents (antigens) by means of antigen antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method (Bancroft *et al.*,2012).

2.7.2.3.1 Cytokeratin 5/6:

Cytokeratins 5/6 (CK5/6) are medium sized neutral polypeptides that are part of the cytokeratin family of polypeptides. They are expressed in keratinized and non-keratinized squamous epithelium of prostate, mammary, and salivary glands (Ma *et al.*,2015).

CK 5/6 expression has been linked to phenomenon known as epithelial mesenchymal transition (EMT). This is a process by which cells of epithelial origin lose epithelial characteristics and polarity, and acquire a mesenchymal phenotype with increased migratory behavior that is associated with increased aggressiveness, and invasive and metastatic potential (Nielsen *et al.*, 2004).

The normal resting breast tissue is composed of luminal cells which express CK 8/18, CK 7, CK19. The basal/ myoepithelial cells express CK 5/6, CK 14, CK 17 and smooth muscle actin. A small subset of cells, comprising less than 5% of entire cell population, expresses CK 5. These cells are dispersed in the inner layer of ductal system and differentiate into myoepithelial or glandular cells via intermediary cells (Chu*et al.*,2002).

The expression of CK5/6 is used in the differential diagnosis of basal-like breast cancer from other triple negative (TN) breast cancer. And its expression in TN breast cancer is correlated with poor prognosis, high grade differentiation and axillary lymph node metastasis (Liu *etal.*,2008).

2.7.2.3.2 Smooth Muscle Actin (SMA):

Smooth Muscle Actin (SMA / ACTA2) is a myoepithelial marker for normal and cancerous breast tissue used alongside smooth muscle myosin heavy chain (SMMHC), calponin and cytokines like CK5 and CK17 to distinguish myoephithelial cells from ductal carcinoma cells, which are negative for these proteins (Zaha, 2014).

In breast tissues, the contractile protein SMA has been demonstrated in the normal myoepithelial cells (MEC) in 88% to 100% of cases, in normal luminal/epithelial cells in 37% of cases, and in the MEC associated with benign lesions in 95.6% of cases. Although most invasive breast carcinomas are SMA negative, the diagnostic utility of this marker is limited by the frequent positivity of stromal myofibroblasts and vascular smooth muscle as well as subsets of the tumor cells in a number of histotypes (notably 10%–16.1% of invasive ductal carcinomas) (Dewar *et al.*,2011).

Loss of the myoepithelial/basal layer is the gold standard for the diagnosis of invasive breast carcinomas. Immunohistochemical markers used are commonly directed against muscle and myoepithelial cell layer related antigens, including smooth muscle actin (SMA), p63, myosin, and calponin, to distinguish the myoepithelial cell layer from the epithelial layer and surrounding fibroblasts (Kalof *et al.*,2004).

2.8 Treatment and management of TNBC:

TNBCs do not drive their growth with the hormones estrogen or progesterone or with the oncogene HER2 and, correspondingly, are characterized by their lack of ER, PR, and HER2 expression in IHC. Without a known driver, there has been no ‘‘target’’ for developing targeted therapy. Women with TNBCs do not benefit from endocrine therapy or trastuzumab. Currently, conventional chemotherapy is the main treatment modality for TNBC in a neoadjuvant or adjuvant setting. However, more than one-half of TNBCs do not respond to chemotherapy (Oakman *et al.*,2011).

Several therapies are being developed that target specific biomarkers of TNBC or basal-like subtype. These strategies include EGFR targeted agents, androgen receptor targeted agents, anti-antigenic agents, and PARP inhibitors are offering an option in triple negative disease (Penault *et al.*,2012).

TNBC with BRCA1 gene mutations may be more sensitive to agents that cause DNA damage, such as Cisplatin (Gonzalez *et al.*,2011).

2.9 Prognosis of TNBC:

The inferior prognosis associated with triple-negative breast cancer was originally recognized in the initial studies examining outcome by intrinsic subtype. These studies uniformly demonstrated a poorer prognosis among patients with breast cancer classified as "basal-like," particularly compared to those in good-prognosis subclasses (i.e. luminal A) via gene expression profiling (Foulkes *et al.*,2004).

Canadian reported series evaluating prognosis in over 1,500 women illustrated an increased likelihood of distant recurrence (hazard ratio [HR] = 2.6, $P < .0001$) and death (HR= 3.2, $P < .0001$) among women with triple-negative breast cancer compared to non–triple-negative disease. Interestingly, the pattern of recurrence over a 5-year follow-up period was substantially different among groups (Foulkes *et al.*,2004).

Chapter Three

Materials and Methods

Chapter Three
Materials and Methods

3.1 Materials:

Archived tissue blocks previously diagnosed as invasive ductal breast carcinoma with triple negative phenotype were used in this study.

3.2 Methods:

3.2.1 Study design:

This is hospital based descriptive retrospective study aimed to detect the expression of CK5/6 and SMA among Sudanese triple negative breast carcinoma patients using immunohistochemical method.

3.2.2 Study sample:

Thirty tissue blocks obtained from breast samples previously diagnosed as invasive ductal carcinoma with triple negative phenotype.

3.2.3 Study area:

This study was held in Radiation and Isotopes Center-Khartoum histopathology laboratory during the period from March2019 to June 2019.

3.2.4 Blocks preparation for tissue microarray:

Target area from origin blocks was identified on the H&E ready stained sections using permanent marker so that the corresponding area on the tissue block can be sampled. Origin block was then subjected to 3 mm skin punch (Miltex biopsy punch, Germany) and tissue was carefully punched. The selected core was then brought in to a recipient paraffin block. The surface of TMA blocks were then pressed by preheated clean glass slide until the surface became smooth, then the blocks were placed in refrigerator until cooling. Glass slide was then detached and the block was ready for cutting.

3.2.5Section preparation and staining:

Tissue microarray block was sectioned by using rotary microtome (Leica RM 2125) and low profile disposable knives by using 4 micron as thickness of choice.

Sections were then floated on a floating water bath adjusted to 45 ^{o}C. Finally clean coated glass slides in addition to ordinary slides were used to pick up the floated sections and slides were left in a 60 ^{o}C for 2 hours, the slides and positive controls were dewaxed in xylene, dehydrated through graded alcohols to distilled water. Slides were then placed in preheated buffer at 97^{o} C in a water bath for 10 minutes, after completion of the retrieval the coplin jar that contained the slides were removed from water bath and allowed to cool to room temperature.

The staining procedures were carried out by using Zytomed System kits as following:

After slides reached the room temperature, they were washed in phosphate buffer saline with Tween 20 pH 7.6 for 5 minutes. After that circle made around the sections by using Dako pen (Dako Denmark A/S). The sections were then covered with 3% hydrogen peroxidase blocker and incubated 30 minutes at room temperature for endogenous peroxidase enzymes blocking , then washed in phosphate buffer saline for 5 minutes, and the primary antibody applied on the sections for 30 minutes,. The slides then were washed in phosphate buffer saline for 5 minutes, and then covered by anti-rabbit/anti-mouse IgG- polymer HRP for 30 minutes, and wash by phosphate buffer saline for 10 min. The DAB chromogen was then applied on to the slides (1ml substrate buffer+ 2drop of DAB chromogen). After that sections were washed in distilled water and counterstained with Mayer's Haematoxlyin for 1 min, washed in distilled water and left to air dry for 5 minutes. Finally slides were cleared in xylene and mounted with a cover glass using DPX (Bancroft *et al.*,2012).

3.2.6 Result interpretation:

All quality control measures were adopted during sample staining and immunohistochemical results assessment. Positive and negative controls were used to confirm location of positivity of expression.

3.2.7 Statistical analysis:

Data were analyzed using SPSS version computer program, frequencies, means and Chi-square tests were calculated.

3.2.8 Ethical considerations:

Hospital administration agreements were taken ethically for archive samples and patients data collection.

Chapter Four

Results

Chapter Four

Results

4. Results :

Thirty samples previously diagnosed as high grade invasive ductal carcinoma with triple negative expression were used in this study. The patient ages were 22 (73.3 %) less than 50 years old and 8 (26.7 %) were more than 50 years old (Table 4-1).

Positive expression of cytokeratin 5/6 among study samples was found in 25/30(83.3 %) and the negative expression was 5/30 (16.7 %) (Table 4-2).

Positive expression of smooth muscle actin among study samples was 6/30 (20 %) and negative expression was 24/30 (80%) (Table 4-3).

The correlation between cytokeratin 5/6 and smooth muscle actin expression among TNBC showed on (Table 4-4) in which we found that the lack of SMA among the positive CK 5,6 was 21/25 (84 %) to correlate between the lack of SMA in basal-like and deregulate control of CK expression.

Table 4- 1: Distribution of age groups among study samples:

Age group (Years)	Frequency	Percent
Less than or equal to 50	22	73.3%
More than 50	8	26.7%
Total	30	100%

Table 4-2: Expression of Cytokeratin 5/6 among study samples:

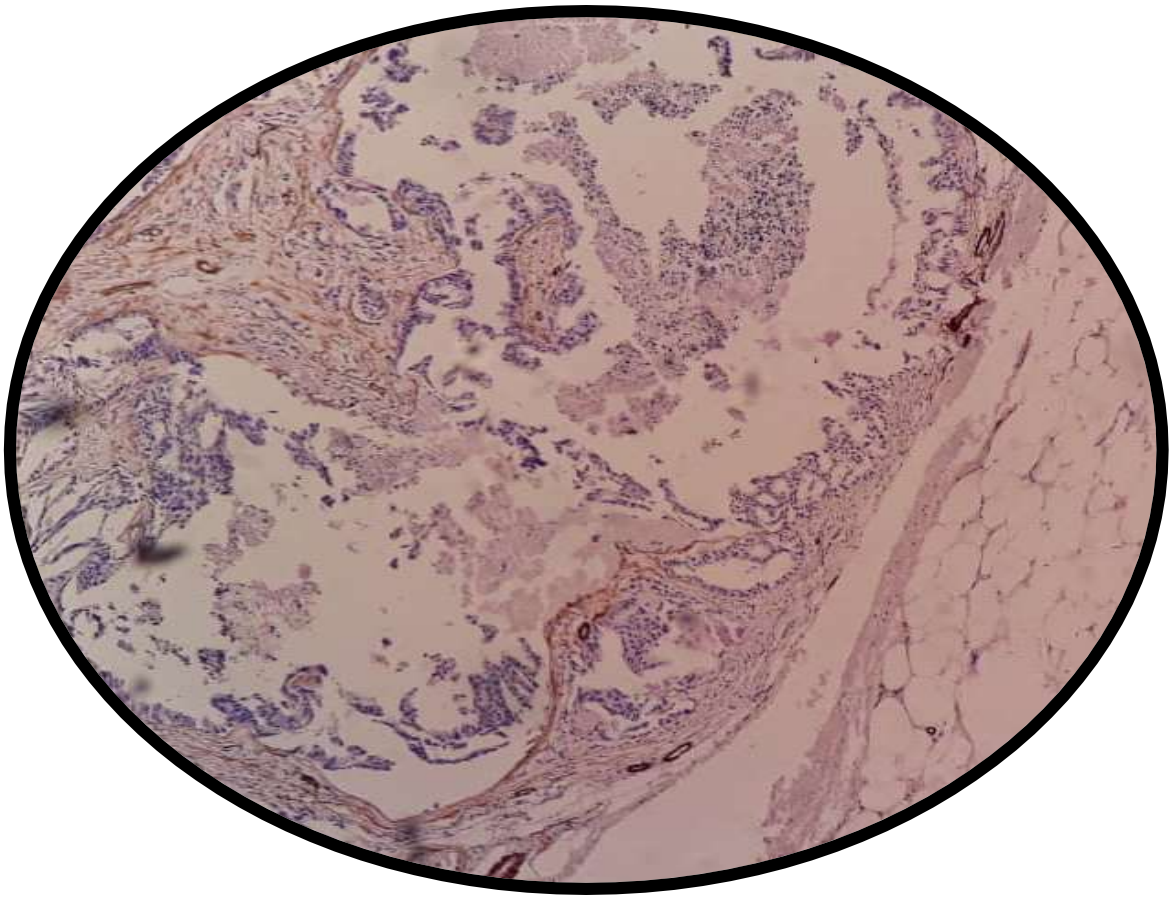
Expression of CK 5/6	Frequency	Percent
Positive	25	83.3%
Negative	5	16.7%
Total	30	100%

Table 4-3: Expression of Smooth Muscle Actin among study samples:

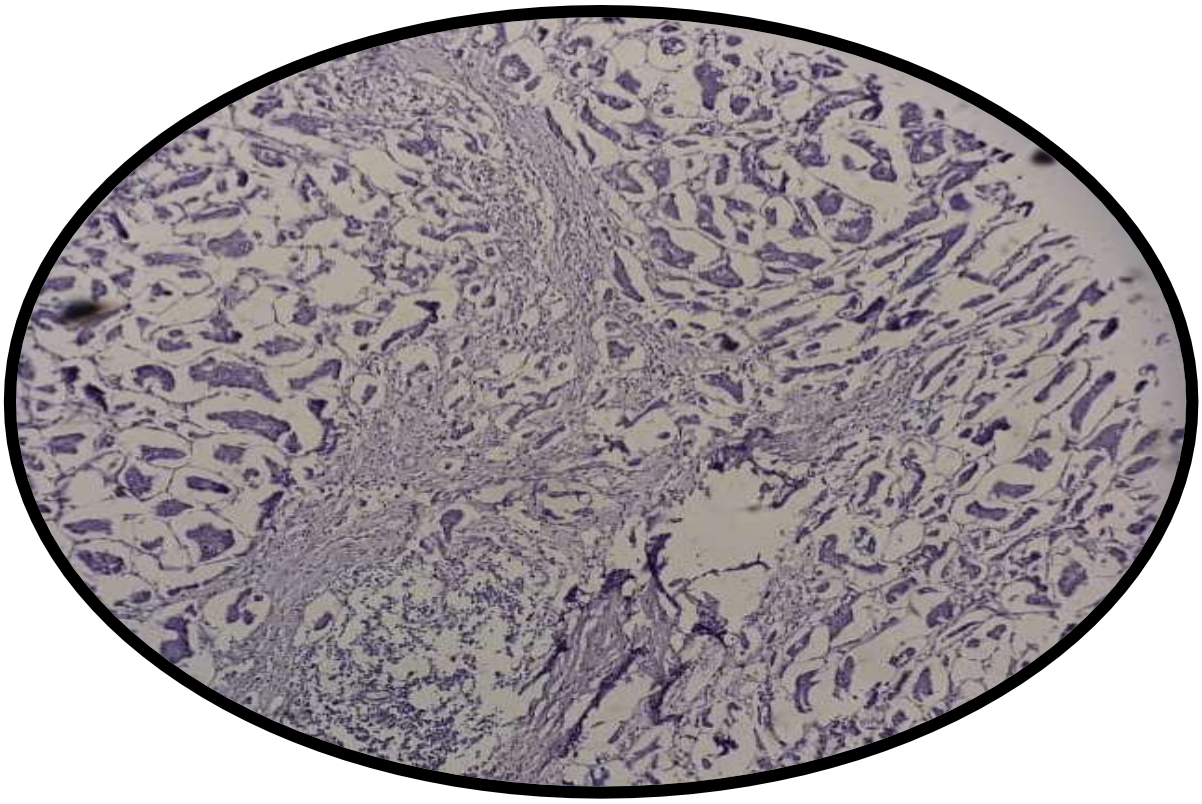
Expression of SMA	Frequency	Percent
Positive	6	20%
Negative	24	80%
Total	30	100%

Table 4-4: Relation between cytokeratin 5/6 and smooth muscle actin:

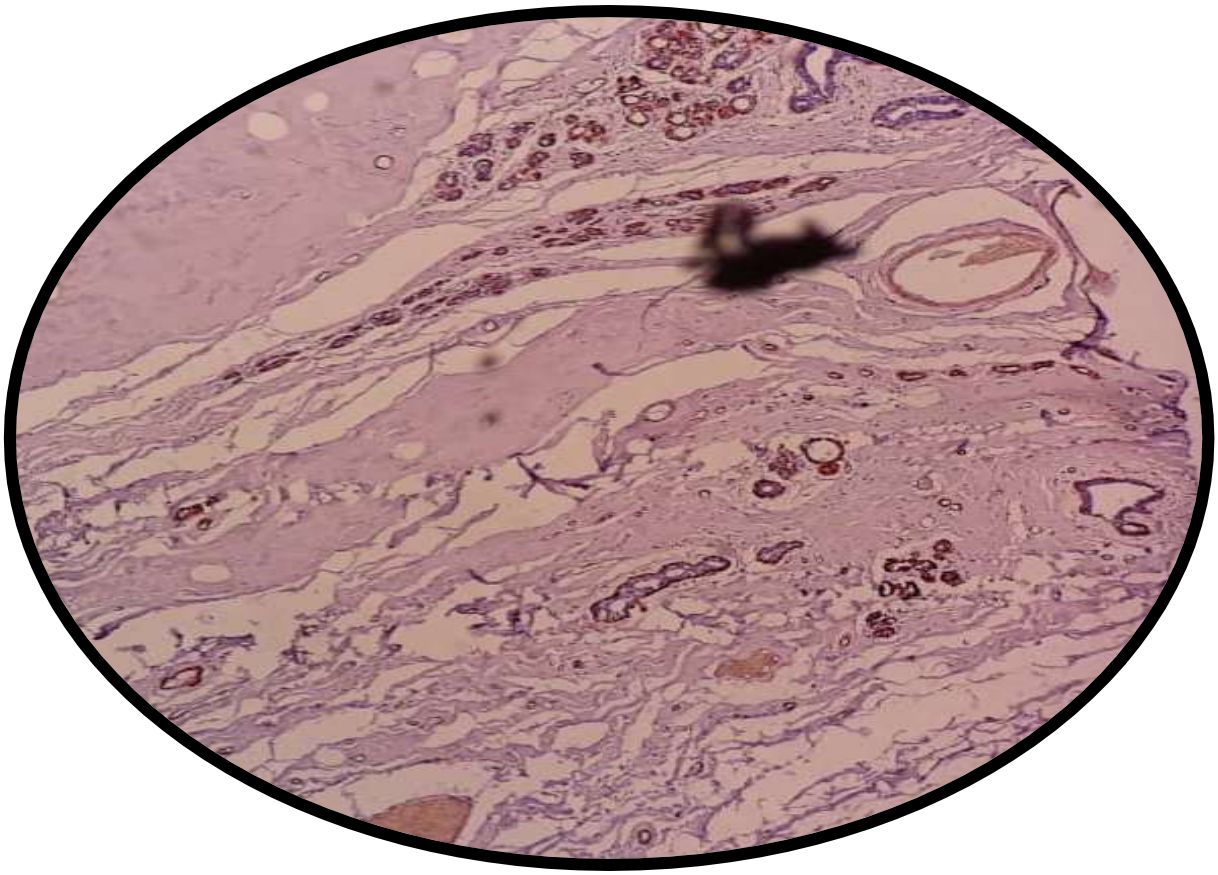
Expression		SMA		Total	P.value
		Positive	Negative		
CK 5,6	Positive	4	21	25	0.221
	Negative	2	3	5	
Total		6	24	30	



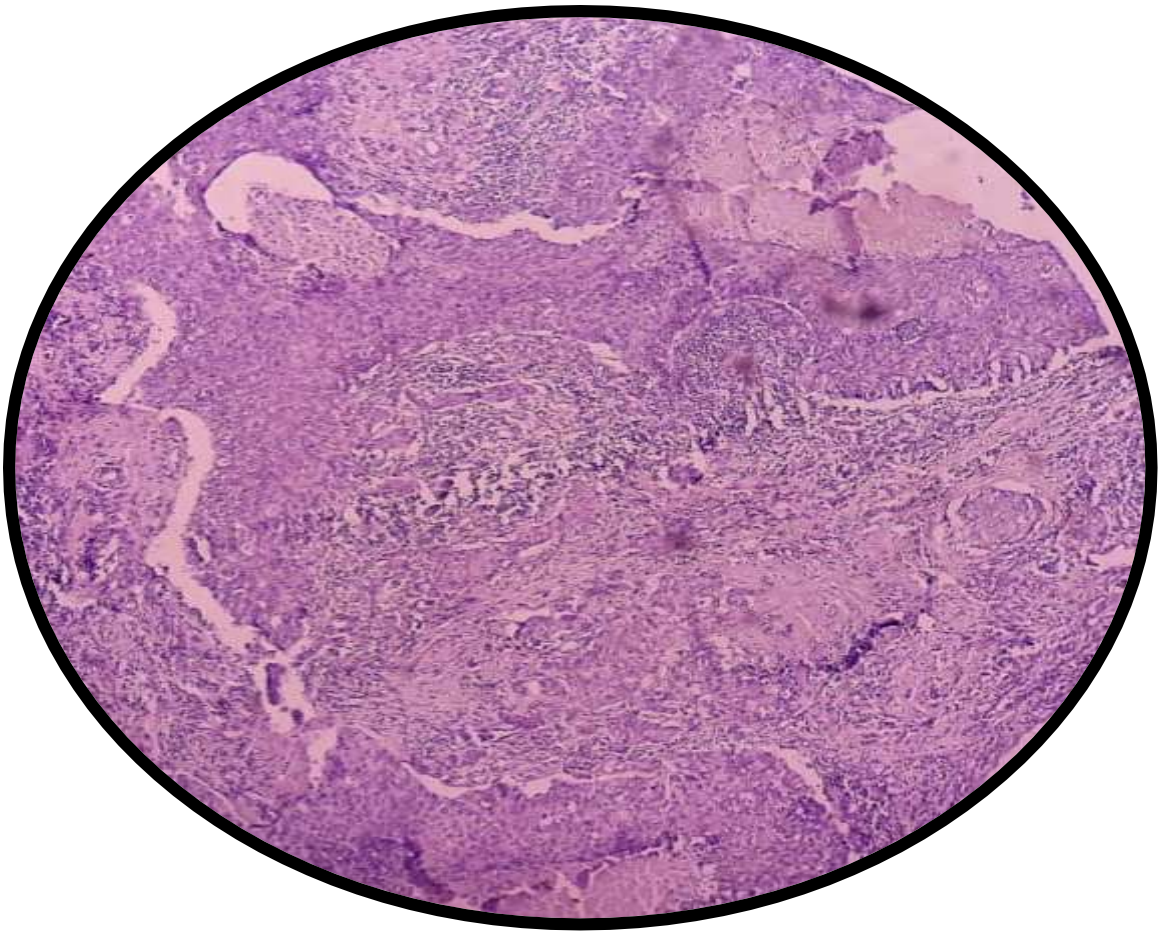
Graph 4.1: Invasive ductal breast carcinoma with triple negative phenotype showing cytoplasmic expression of cytokeratin 5/6 (40x).



Graph 4.2: Invasive ductal breast carcinoma with triple negative phenotype showing loss of expression of SMA (40x).



Graph 4.3: Invasive ductal breast carcinoma with triple negative phenotype showing cytoplasmic expression of SMA (40 xs).



Graph 4.4: Invasive ductal breast carcinoma with triple negative phenotype showing loss of expression of cytokeratin 5/6 (40 xs).

Chapter Five

Discussion, conclusion and recommendations

Chapter Five

Discussion, conclusion and recommendation

5.1 Discussion:

The present study involves thirty cases of high grade invasive ductal breast carcinoma with triple negative phenotype for immuohistochemical staining by cytokeratin 5/6 and smooth muscle actin.

Regarding the age of the population, the study revealed that the most of patients were less than 50 years. So, it proves that TNBC is more common in younger patients which is compatible with Pal *et al* (2011), who reported that TNBC is more frequently affects younger patients.

The positive expression of cytokeratin 5/6 was found in 83.3 % of TNBC, which reflect the basal-like phenotype among TNBC as cytokeratin 5/6 has been employed as a marker of basal differentiation resulting in association with triple negative molecular subtype according to Pillai *et al* (2012). And it also reflects that the basal-like is more frequently than the non-basal-like among TNBC. Livasy *et al* (2006) reported that cytokeratin 5/6 expression in tumors was significantly associated with the basal-like subtype, P = 0.0001.

This result was disagree with Hashmi *et al* (2008) who reported that positive CK5/6 expression was only noted in 8% (12 cases) of TNBC while 2.4% (4 cases) showed focal positive and 89.3% (134) were negative with CK5/6.

The positive expression of smooth muscle actin was found in 20% of TNBC which reflect the loss of the myoepithelial/basal layer and invasion ,since benign and early lesions have an intact myoepithelial layer surrounding breast glands which agree with Livasy *et al* (2006) who reported that only 4/18 (22%) of the basal-like tumors showed immunoreactivity for SMA .

This result was disagree with (Jeon,2016) who states that high levels of smooth muscle actin expressions are correlated with an increase in invasive potential through its functions in the epithelial to mesenchymal transition.

The negative expression of SMA was in 80% of TNBC, and does not reflect any significance alone, but when correlated with the positive expression of cytokeratin 5/6 it appears to have more values, we found that the lack of SMA among the positive CK 5,6 was 21/25 (84 %) which agreed with Gorski *et al* (2010) who found that the lack of SMA in basal-like breast cancers

could reflect the activation of mechanisms responsible for the development of squamous metaplasia that also deregulate control of CK expression.

5.2 Conclusion:

On the basis of this study we conclude: the expression of CK 5/6 is high (83.3%) among TNBC, and the expression of SMA is low (20%) among TNBC.

The lack of the expression of SMA in positive CK 5/6 expressed tissues was (84%) among TNBC.

5.3 Recommendations:

Further studies with large sample size should be done on the expression of SMA and CK 5/6 in TNBC.

Other tumor markers should be applied for TNBC like human epidermal growth factor receptor (EGFR) to assist identification of basal-like phenotype among TNBC, and as new treatment strategy target.

References

References

1. Abdelrahman A , Rashed H, AbdelgawadM , and Abdelhamid M (2017) .Prognostic impact of EGFR and cytokeratin 5/6 immunohistochemicalexpression in triple-negative breast cancer .*Annals of Diagnostic Pathology*.**28**:43-53.
2. Anbazhagan R, Osin P, Bartkova J, Nathan B, Lane E, and Gusterson B (1998). The development of epithelial phenotypes in the human fetal and infant breast. *J Pathol.***184**:197-206.
3. Atchley D, Albarracin C, Lopez A, Valero V, Amos C, Gonzalez-Angulo A *et al* (2008). Clinical and pathologic characteristics of patients with BRCA-positive and BRCA negative breast cancer. *J Clin Oncol.***26**(26):4282-8.
4. Bancroft J, Suvarna K, and Layton C (2012). Theory and practice of histological techniques, 7th edition. London, New York, Oxford Philadelphia, St Louis Sydney Toronto. PP. 381-427.
5. Bast C, Ravdin P, Hayes F,Bates S , Fritsche H, and Jessup M *et al* (2001). American Society of Clinical Oncology Tumor Markers Expert Panel. 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol.* **19**(6):1865–1878.
6. Bertucci F, Finetti P, Cervera N, Esterni B, Hermitte F, and Viens P *et al* (2008). How basal are triple-negative breast cancers?. *Int JCancer.***123**:236–240.
7. Carey L, Perou C, Livasy C, Dressler L, Cowan D, and Conway K *et al* (2006). Race breast cancer subtypes, and survival in the carolina breast cancer study. *JAMA.***295**:2492–2502.
8. Chu G and Weiss M (2002). Keratin expression in human tissues and neoplasms. *Histopathology.***40**:403-39.
9. Cooper D, Schermer A, and Sun T (1985). Classification of human epithelial and their neoplasms using monoclonal antibodies to keratins: Strategies, applications, and limitations. *Lab Invest.***52**:243-56.
10. Dabbs D, Chivukula M,Carter G, and Bhargava R (2006).Basal phenotype of ductal carcinoma in situ: recognition and immunohistologic profile. *Modern Pathology.* **19**, 1506–1511.

11. Dewar R,Fadare O, Gilmore H, and Gown A (2011). Myoepithelial Markers in Breast Pathology. *Arch Pathol Lab Med.***135**:422–429.
12. Dolle J, Daling J, White E, Brinton L, Doody D, Porter P *et al* (2009). Risk factors for triple-negative breast cancer in women under the age of 45 years. *Cancer Epidemiol Biomarkers Prev.* **18**(4):1157–1166.
13. Foulkes W, Brunet J, Stefansson I, Straume O, Chappuis P, and Bégin L*et al* (2004) The prognostic implication of the basal-like (cyclin E/p27/p53/glomeruloid-microvascular-proliferation) phenotype of BRCA1-related breast cancer. *Cancer Res*.**64** (3):830–835.
14. Foulkes W, Smith I, and Reis-Filho J (2010). Triple-negative breast cancer. *N Engl JMed.* **363**(20):1938–1948.
15. Gonzalez-Angulo A, Timms K, Liu S, Chen H, Litton J,and Potter J*et al* (2011).Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer. *Clinical Cancer Res.***17**:1082–1089.
16. Gorski J, James C, Quinn J, Stewart G, Staunton K, and Buckley N*et al* (2010). BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer. *Breast Cancer Res. Treat*.**122**, 721–731.
17. Hashmi A, Naz S, Hashmi S, Hussain Z, Irfan M, Abu Bakar S *et al*(2018). Cytokeratin 5/6 and cytokeratin 8/18 expression in triple negative breast cancers: clinicopathologic significance in South-Asian population.*BMC Res Notes.***11** (1):372.
18. Jeon M, You D, Bae S, Kim S, Nam S, Kim H *et al* (2016). Dimerization of EGFR and HER2 induces breast cancer cell motility through STAT1-dependentACTA2 induction. *Oncotarget.* **8**(31):50570-50581.
19. Kalof AN, Tam D,Beatty B,and CooperK (2004).Immunostaining patterns ofmyoepithelial cells in breast lesions: acomparison of CD10 and smoothmuscle myosin heavy chain. *J ClinPathol*.**57** (6):625-9.
20. Kerlikowske K, Hubbard R, Miglioretti D,Geller B, Yankaskas B, and Lehman C *et al* (2011). Breast Cancer Surveillance Consortium. Comparative effectiveness of digital versus film-screen mammography in community practice in the United States: a cohort study. *Ann Intern Med.***155**(8):493–502.

21. Kohler B, Sherman R, Howlader N, Jemal A, Ryerson A, Henry K *et al* (2015). Annual report to the nation on the status of cancer, 1975-2011, featuring incidence of breast cancer subtypes by race/ ethnicity, poverty, and state. *J Natl Cancer Inst.***107** (6).
22. Lakhani S, Reis-Filho J, Fulford L, Penault-Llorca F, Vijver M, and Parry S*et al* (2005). Prediction of BRCA1 status in patients withbreast cancerusing estrogen receptor and basal phenotype. *Clin Cancer Res***11**:5175–5180.
23. Lehmann B, Bauer J, Chen X, Sanders M, Chakravarthy A, and Shyr Y*et al* (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest.***121** (7):2750–2767.
24. Lim, E, Vaillant F, Wu D, Forrest N, Pal B, and Hart A *et al* (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nat. Med.***15**, 907–913.
25. Linzell J (1952). The silver staining of myoepithelial cells, particularly in the mammary gland, and their relation to the ejection of milk. *J. Anat*. **86**, 49–57.
26. Liu Z, Wu J, Ping B, Feng L, Shen Z,and Shao Z (2008). Expression of CK5/6 and CK17 and its correlation with prognosis of triple-negative breast cancer Patients. *Chinese journal of oncology*.**30** (8):610-614.
27. Livasy C, Karaca G, Nanda R, Tretiakova M, Olopade O, and Moore D *et al* (2006).Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Modern Pathology*.**19**, 264–271.
28. Ma Y, Fan M, Dai L, Kang X, Liu Y, Sun Y *et al*(2015). Expression of p63 andCK5/6 in early-stage lung squamous cell carcinoma Is not only an early diagnostic indicator but also correlates with a good prognosis. *Thoracic Cancer*.**6** (3):288-295.
29. Millikan R, Newman B, Tse C-K, Moorman P, Conway K , and Dressle L *et al* (2008). Epidemiology of basal-like breast cancer. *Breast Cancer Res Treat.***109**:123–139.
30. Morris G, Naidu S, Topham A, Guiles F, Xu Y, and McCue P*et al* (2007). Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: A single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology and End Results database. *Cancer*.**110** (4) -876-884.

31. Nielsen T, Hsu F, Jensen K, Cheang M, Karaca G,Hu Z *et al* (2004). Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clinical cancer research.* **10**(16):5367-5374.
32. Oakman C, Moretti E, Galardi F, Biagioni C, Santarpia L, and Biganzoli L *et al* (2011). Adjuvant systemic treatment for individual patients with triple negative breast cancer. *Breast.* **20** (suppl 3):S135–S141.
33. O'Connor M, Rhodes D, and Hruska C (2009). Molecular breast imaging. *Expert Rev Anticancer Ther*.**9** (8):1073–1080.
34. Partridge A, Hughes M, Warner E, Ottesen R, Wong Y, Edge S *et al* (2016). Subtype-dependent relationship between young age at diagnosis and breast cancer survival. *J Clin Oncol*.**34** (27):3308-14.
35. Pal S, Childs B, and Pegram M (2011). Triple negative breast cancer: unmet medical needs. *Breast Cancer Res Treat*.**125** (3):627–636.
36. Palmer M, and Tsangaris T (1993). Breast biopsy in women 30 years old or less. *Am J Surg.* **165**(6):708–712.
37. Penault F, and Viale G (2012). Pathological and molecular diagnosis of triple-negative breast cancer: a clinical perspective. *Ann Oncol.***23**:vi19–vi22.
38. Pillai S, Tay A, Nair S, Leong C-O *et al* (2012). Triple-negative breast cancer is associated with EGFR, CK5/6 and c-KIT expression in Malaysian women.*BMC Clin Pathol.***12**(1):p.18.
39. Putti T, El-Rehim D, Rakha E, Paish C, Lee A, Pinder S *et al* (2005). Estrogen receptor-negative breast carcinomas: A review of morphology and immunophenotypical analysis. *ModPathol.***18**:26-35.
40. RakhaE and Reis-Filho J (2009). Basal-like breast carcinoma: From Expression Profiling to Routine Practice. *Arch Pathol Lab Med.***133**(6):860-868.
41. Shah S, Roth A, Goya R , Oloumi A, Ha G, Zhao Y*et al* (2012). The clonal and mutational evolution spectrumof primary triple-negative breast cancers. *Nature.* 486:395.
42. Sorlie T, Perou C, Tibshirani R, Aas T , Geisler S, Johnsen H *et al* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA.***98**:10869–10874.

43. Sutton L, Han J, Molberg K, Sarode V, Cao D, Rakheja D *et al* (2010). Intratumoral expression level of epidermal growth factor receptor and cytokeratin 5/6 is significantly associated with nodal and distant metastases in patients with basal-like triple-negative breast carcinoma. *Am J Clin Pathol.***134**(5):782–787.
44. Van Goethem M, Tjalma W, Schelfout K, Verslegers I, Biltjes I, and Parizel P. (2006). Magnetic resonance imaging in breast cancer. *Eur J Surg Oncol.***32**(9): 901–910.
45. Yang X, Sherman M, Rimm D, Lissowska J, Brinton L, Peplonska B *et al* (2007). Differences in risk factors for breast cancer molecular subtypesin a population-based study. *Cancer Epidemiol Biomarkers Prev.***16**:439–443.
46. Zaha D (2014). Significance of immunohistochemistry in breast cancer. *World Journal of Clinical Oncology.* **5**(3):382-392.

Appendix

Appendix

Materials and Instruments

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

Disposable gloves.

Rotary microtome.

Microtome knifes.

Coated slides.

Cover glasses.

Oven.

Water bath.

Re embedding paraffin block.

Humidity chamber.

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

Xylene.

Mayer's haematoxylin (1 gm haematoxylin, 50 gm aluminum ammonium sulphate, 0.2 sodium iodate, 50 gm chloral hydrate, 1 gm citric acid and 1 liter distilled water).

Sodium citrate buffer :(10 Mm sodium citrate, 0.05% Tween20, PH 6.0 prepared as the following: Tri-sodium citrate (dehydrate) 2.94 g, 1 liter distilled water mix to dissolve and add 0.05 ml of Tween20 and mix).

Phosphate buffer (PH 7.4).

Hydrogen peroxidase blocking solution.

Primary antibodies:

Mouse anti-Actin, smooth muscle:

Antibodies to alpha smooth muscle actin, don't detect the other actin isoforms, also called smooth muscle actin SMA, clone 1A4 or sm-1 (16 ml ready to use).Identify smooth muscle cells and myoepthelial cells in normal, reactive, or neoplastic tissue. The positive result interpreted as membranous or cytoplasmic staining.

Mouse anti-Cytokeratin 5/6:

Basic (type II) cytokeratins of molecular weight 58 kDa (CK5) and 56 kDa (CK6) common antibodies which directed against both cytokeratin 5 and 6 (16 ml ready to use)**.** Stains

basal/myoepithelial cells of breast. And the positive result interpreted as diffuse cytoplasmic staining with perinuclear enhancement

Secondary antibody.

DAB (3-3 diaminobenzidine).

DAB substrate buffer.