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Immunohistochemical Detection of Estrogen Receptor among Sudanese Females with Ovarian cancer

A thesis submitted for partial fulfillment of the requirements of the M.Sc. degree in Medical laboratory science (Histopathology and Cytology)

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الايـة

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيم

قَالَ تَعَالَى :

(اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ ۚ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ ۖ الْمِصْبَاحُ فِي زُجَاجَةٍ ۖ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِنْ شَجَرَةٍ مُبَارَكَةٍ زَيْتُونَةٍ لَا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ ۚ نُورٌ عَلَىٰ نُورٍ ۗ يَهْدِي اللَّهُ لِنُورِهِ مَنْ يَشَاءُ ۚ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ ۗ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ)

صدق الله العظيم

سورة النور-{ 35}

Dedication

To whom did you suffer,, I learned and changed..

To those who said it was impossible and I believed in hope.

To myself

To who knocked the doors of the seventh heaven in the dead of night by the most earnest invitations to me, and the thinner of tears shed my love for me,

To the light of the eyes of my dear mother Salma.

To who planted in my soul the hope and spirit of life and struggle, and that it is not impossible to exist, a permanent guide to my dear father Jamal.

To whom I have forged with them the most beautiful memories and the best wishes and the darkest sorrows.

To the eternal bond, the source of strength of human beings: my brothers Mohammed, Ayman, Zafer and Eman.

To those who listened to me, advised me and shared my tears before I laughed.

To my friends and fellow companions.

To my dear sons in the future.

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List of contents

الآية ……..,……..……...I

Dedication ……...II

Acknowledgment…….III

List of contents……..……... IV

List of figures …….....VI

List of tables ……....VII

List of abbreviatio……VIII

Abstracts ……..……..……X

Chapter one-Introduction

1.1 Introduction…….……...…….…1

1.2 Rationale……...…….……..3

1.3 Objectives…….…….....4

Chapter two-Literature review

2.1. Ovarian cancer..……..…….5

2.2. Promoter ER...……...……8

2.3. ER and ovarian cancer..……..……9

2.4. Epidemiology……..…… ……...…..…….10

2.5. Ovarian cancer in Sudan ...…..…….……....12

2.6. Immunohistochemistry method…….……..…13

Chapter three-Materials and methods

3.1. Study design …….……....14

3.2. Study duration ……..14

3.3. Study area …….……..……..14

3.4. Study population, samples and samples size……..……...14

3.4.1. Inclusion and exclusion criteria ……..……..14
3.4.2. Study sample and samples size ……...……...…..14
3.5. Sampling technique …….…….....14
3.6. Data Collection tools and variables ……..14
3.7. Quality controls……..……...14
3.8. Sampling processing …….……...……..14
3.9. Methods of detection ……..…….....15

Chapter four-Results

4. Results…….…….....18

Chapter five-Discussion, conclusion and recommendations

5.1. Discussion…….…….29
5.2. Conclusion…….31
5.3. Recommendations…….....32

References

References……...……...33

List of figures

Figure No.	Title	Page No.
1	Frequency of age group among study populations	20
2	Frequency of cancers types	21
3	Frequency of tumor grades	22
4	Expression of estrogen receptor	23
5	Frequency of degree of estrogen receptor expression	24

List of tables

Table No.	Title	Page No.
1	Correlation of ER expression with cancer types	25
2	Correlation of ER expression with tumor grade	26
3	Correlation of ER expression score with tumor grade	27
4	Correlation of ER expression with patient's age	28

List of abbreviations

AR …….…….....Androgen Receptor

ASR …….…….....Age Standardized rate

CT …….…….....Computed Tomography

DAB……3.3 diaminobenzidine tetrahydrochloride

DPX……..Disterene, aplasticizer (polystyrene) and xylene

DW ……...……..…Distilled Water

EOC……..……...Epithelial Ovarian cancer

ER…….…….……Estrogen Receptor

FFPE…….……...……...Formalin Fixed Paraffin Embedded

FIGO……International Federation of Gynecology and Obstetrics

HRP …….……..Horse Radish Peroxidase

IHC…….…….……...Immunohistochemistry

IP…..**.....**Intraperitoneal

IV …….……..Intravenously

MLS …….…….Medical Laboratory Sciences

MRI …….……...Magnetic Resonance Imaging

NACT…….……..Neo Adjuvant Chemotherapy

NCI…….…….....National Cancer Institute

PBS…….……...Phosphate Buffer Saline

PET …….……Positron Emission Tomography

PI…….……..…….Proliferation index

PR ……...Progesterone Receptor

RT…….……Room Temperature

RTW…….……...Running tap water

SEOC…….……..Surface Epithelial Ovarian Carcinoma

SPSS…..Statistical Package for Social Sciences

STABC …….……....streptomyces Avidin biotin Complex

TRB…….……..……...Target Retrieval Buffer

TMA…….……...Tissue Microarray

Abstract

Background; Ovarian cancers are classified into three very broad categories, epithelial ovarian tumors, germ cell ovarian tumors, and sex cord stromal ovarian tumors. Most commonly, tumors arise from the epithelium, or lining cells, of the ovary. Estrogen receptor is one such recommended prognostic marker and is a nearly new method for investigating the proliferative index (PI) of a tumor lesion. Over expression of estrogen receptor antigens is associated with subtype of Estrogen receptor, tumor type, tumor aggression, vascular invasion, tumor spread, reserved prognosis and poor response to chemotherapy.

Materials and methods; This was a descriptive cross sectional study conducted in Khartoum state-Sudan during period from October 2017 to June 2018. Forty paraffin-embedded tissue blocks with ovarian tumors were subjected to detect estrogen receptor antigen using Immunohistochemistry technique.

Result; ER antigen was detected in 24 cases (60%) with no statistically significant different, the p value was 0.643.

There was no relation between tumor grade and estrogen receptor expression as the p. value was 0.915. Also there was no relation between age and estrogen receptor expression as the p.value was 0.890.

Conclusions; There was no statistically significant correlation between tumors type and estrogen receptor expression. There was no correlation between tumor grades, age of populations and expression of estrogen receptor.

المستخلص

الخلفية؛ تصنف أورام المبايض إلى ثلاث فئات واسعة، و هي أورام المبيض الطلائية، وأورام المبيض الخلوية الجرثومية، أورام المبيض اللحمية الحبلية الجنسية. تنشأ هذه الأورام في الغالب الأعم من الطلاء أو الخلايا المبطنة للمبيض. يعتبر مستقبل الأستروجين أحد موسمات الأورام النزيرية التي يوصي بها وهو حديثا طريقة حديثه لتشخيص مؤشر التكاثرللأورام. يرتبط الأفراز العالي لأنتجين مستقبل الأستروجين مع أنواع مستقبل الأستروجين, نوع الورم, عدوان الورم, أختراق الأورام للأوعية الدموية, انتشار الورم, التكهن المحجوز، والأستجابة السيئة للعلاج الكيميائي .

الطرائق والمواد؛ أجريت هذة الدراسة الوصفية المقطعية في ولاية الخرطوم-السودان في الفترة ما بين أكتوبر 2017 إلى يونيو 2018. أشتملت هذة الدراسة علي 40 قطعة نسيجية معمولة بطريقة شمع البرافين مصابة بأورام المبيض لتحديد أنتجين مستقبل الأستروجين بإستخدام تقنية الانسجة المناعية.

النتائج؛ تم تحديد أنتجين مستقبل الأستروجين في 24 حالة (60%) مع عدم وجود إختلاف ذو دلالة إحصائية, كانت القيمة الإحتمالية 0,890

الخلاصة؛ لم تكن هنالك علاقة مهمة إحصائية ما بين نوع الأورام وإفراز مستقبل الأستروجين. ليس هنالك علاقة بين مستوى الورم ، عمر المستهدفين في الدراسة و إفراز مستقبل الأستروجين.

CHAPTER ONE
INTRODUCTION

1.1. Introduction

Ovarian cancer causes more than 140,000 deaths worldwide every year and is the most lethal gynecological malignancy in developing countries. (Jemal *et al*., 2011)

Ovarian tumors are classified into three very broad categories, epithelial Ovarian tumors, germ cell ovarian tumors, and sex cord stromal ovarian tumors. (General *et al*., 2013) The ovaries are made up of 3 main kinds of cells. Each type of cell can develop into a

Different type of tumors: Epithelial tumors start from the cells that cover the outer surface of the ovary. Most ovarian tumors are epithelial cell tumors. Germ cell tumors start from the cells that produce the eggs (ova) Stormy tumors start from structural tissue cells that hold the ovary together and produce the female hormones estrogen and progesterone. Most of these tumors are benign (non-cancerous) and never spread beyond the ovary. Benign tumors can be treated by removing either the ovary or the part of the ovary that contains the tumor. Malignant (cancerous) or low malignant potential ovarian tumors can spread (metastasize) to other parts of the body and can be fatal.

The symptoms of ovarian cancer are often vague or attributed to or blamed on other more common conditions.

Ovarian cancer ranks ninth in the most common cancers among women and fifth in cancer deaths among women. It is the cause of more deaths than any other cancer of the female reproductive system. Cancer starts when cells in the body begin to grow out of control. Cells in nearly any part of the body can become cancer, and can spread to other areas of the body. Ovarian cancer is frequently hard to diagnose in its early stages.

No publish data that correlated estrogen receptor with ovarian tumor in Sudan this was the first study in Sudan conducted in correlation between

Estrogen Receptor and ovarian tumor prognosis marker. Estrogen Receptor (ER) status are used as an important prognostic marker for ovarian tumor. ER is known to induce responses in the reproductive tract, mammary tissue and pituitary but also affects non-reproductive processes such as bone formation and cardiovascular health.ER is a soluble term labile protein easily destroyed by proteases, (Jensen *et al*., 1972) and degraded at a rapid turnover rate in both uterus and human breast cancer cells. (Nardulli *et al*., 1986) Immunohistochemical analysis of the expression of ER α and ER β protein in a normal ovary indicate that, although both ERs are present.

1.2. Rationale

Ovarian cancer is common in Sudan. Progression, diagnostic and therapeutic bio markers may play an important role in the detection and treatment of female ovary cancer. Usually women present late in disease due to lack of efficient education, efficient health care system and low socio economic status. No publish data that correlated estrogen receptor with ovarian tumor in Sudan this was the first study in Sudan conducted to correlate between Estrogen Receptor and ovarian cancer, and to evaluate the Estrogen Receptor (ER) status as prognostic and differential marker for ovarian tumors.

1.3. Objectives

1.3.1. General Objective

1. To detect immunohistochemical expression of ER among Sudanese females with ovary cancer.

1.3.2. Specific objectives

1. To correlate ER immunohistochemical expression with histological subtypes.
2. To correlate between score of ER immune stain and tumor grade.
3. To correlate ER immune expression with patients age.

2.1. Ovarian cancer

The exact cause of ovarian cancer is unknown. According to the American Cancer Society in the year 2013 in the United States approximately 22 240 women will be diagnosed with ovarian cancer and about 14 230 women will die from ovarian cancer. Ovarian cancer ranks ninth in the most common cancers among women and fifth in cancer deaths among women. It is the cause of more deaths than any other cancer of the female reproductive system. Many risk factors have been attributed to ovarian cancer and include the following: Age of the women; older women are at the highest risk of developing ovarian cancer. reproductive history; women who have fewer children and the later in life she gives birth are at a higher risk of developing ovarian cancer, estrogen only replacement taken for more than five years will increase the risk but birth control pills will decrease the risk, fertility drugs may or may not increase the risk of developing ovarian cancer, family history of ovarian cancer; breast cancer or colorectal cancer can also increase the risk of developing ovarian cancer, personal history of breast cancer will also increase the risk especially if there is mutation in the BRCA1 or BRCA2 genes. (Pieta *et al*., 2012)

2.2. Treatment

After diagnosis of ovarian cancer several treatment options are available. The main treatments for ovarian cancer are surgery, chemotherapy, hormone therapy, targeted therapy and radiation therapy. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013) Quite often 2 or more different types of treatments are used. (National Library of Medicine website, 2013). Surgery is used to treat all stages and types of ovarian cancer. Surgery may be the only treatment needed for some early stage ovarian cancers. Surgery may include any one or all of the following: Total hysterectomy, removal of the uterus.

Bilateral salpingo-oophorectomy, removal of both ovaries and fallopian tubes, however if the cancer is only in one ovary and the patient wishes to

try and get pregnant the unaffected ovary and fallopian tube will not be removed Complete or partial removal of the momentum, the fatty layer that covers and pads organs in the abdomen Lymph nodes and other tissues in the pelvis and abdomen will be examined, biopsied and/or removed Debunking, removing as much of the tumor and/or tumors as possible. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013) Chemotherapy is the use of drugs to treat any cancer that may remain after surgery or if cancer comes back. Chemotherapy for ovarian cancer is typically a combination of 2 or more drugs given intravenously (IV), into the veins, or intraperitoneal (IP), directly into the abdominal cavity. The drugs used will depend on the stage and classification of the ovarian cancer. Epithelial and germ cell tumors are treated with chemotherapy while stoma tumors are more often treated with hormone therapy. Hormone therapy is the use of hormone-blocking drugs or hormones to fight cancer. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013)

2.3. Prognosis and Follow-up care

Survival rates of patients with ovarian cancer are determined by looking at the 5-year survival rate of other patients diagnosed with ovarian cancer. Combined together the 5-year survival rate for all ovarian cancers is 44%. Patients diagnosed younger than 65 do better and have better survival rates than women diagnosed after 65. For all stage I ovarian cancers diagnosed and treated the patients survival rate is 92%, however, only about 15% of all ovarian cancers are found at this early stage. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013) Each type of ovarian cancer will have its own very different 5-year survival rating but all show that early diagnosis dramatically increases a patient's chance of surviving ovarian cancer.

Careful follow-up care is highly recommended for patients whose ovarian cancer has gone into remission. A physical exam every 2-4 months for the first 2 years followed by every 6 months for 3 years and then every year is recommended. (Ovarian cancer. American Cancer Society, 2013) Blood tests to monitor CA-125 levels will also be ordered if the patient's levels started out high when diagnosed with ovarian cancer. CT scans of the chest; abdomen and pelvis may also be ordered periodically. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013) Any changes or concerns a patient might have should be discussed with their doctor. Side effects of treatments may last several weeks to months while others may not go away at all. Lifestyle changes may also be recommended, such as eating better and getting plenty of rest and exercise. Surgery is used to treat all stages and types of ovarian cancer. Surgery may be the only treatment needed for some early stage ovarian cancers. Surgery may include any one or all of the following: Total hysterectomy, removal of the uterus. Bilateralsalpingo-oophorectomy, removal of both ovaries and fallopian tubes, however if the cancer is only in one ovary and the patient wishestotry and get pregnant the un affected ovary and fallopian tube will not be removed Complete or partial removal of the momentum, the fatty layer that covers and pads organs in the abdomen Lymph nodes and other tissues in the pelvis and abdomen will be examined, biopsied and/or removed Debunking, removing gas much of the tumor and/or tumors as possible. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013) Chemotherapy is the use of drugs to treat any cancer that may remain after surgery or if cancer comes back. Chemotherapy for ovarian cancer is typically a combination of 2 or more drugs given intravenously (IV), in to the veins, or intra peritoneal (IP), directly in to the abdominal cavity. The drugs used will depend on the stage and classification of the ovarian cancer. Epithelial and germ cell tumor sere

treated with chemotherapy while stomal tumors are more often treated with hormone therapy. Hormone therapy is the use of hormone-blocking drugs or hormone stofight cancer. (National Library of Medicine website, 2013; ovarian cancer. American Cancer Society, 2013)

2.4. Promoter ER

Estrogen is important hormones secreted by the ovary acting through specific receptors. Tumor tissue expression profiles of these have demonstrated prognostic value in malignancies such as breast, uterine and prostate cancer. Estrogen and progesterone are important hormones secreted by the ovary acting through specific receptors. A putative direct action of gonad steroids on ovarian carcinogenesis has been suggested, supported by findings of mRNA transcripts and translated proteins of estrogen receptors (ER) and progesterone receptors (PR) in both normal ovarian tissue and malignant ovarian tumors.(Lindgren *et al.*, 2004; Lee *et al.*, 2005)The action of ER is believed to be mediated by the two ER receptors, ER-α and ER-ß, which through differential regulation of gene transcription may exert opposite actions on OC growth and survival. (Syed *et al.*, 2005; Lazennec, 2006) It is thus conceivable that the expression profiles of ER are related to ovarian tumor behavior or prognosis,(Munstedt *et al*., 2000; Lee *et al*., 2005) as it has been shown for other tumors such as breast, endometrial and prostate cancer. (Muntedt *et al.*, 2000; Lindgren *et al*., 2004; Syed *et al*., 2005)

Molecular weight of ER is 66 KDa. (Green *et al.*, 1986) ERs are members of a family of nuclear transcription factors including receptors for sex steroids, thyroid hormone, and retinoid as well as many receptors, for which on ligands have been identified. (Mangelsdorf *et al.*, 1995) A second ER is known to induce responses in the reproductive tract, mammary tissue and pituitary but also affects non-reproductive processes such as bone formation and cardiovascular health.ER is a soluble term labile protein

easily destroyed by proteases, (Jensen et al., 1972) and degraded at a rapid turnover gene was cloned from prostate tissue in 1996, (Koper *et al.*, 1996) and thus, there are two ER molecules; the original ER "α" and the recently discovered ER"β". Immunohistochemical analysis of the expression of ER α and ER β protein in a normal ovary indicate that, although both ERs are present, their distribution differs; with ER β predominantly in the granulose cells of the follicles and ER α localized in the cognitional and interstitial regions of the ovary. The knownestrogen receptor variants are coded from two separate genes, ER_ and ER_, located on chromosomes 6q25.1 and 14q22-24, respectively. (Greene *et al*., 1986; Enmark *et al.,* 1997) The trans-activating mode of action of ER_ is similar to ER_, (Pettersson *et al.*, 1997) and anti-estrogens can inhibit this effect. (Mosselman *et al.*, 1996; Tremblay *et al*., 1997) Moreover, ER_ and ER_ are usually, but not always, co-expressed in many human tissues. (Enmark *et al*., 1997; Taylor and Al-Azzawi, 2000)

2.5. ER and ovarian cancer

Tumor cells do interact with the environment around them. (Giles *et al.* 2010) In recent years, with the development of the molecular biology and immunologic methods, molecules such as the human epidermal growth factor receptor type 2 (HER2), and the steroid receptors, estrogen (ER),progesterone (PR) and androgen (AR), have been tested as potential biomarkers of individualized clinical behavior of cancer. The effect of steroid hormones in carcinogenesis has been studied specially for breast and endometrial cancer with well-known and promising results for therapy. However for ovarian cancer these results have been conflicting and unclear. (Kommoss *et al*., 1991; Liun *et al.,* 2011)

While the expression of ER has been shown to predict better prognosis with apoptotic effect, (Lee *et al*., 2005) a positive ER status has shown discrepant result, (Halon *et al*., 2011; De stafano *et al.,* 2011) depending

on the subtype of ER and tumor type. (Burges *et al.*, 2010) In recurrent low-grade carcinoma of the ovary or peritoneum, therapies had a greater anti-tumor activity in ER+/PR+ patients than in ER+/PR- patients.

Although limited information is available regarding the role of ER_ in ovarian epithelial cancer. (Brandenberger *et al.*, 1998; Pujol *et al.*, 1998) Previous studies have shown that; ER_ and PR are commonly found in more than 50% of carcinomas. (Rao and Slotman, 1991; Clinton and Hua, 1997; Miller and Langdon, 1997; Emons and Kavanagh, 1999) However, their significance in relation to the development of ovarian cancer tumors and patient prognosis is still unclear. With the incentive to search for differences between various ovarian tumor groups, we used immunohistochemical methods to characterize the pattern of expression for ER in different malignant tumors of ovary.

2.6. Epidemiology

The American Cancer Society estimates for ovarian cancer in the United States according to the National Cancer Institute (NCI), in 2015, concluded that; there were an estimated 21,290 new cases of ovarian cancer and 14,180 deaths from the disease. The vast majorities of the cases are EOC and are found at stage 3 or later, meaning the cancer has spread beyond the pelvis or to the lymph nodes. This is mostly due to the lack of definite symptoms at the early stages of cancer growth. Around 1.3% of women will be diagnosed with cancer of the ovary at some point in life, thus it is relatively rare. The median age of diagnosis is 63. However, approximately 25% of cases are diagnosed between ages 35 and 54. Caucasian women have the highest rate of diagnosis. And in the United States 20,749 ovarian cases were diagnosed in 2007 (the most recent year for which data are available), for an incidence rate of 12.2 per 100,000 women, (Cancer Statistics Working Group, US, 2010) Koper *et al*., 1996) reported a rate of 14.9 in the Netherlands, similar to that found in the United States.) In 2017

there are about 22,440 women will receive a new diagnosis of ovarian cancer. (Kuiper *et al*., 1996)

About 14,080 women will die from ovarian cancer, ovarian cancer ranks fifth in cancer deaths among women, accounting for more deaths than any other cancer of the female reproductive system. A woman's risk of getting ovarian cancer during her lifetime is about 1 in 75. Her lifetime chance of dying from ovarian cancer is about 1 in 100. This cancer mainly develops in older women. About half of the women who are diagnosed with ovarian cancer are 63 years or older. It is more common in white women than African-American women. The rate at which women are diagnosed with ovarian cancer has been slowly falling over the past 20 years. (National Library of Medicine website, 2013)

The Chinese Shanghai Cancer Registry also reported an increase in ovarian cancer incidence from 1979-1989. Some of these increases may be due to increases in population coverage or completeness of data within the registry; however, the Chinese increase is thought to be a birth cohort effect in women born between1925-1935. (Jin *et al*., 1993) An Italian network of cancer registries reported 7,690 cases of ovarian cancer from 1986 through 1997. (Zambon *et al*., 2004) Few countries publish trends in ovarian cancer incidence over time. This may be due to differing methods of data collection and data quality issues, especially for countries that do not have a national registry. In the United States, a recent report estimates that ovarian cancer incidence has been decreasing since 1998, with a significant decline of 2.3% per year from 2003-2007. (Kohler *et al*., 2011) The reasons for this decrease are unclear, but are likely not art fact due to the long-standing high-quality data available for the United States. In The Egypt, (Dey *et al*., 2010) and Italy (Minelli *et al*., 2007), have found ovarian cancer rates to be higher in urban compared to rural areas. Globally, a lack of reliable screening modalities has restricted the opportunities for early

diagnosis and cancer detection, leading to a significant proportion of women worldwide presenting at an advanced stage of the disease. Due to this late presentation, available treatments are ineffective, and the majority of patients relapse following treatment-induced regression. (Holschneider *et al*., 2000) Furthermore, there is substantial geographic variation in the incidence of ovarian cancer and mortality, with higher incidence observed in developed countries (9.4 per 100,000 women) compared with women living in the developing world (5.0 per 100,000 women) (Jemal *et al*., 2011). Ovarian cancer accounts for 5% of all cancer deaths in Western countries and is the most frequent cause of gynecological cancer mortality. The incidence varies with age between 1% and 14%, with a peak rate in the eighth decade and, in the majority of cases, the disease has already spread beyond the pelvic cavity at the time of diagnosis.

2.7. Ovarian cancer in Sudan

the incidence rate of ovarian cancer in the entire Sudan has yet to be identified; however, in a hospital-based data set from the National Cancer Institute, Gezira University, Central Sudan and Radiation Isotopes Center in Khartoum, collected between 2000 and 2006, ovarian cancer accounted for 6.8%(949) of all recorded cancers (n=226,652), and it was ranked the sixth most common cancer for both genders. (Mohammed *et al*., 2014) Additionally, in a more recent data set (2009–2010) from the National Cancer Registry for Khartoum State alone, ovarian cancer was the fourth most common cancer in women, with an estimated incidence rate of 188 per 100,000 populations, a gender-specific rate of 8.0 per 100,000 populations, and an age-standardized rate (ASR) of 7.0 per 100,000 populations. (Saeed *et al*., 2014) Furthermore, neither the morality rate for ovarian cancer nor the survival rate in Sudan has previously been described due to a lack of the availability of death certificates, the majority of patients

presenting with advanced stage disease were not thoroughly investigated or treated symptomatically.

2.8. Immunohistochemistry method

Immunohistochemistry (IHC) 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 a secondary labeling method. The recent introduction of prognostic and predictive markers in IHC has made a tremendous impact on patient treatment and management. Immunohistochemistry is widely employed in establishing diagnosis, predicting prognosis and response to therapy and in the study of disease pathogenesis. (Bancroft and Gamble, 2008)

There are numerous immunohistochemical staining techniques that may be used to localize and demonstrate tissue antigens. The selection of a suitable technique should be based on parameters such as the type of specimen under investigation, the type of preparation under investigation e.g., frozen sections, paraffin sections, resin sections or cytological preparations and the degree of sensitivity required. A technique with high sensitivity is able to detect smaller amounts of antigen than technique with low sensitivity. If used to detect the same amount of antigen, the technique with high sensitivity would sensitivity. The specificity of the antibody for ovarian carcinoma must be high be-cause reaction with other tumors or with non tumorous tissue ccurate diagnosis the sensitivity of oc125 for malignant epithelial ovarian tumors.

3.1. Study design

This study was a descriptive cross sectional study.

3.2. Study duration

This study was conducted during period from October 2017 – June 2018.

3.3. Study area

This study was conducted at Elrahma Medical Center- Khartoum north - Sudan.

3.4. Study populations, samples and samples size

Populations involve in this study were formalin fixed paraffin embedded (FFPE) tissue with ovary cancer.

3.4.1. Including and excluding criteria

All samples were from Sudanese female, malignant lesions of the ovary were included in this study.

3.4.2. Study sample and samples size

Forty FFPE tissues were included in this study.

3.5. Sampling technique

Convenient sampling technique was used to include subjects in this study.

3.6. Data Collection tools and variables

Master sheets were used to record all patients, samples and results data, ER was detected using IHC method.

3.7. Quality controls

All quality issues and precautions were taken as manufacture instructions (Thermo Scientific- Italy).The sample was recorded positive when at least 5 cells showed brown color at nucleus and/or cytoplasm.

3.8. Sampling processing

All forty paraffin blocks were assembled in one tissue microarray (TMA) block, the procedure of TMA microarray technique as followed; conventional mechanical pencil tips of a hollow needle was used to remove regions of interest from donor FFPE tissues. These tissue cores were then inserted in one recipient paraffin block in a precisely spaced, array pattern. After that, microscopic glass slide was placed over this block and then introduced into dry oven at 37^0c, and then cooled at room temperature and refrigerator consecutively. After that 4 μm thick section was cut by using a microtome (MR22150-K2258-1124 Histoline- Italy). The section was

taken to the water bath (LAB TECK, 009222- India) at 40^0c after floatation in 70% alcohol, after that section was placed on a super frost positive charge glass slide (Thermo Scientific-Italy). Then slide contained section was incubated at room temperature (RT) over night, and then slide was transferred to dry oven at 50 °C for drying for 12-24 hours. After that, the slide was stained immunohistochemically.

3.9. Methods of detection

3.9.1. Hematoxylin and Eosin (H&E) staining method

H & E staining technique was carried out twice; firstly each section from the donor blocks was stained with H & E to confirm the diagnosis and to ensure that the selected section for TMA contained the target tumor area. Secondly H & E was taken out to ensure that the cut section from TMA was contained the target tumor cells for preceding IHC technique. H& E was done as followed; slide contained section was rinsed in 3changes of xylene, 2 minutes in each change, after that, slide was rehydrated as followed; placed in absolute alcohol (10 dips), Alcohol, 95%, (2 changes 10 dips in each), the slide then was placed in tap water (rinse until water runs off evenly), after that the slide was stained in Mayer's Hematoxylin for 15 minutes, then rinsed in tap water until the section blued, then counterstained in Eosin (10-20 dips), then dehydrated in ascending grades of alcohol as followed; 70% (10-15 dips), 95% (10-15 dips), absolute alcohol (3 changes 10-15 dips in each). After that the slide was cleared in xylene (3 changes 10-15 dips in each). Finally the slide was mounted in Disterene and a Plasticizer and Xylene (DPX).

3.9.2. Immunohistochemistry staining method (IHC)

The immunohistochemical procedure was done as followed; section (3µm) from TMA block was cut by a microtome and then mounted onto sialinized slide. The slide contained section was stained with IHC as followed; section was deparaffinized in xylene, slide was then rehydrated through a

graded series of alcohol and placed in distilled water. Samples were steamed for antigen retrieval using high pH (9.0) by water bath at 95^{o}C for 40 min., then the slide was washed with phosphate buffer saline (PBS) for 3 min., then the endogenous peroxidase activity in each section was blocked with 3% hydrogen peroxide in methanol for 10 min. After that, slide was washed in PBS for 3 min., then the slide was incubated with (100 µ L) of specific mouse monoclonal antibody for ER (Dako-USA), for 30 min at room temperature in a moisture chamber. After that, the slide was washed with PBS for 3 min., binding of antibodies were detected by incubating slide for 20 min with dextran labeled polymer (Dako-USA). Then the sections were washed in three changes of PBS, followed by adding 3, 3 diamino benzidine tetra hydrochloride (DAB) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for up to 5 min. After that, slide was washed with distilled water for 3 min., then the slide was counterstained with Mayer's Hematoxylin for one min., then washed in running tap water for several minutes 7-10 (for bluing), then the slide was dehydrated in ascending grades of alcohol, cleared in xylene and mounted in DPX. The immune stained slide was interpretated by the investigator, and then the stained results were confirmed by well trained PhD histopathologist.

3.10. Data analysis and presentation

All the collected data and results were analyzed using statistical package for Social Sciences (SPSS) version (22.0), Pearson's chi square test was used to detect significance of the results. All calculated data and results were presented in form of figures and tables, p value used to asses significance of the result was <0.05.

3.11. Ethical considerations

This study was approved from the board of Medical Laboratory Sciences (MLS) and the College Higher studies at Shendi University. A written agreement was a signed prior to sample collection with each hospital and laboratory administrations. Also permission for this study was obtained from the authorities in the area of the study. The aims and the benefits of this study were explained well with assurance on confidentiality.

4. Results

A total of 40 cases (patients with histopathologically confirmed ovarian cancer) were included in this study. The age of patients was ranged from 32-65 years with average mean of 49 years.

The ages were divided into two age groups the first category of less than 45 years represented 13 cases (32.5%), the second age group was older than 45 years represented 27 cases (67.5%) as indicated in figure (4.1).

Figure (4.2) summarized the frequency of cancer types among study samples, adenocarcinoma was the most frequent type found in 29/40 cases (72.5%), followed by granulosa cell tumor 8/40 (20.00%), followed by malignant stromal tissue, immature teratoma and transitional cancers respectively (1 sample for each type) (2.5% each type).

Figure (4.3) demonstrated the frequency of tumor grade; of the 40 cases, grade II observed in 21/40 (52.5%) followed by grade 1 and grade III constituting 14/40 (35%) 5/40 (12.5%) respectively.

Figure (4.4) demonstrated the frequency of ER expression; of the 40 cases 24 cases (60%) were positive and 16 cases (40%) were negative.

Figure (4.5) demonstrated the frequency of ER expression score; of 40 cases, the highest frequency was score I 16/40 (40%), score II 2/40 (5%), score III 6/40 (15%) followed by score (0.00) 16/40 (40%).

Table (4.1) illustrated the association of cancer types with ER expression. Of the 40 tested samples for ER, adenocarinoma was most positive type expression 16/40 cases (40%), followed by Granulosa cell tumor was positive in 5/40 cases (12.5%), followed by immature teratoma 1/40 (2,5%), transitional cell cancinoma 1/40 (2.5%) and malignant stromal tissue 1/40 (2.5%), the p value was 0.680.

Table (4. 2) illustrated the association of tumor grade and ER expression. Grade II was showed the most positive type of expression 12/40 (30%) followed by grade I 9/40 (22.5%) and grade III 3/40 (7.5%),with no statistically significant different, p value was 0.915.

Table (4.3) showed the association between tumor grade and score of ER expression. Among 40 samples, score (0.00) was observed in 16 samples (40%) as followed; 5 samples out of 14 (5/14=35.7%) samples with grade 1 [5/40=12.5%], 9 samples out of 21 (9/21=42.8%) samples with grade 2 [9/40=22.5%], 2 samples out 5 (2/5=40%) samples with grade 3 [2/40=5%]. Score 1 was observed in 16 samples out of 40 (40%) as followed; 6 samples out of 14 (6/14=42.8%) samples with grade 1 [6/40=15%], 8 samples out of 21(8/21=38.1%) samples with grade 2 [8/40=20%], 2 samples out 5 (2/5=40%) samples with grade 3 [2/40=5%)]. Score 2 was observed in 2 samples out of 40 samples (5%) as followed; 0.00 sample out of 14 (0.00/14=0.00%) samples with grade 1 [0.00/40=0.00%)], 1 sample out of 21 (1/21=4.7%) samples with grade 2 [1/40=2.5%)], 1sample out 5 samples (1/5=20%) with grade 3 [1/40=2.5%]. Score 3 was observed in 6 samples out of 40 samples (15%) as followed; 3 samples out of 14 (3/14=21.4%) samples with grade 1 [3/40=7.5%], 3samples out of 21 (3/21=14.2%) samples with grade 2 [3/40=7.5%], 0.00 sample out 5 (0.00/5=0.00%) samples with grade 3 [0.00/40=0.00%]. With no statistically significant different,P value was 0.643.

Table (4.4) illustrated the association between age of patients and ER expression. 8/40 (20%) cases were showed positive expression in the age group up to 45 years and 16/40 (40%) cases were showed positive expression in the age group above than 45 years. With no statistically significant different, p value was 0.890.

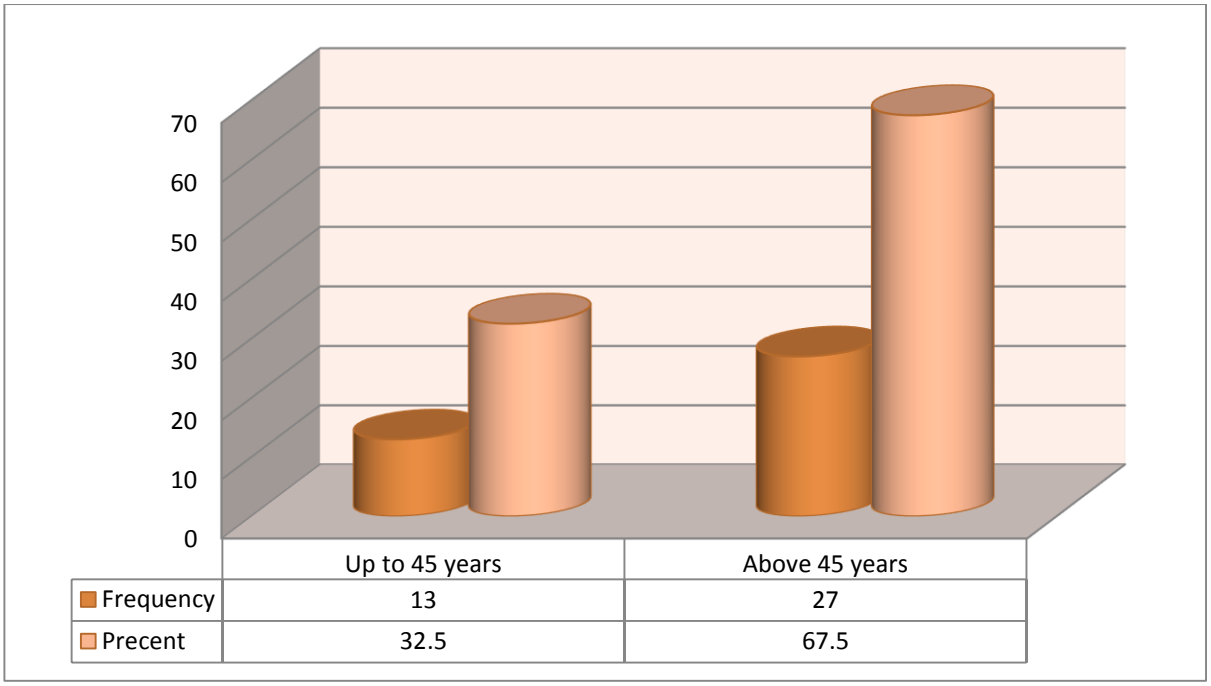
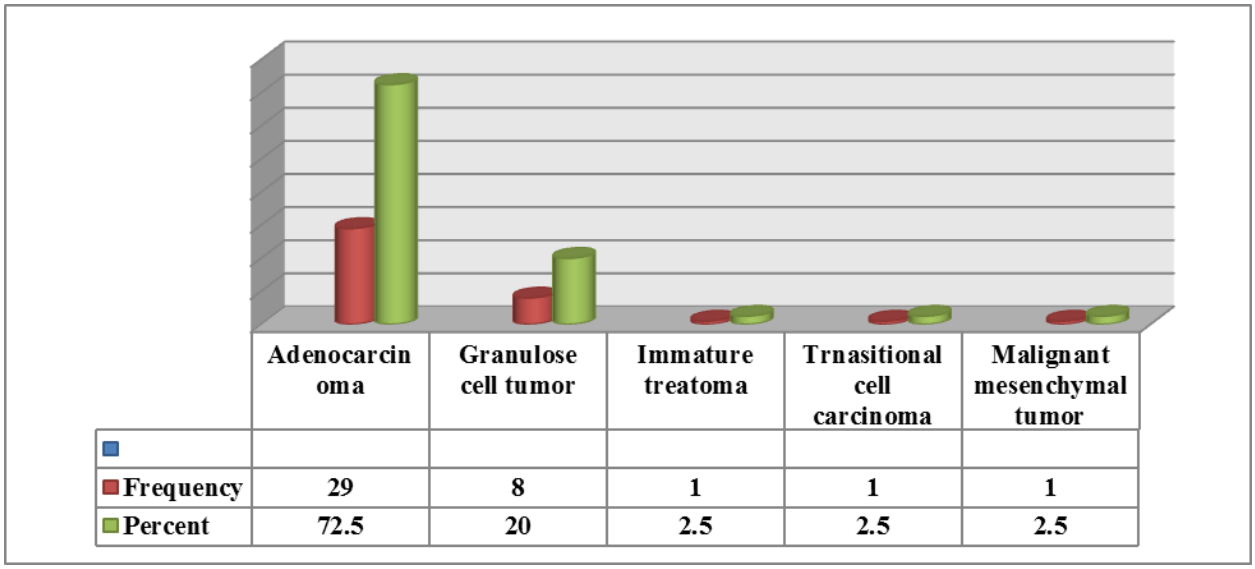


Fig 1: Shows frequency of age group among study populations.

- **N=40.**



	Adenocarcinoma	Granulose cell tumor	Immature treatoma	Trnasitional cell carcinoma	Malignant mesenchymal tumor
Frequency	29	8	1	1	1
Percent	72.5	20	2.5	2.5	2.5

Fig 2: Shows frequency of cancer types.

- **N=40.**

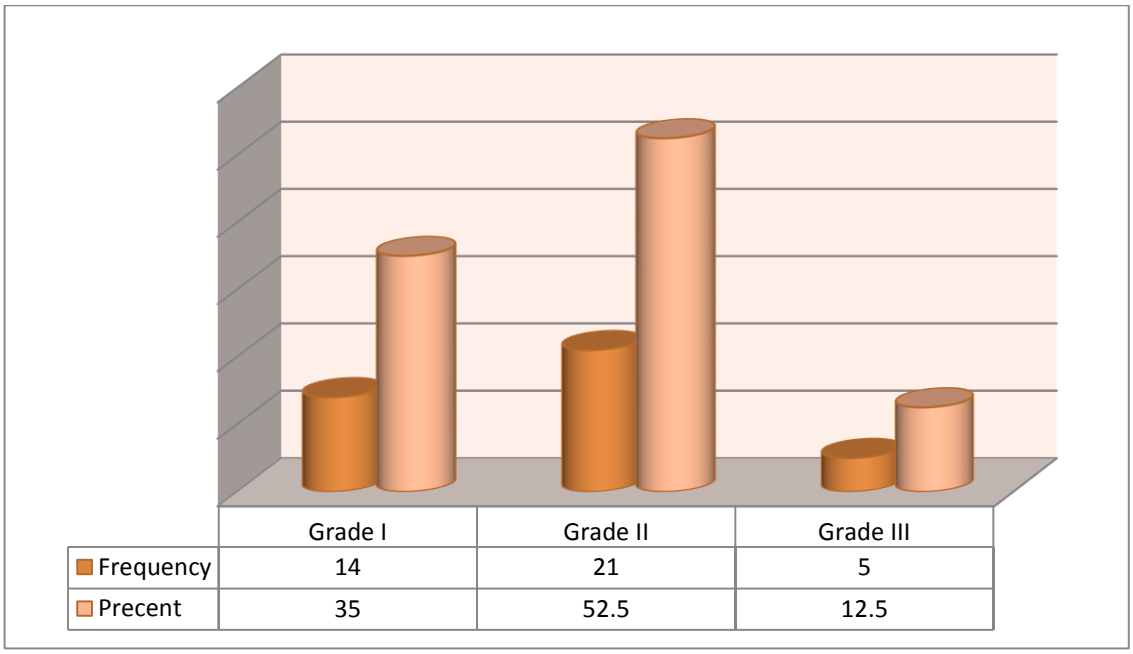
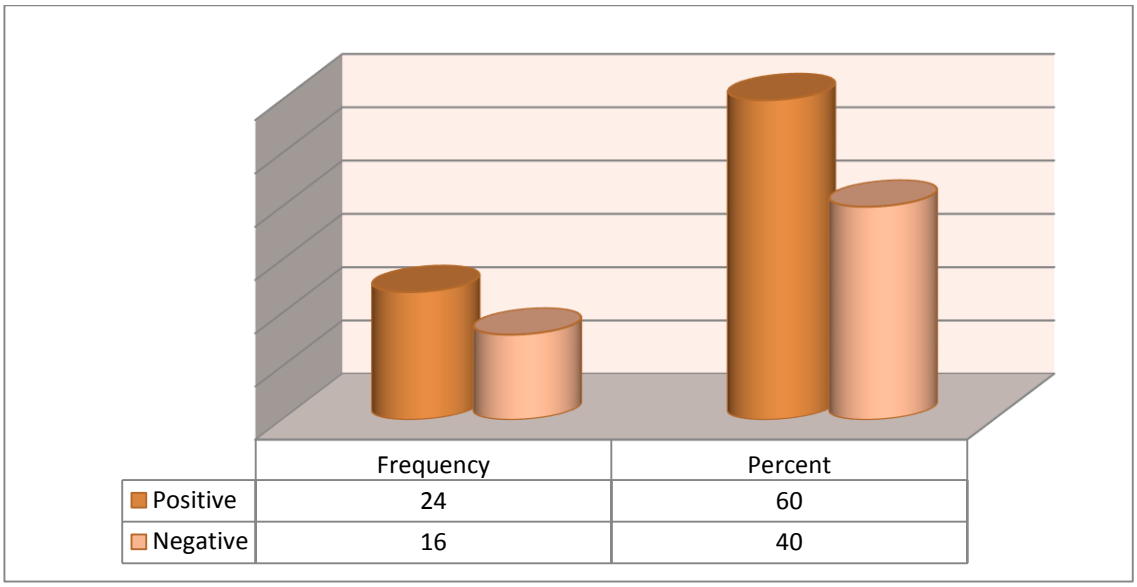


Fig 3: Shows frequency of tumor grades.

- **N=40.**



	Frequency	Percent
Positive	24	60
Negative	16	40

Fig 4: Shows expression of estrogen receptor.

- **N=40.**

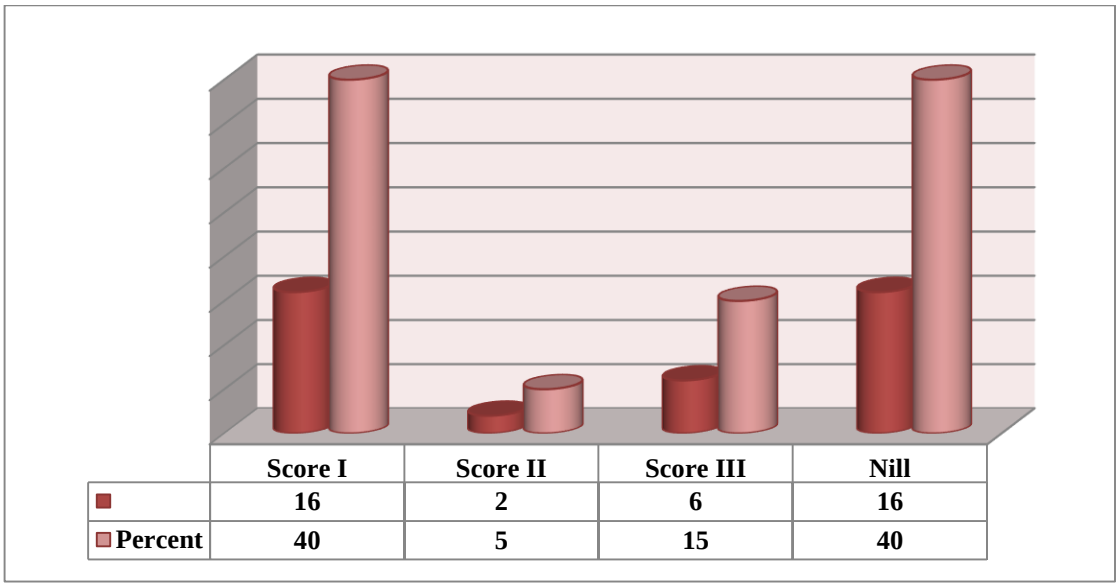


Fig 5: Shows frequency of estrogen receptor expression score.

- **N=40.**

Table 1: Correlation of ER expression with cancer types.

Tumor types	Estrogen Receptor expression		Total	P. value
	Positive	Negative		
Adenocarcinoma	16	13	29	0.68
Granulose cell tumor	5	3	8	
Immature teratoma	1	0	1	
Transitional cell carcinoma	1	0	1	
Malignant stromal tissue	1	0	1	
Total	24	16	40	

Table 2: Correlation of ER expression with tumor grade.

Tumor grade	Expression estrogen receptor		Total	p. value
	Positive	Negative		
Grade I	9	5	14	0.915
Grade II	12	9	21	
Grade III	3	2	5	
Total	24	16	40	

Table 3: Correlation of ER expression score with tumor grade.

Score of ER expression	Tumor grade			Total	p. value
	Grade I	Grade II	Grade III		
Score I	6	8	2	16	0.643
Score II	0	1	1	2	
Score III	3	3	0	6	
Score 0	5	9	2	16	
Total	14	21	5	40	

Table 4: Correlation of ER expression with patient's age.

Age	ER expression		Total	p. value
	Positive	Negative		
Up to 45 years	8	5	13	0.890
Above 45 years	16	11	27	
Total	24	16	40	

5.1. Discussion

Ovarian cancer ranks ninth most common cancers and fifth in cancer deaths among women. It is the cause of more deaths than any other cancer of the female reproductive system. Cancer starts when cells in the body begin to grow out of control. Cells in nearly any part of the body can become cancer, and can spread to other areas of the body. Estrogen Receptor (ER) status are used as an important prognostic marker for ovarian cancer (Jensen *et al*., 1972), and degraded at a rapid turnover rate in both uterus and human breast cancer cells (Nardulli *et al*., 1986). In a more recent data set (2009–2010) from the National Cancer Registry for Khartoum State alone, ovarian cancer was the fourth most common cancer in women (Saeed *et al*., 2010).

In Sudan there is no publish data that correlated estrogen receptor with ovarian tumor and this is the first study in Sudan conducted to correlate between ER and ovarian cancer.

In this study we found that; the most patients are elderly women (67.5%), this has been supported by another study conducted in 2015 in the United States according to the National Cancer Institute (NCI) concluded that; the median age among ovarian cancer patients was 63 (NCI, 2015). In 1993 (Jin *et al*, 1993) was found about half of the women who are diagnosed with ovarian cancer are of 63 years or older.

Regarding frequency of cancer types, we summarized that, adenocarcinoma was the most common samples present in one to quarter of study samples followed by granulosa cell tumors present in quarter of study samples; the rare types included immature teratoma, malignant stromal tissue and transitional cell cancer.

Regarding immunoexpression of ER we found two thirds of the study samples with positive expressions, this finding near to previous studies showed that; ER are commonly found in more than 50% of carcinomas

(Rao and Slotman, 1991; Clinton and Hua , 1997; Miller and Langdon, 1997; Emons and Kavanagh, 1999).

Concerning correlation of ER expression with cancer subtypes our results showed no significant association was found between ER expression and cancer subtypes, this result is consistent with other study in 2017 by (Zhaojun *et al*., 2017).

In our study there is no significant association was found between tumor grade and ER expression, which agree with other study in 2017 by (Mustapha *et al*., 2017) they concluded that; no significant association was found between cancer grade and ER expression.

5.2. Conclusion

On the basis of this study we conclude that;

- Ovarian cancers are common in the elder women.
- The most common ovarian type is adenocarcinoma.
- The most common grade among ovarian cancer is grade two.
- ER is present in two thirds of samples.
- There is no significant relation between cancer types and ER expression.
- There is no significant relation between tumor grade and ER expression and it' s score.
- There is no significant relation between age of populations and expression of ER.

5.3. Recommendation

On the basis of this study we recommended;

- Further studies are needed with large sample size to confirm the exact role of ER expression in ovarian cancers.
- Future studies should compare benign, borderline and malignant ovarian tissues with ER expression.
- Future studies should perform using advanced techniques such as PCR and ISH to confirm efficiency of IHC in detection ER expression.

References

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Appendix-1

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

- Disposable gloves
- Microtome knife
- Positively charged slide
- Cover glass
- Dry oven
- Water bath
- Embedding center
- Coplin jars (plastic)
- Humidity chamber
- Ethanol (100%, 90%, 70%)
- Reaction buffer
- Primary antibody (ER)
- Tris EDTA buffer (pH 9)
- Phosphate buffer saline (pH 7.4)
- Peroxides blocker (3% hydrogen peroxide in methanol)
- Secondary anti body (dextran polymer conjugated secondary – HRP)
- DAB (3,3 di amino benzidin tetra hydrochloride) substrate solution
- Bluing Reagent (Running Tap Water)
- Xylene

Appendix-2

Preparation of Mayer's Hematoxylin:

Hematoxylin……1 g

D.W…….1000 ml

Sodium iodate…….0.2 g

Potassium alum……..50 g

Citric acid……1 g

Chloral hydrate…….50 g

Preparation of eosin Y

Eosin Y……...1 g

D.W……..……100 ml

Glacial acetic acid……...0.05 ml

Crystal thymol…….small amount

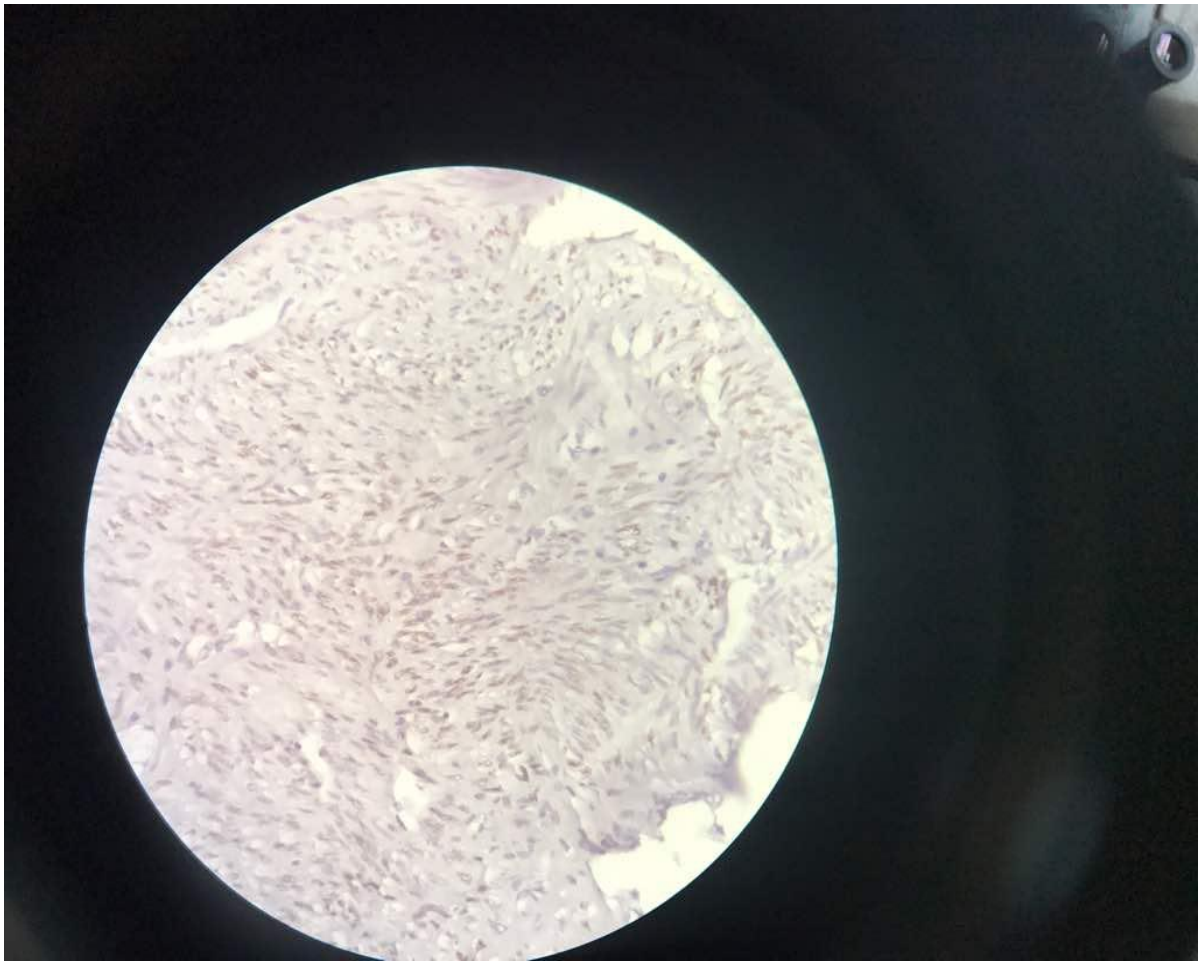
Shendi University

Faculty of Graduate Studies and Scientific Research

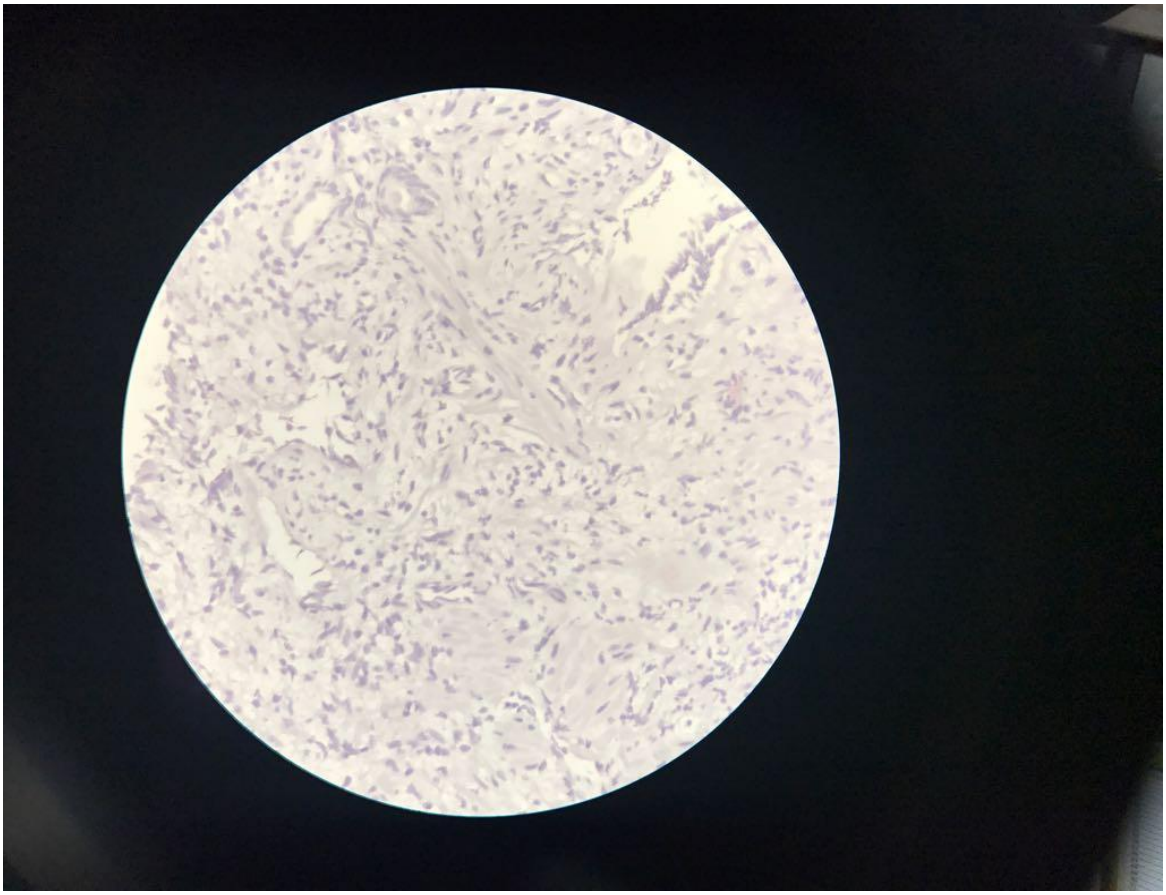
Immunohistochemical Detection of Estrogen Receptor among Sudanese Females with Ovarian cancer

Questionnaire sheet

NO.	Age	Cancer type	Grade	ER result	Expression score



Picture 1: Shows positive estrogen receptor immunohistochemical expression.



Picture 2: Shows negative estrogen receptor immunohistochemical expression.