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Immunohistochemical Expression of Ki-67 among Sudanese Females with Ovarian Tumors in Khartoum State
A thesis submitted for partial fulfillment of the requirements of M.Sc. degree in Medical Laboratory Sciences (Histopathology and Cytology)

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الآية

بسم الله الرحمن الرحيم
قال تعالى:
{و قُل رَبَّ زَدْنِي عَلْماً}
صدق الله العظيم
(طه - 114)
Dedication

To my mother and my father

To my family… always…

To my daughter

To all my friends,

Sahar
Acknowledgment

All the thanks to Allah for the gift of life, health and faith and for the strength and desire that he gave me to continue this way, and look always for the best.
I am grateful to my supervisor Dr. Mohammed Abdelgader Elsheikh Mohammed for his help, patience, care, invaluable support and for providing me with the materials that I need to carry out this study.
Special thanks to Dr. Ibrahim Bakheet and Mr. Mahmoud Ibrahim Osman for their help, care and support. A lot of thanks extended to Elrahma Medical Center staff.
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List of abbreviations

DAB..........................................................diaminobenzidine tetra hydrochlorid
DPX..........................................................Distyrene plasticizer xylene
EOC..........................................................Epithelial Ovarian Cancer
FFPE..........................................................Formalin Fixed Paraffin Embedded
FIGO..........................................................International Federation of Gynecology and Obstetrics
HGSOC.......................................................High-grade serous ovarian carcinoma
IHC............................................................Immunohistochemistry
ISH..............................................................In situ hybridization
MLS............................................................Medical Laboratory Sciences
MIB-1.........................................................Mind bomb E3 ubiquitin protein ligase 1
NACT..........................................................Neo Adjuvant Chemotherapy
NCI............................................................National Cancer Institute
PBS.............................................................phosphate buffer saline
PCR............................................................Polymerase chine reaction
PI...............................................................Proliferation index
SEOC..........................................................Surface Epithelial Ovarian Carcinoma
SPSS..........................................................Statistical Package for the Social Sciences
TMA.............................................................tissue microarray
Abstract

Background; Ovarian tumors include several different types of tumors that all arise from cells of the ovary. Most commonly, tumors arise from the epithelium, or lining cells, of the ovary. Ki-67 tumor maker immunostaining to detect proliferation of tumor cells 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 Ki-67 antigens is associated with 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 the period from September 2017 to May 2018. Forty paraffin-embedded tissue blocks with ovarian tumors were included in this study; samples were subjected to detect Ki67 antigen using immunohistochemistry technique.

Results; Ki67 antigen was detected in 30 cases (75%) with no statistically significant different, the p value was 0.664. There was no statistical correlation of tumor grade with marker expression, as the p.value was 0.579. Also there was no statistical correlation between age and marker expression, as the p.value was0.330. Conclusions; There was no relationship between tumors type and ki67 expression. There was no relationship between grade of tumors and ki67 expression.
النتائج

ظهر التحذير (كي أي 67) في ثلاثة عينات والتي تمثل نسبة 75% مع عدم وجود دلالة ذات قيمة إحصائية وكانت القيمة الإحتمالية 0.664. أيضاً أضحى أنه ليست هناك علاقة بين تدرج العمر وظهور التحذير (كي أي 67) وكانت القيمة الإحتمالية 0.579. أيضاً أضحى أنه ليست هناك علاقة بين العمر وظهور التحذير (كي أي 67) وكانت القيمة الإحتمالية 0.330.

الاستنتاج: لا توجد علاقة بين ظهور التحذير (كي أي 67) ودرج العمر أو العمر.

الطرق والمواد: أجريت هذه الدراسة المتعلقة بالمشاهدة والتي شملت مجموعة الحالة بولاية الخرطوم في الفترة من سبتمبر 2017 إلى مايو 2018. أشتملت الدراسة على 40 عينة نسيجية من المبيض في شمع البرافين جميع العينات شنست على أنها أورام مبسطة. أخبرت هذه العينات للكشف عن التحذير (كي أي 67) عن طريق تقنية الصبغ المناعي.

المستخلص

الخلفية: تشمل أورام المبيض على عدة أنواع مختلفة من الأورام تنشأ من خلايا المبيض. وتغطي الأورام التي تنشأ من خلايا المبطنة للمبيض هي الأكثر شيوعا. الكشف عن الأنتيجن (كي أي 67) عن طريق تقنية الصبغ المناعي إحدى الوسائل الجديدة للكشف عن تكاثر الخلايا وهو إحدى العلامات التنظيمية الموصى بها للكشف عن عامل تكاثر الخلايا. ظهور الأنتيجن (كي أي 67) بكثرة يترافق مع انتشار الورم وأنتقاله عن طريق الأوعية الدموية أيضاً له دلالة على استجابة الورم للعلاج.
CHAPTER ONE
INTRODUCTION
1.1. Background

Ovarian tumors include several different types of tumors that all arise from cells of the ovary. Most commonly, tumors arise from the epithelium, or lining cells, of the ovary. These include epithelial ovarian, fallopian tube, and primary peritoneal cancer. These are all considered to be one disease process. There is also an entity called ovarian low malignant potential tumor; these tumors have some of the microscopic features of a cancer, but tend not to spread like typical cancers. (Green et al., 2013) There are also less common forms of ovarian tumor that come from within the ovary itself, including germ cell tumors and sex cord-stromal tumors. (Green et al., 2013) High-grade serous ovarian carcinoma (HGSOC) accounts for 67% of all ovarian tumors and is the most aggressive subtype. These cancers arise due to DNA changes in cells that lead to the development of cancer. (Chen, 2014) Ovarian cancer is the fifth most frequent female cancer type. (Jemal et al., 2004) Ovarian tumors account 30% of all female genital tract cancers. More than 90% of the tumors cases of the ovary are SEOS and is the common cause of death of gynecologic neoplasms. (Prat, 2012; Kurman et al., 2014; Khandakar et al., 2014) Ovary tumors are ten times less common than breast cancer; it is associated with late stage where the tumors are metastasized beyond the pelvis in 70-75% of cases. The presence of recurrence after surgical removal and chemotherapy is high with high number of deaths in due course of cancer. (Stewart and Kleihues, 2003; Khandakar et al., 2014) Early detection plans and new therapeutic approaches to minimize death have been mainly ineffective due to absence of specific tumorigenesis and diagnostic aids for screening. (Kurman and Shih, 2010; Liu et al., 2012, Heeran et al., 2013, Ezzati et al., 2014) There are no proven methods of prevention. If diagnosed and treated while the
disease is confined to the ovary, the 5-year survival rate is 95%; unfortunately, 70% of cases are diagnosed at late stages and prognosis is dismal. (Niloff et al., 1984) Most patients initially respond to chemotherapy, the majority succumb to recurrent chemoresistant tumors. Efforts to overcome chemoresistance have been largely unsuccessful. The mortality rate remains high. (Jemal et al., 2004) Cell proliferation exhibits an important role in the clinical behavior and aggressiveness of tumor of the ovary. Detection of proliferation activity has been demonstrated to be of a diagnostic with prognostic value and many methods are used to investigate the number of proliferating cells. (Mita et al., 2004; Gursan et al., 2009; Heeran et al., 2013; Ezzati et al., 2014)

Ki-67 tumor maker immunostaining to detect proliferation of tumor cells is one such recommended prognostic marker and is a nearly new method for investigating the proliferative index (PI) of a tumor lesion by the Mind bomb E3 ubiquitin protein ligase 1 (MIB-1 antibody) immunohistochemistry (IHC). Ki-67 tumor marker over expressed in ovarian tumor compared to benign or borderline tumors of surface epithelial origin. Over expression of Ki-67 antigens is associated with tumor aggression, vascular invasion, tumor spread, reserved prognosis and poor response to chemotherapy. Its expression can be used to direct the clinical management of ovary cancer both as diagnostic and prognostic tool. (Scholzen et al., 2000; Mita et al., 2004; Kritpracha et al., 2005; Aune et al., 2011, Prat, 2012; Khandakar et al., 2014) This study aimed to correlate the Ki-67 antigen expression with histological subtypes, grades and investigating the clinical value of Ki-67 antigen as a proliferative marker for diagnostic purpose.
1.2. Rationale

Ovarian cancer is the fourth common cancer in Sudan. Usually women present late in disease due to lack of efficient education, efficient health care system and low socioeconomic status. No published data in Sudan that correlate Ki-67 proliferating gene with ovarian cancer development, progression and prognosis. Detection of KI 67 in ovarian cancer tissues may play important role in reliable diagnosis and further detection of tumor stage, progression, prognosis and thus may lead to sufficient treatment and follow up.
1.3. Objectives

1.3.1. General Objective
1- To detect Ki-67 in tissues taken from Sudanese females with ovarian tumors using immunohistochemical monoclonal MIB-1 antibody.

1.3.2. Specific Objectives
1- To correlate ki-67 immunoexpression with histological subtypes.

2- To correlate Ki-67 expression score with tumor grade.

3- To correlate Ki-67 immune stain with patient’s age.
CHAPTER TWO
LITERATURE REVIEW
2.1. Ovarian tumors

Epithelial ovarian tumor accounts for a majority of all ovarian tumors. It is generally thought of as one of three types of tumors that include ovarian, fallopian tube, and primary peritoneal tumor. All three tumors behave, and are treated the same way, depending on the type of cell that causes the tumor. The four most common cell types of epithelial ovarian tumors are serous, mucinous, clear cell, and endometrioid. These tumors arise due to DNA changes in cells that lead to the development of tumor. The serous cell type is the most common variety. It is now thought that many of these tumors actually come from the lining in the fallopian tube, and fewer of them from the cells on the surface of the ovary, or the peritoneum. However, it is often hard to identify the sources of these tumors when they are found at advanced stages, which is very common. (Chen, 2014) The type of cell where the cancer begins determines the type of ovarian tumors you have. Ovarian tumors types include; epithelial tumors, which begin in the thin layer of tissue that covers the outside of the ovaries. About 90 percent of ovarian tumors are epithelial tumors. The second type is stromal tumors, which begin in the ovarian tissue that contains hormone-producing cells. These tumors are usually diagnosed at an earlier stage than other ovarian tumors. About 7 percent of ovarian tumors are stromal. The last type is germ cell tumors, which begin in the egg-producing cells. These rare ovarian cancers tend to occur in younger women. (Desai et al., 2014)

Ovarian cancer may not cause any specific symptoms, particularly in its early stages. When it does cause symptoms, these may be nonspecific and vague. Symptoms can include; abdominal enlargement or swelling, abdominal fullness, early satiety (feeling full early), changes in bowel or bladder habits or clothes not fitting well. Other signs and symptoms can
include shortness of breath, leg swelling, and pain in the abdomen or pelvis. Fatigue may be present, but it is considered another nonspecific symptom. (Rochester, 2013)

2.2. Risk factors of ovarian tumors
Risk factors associate with the development of ovarian cancer are; age, obesity, reproductive history, birth control, gynecologic surgery, fertility drugs, androgens, estrogen therapy and hormone therapy, family history of ovarian cancer, breast cancer, or colorectal cancer, family cancer syndromes, diet analgesics, smoking and alcohol use. (Havrilesky et al., 2013) Although the etiology of ovarian tumors is not clear, certain factors are implicated in the etiology of this disease, such as ovulation, gonadotropic and steroid hormones, germ cell depletion, oncogenes and tumor suppressor genes, growth factors, cytokines, and environmental agents. Family history of breast or ovarian cancer is a prominent risk factor for ovarian cancer, with 5–10% of ovarian tumors due to heritable risk. (Fariba et al., 2008)

2.3. Ki-67 and ovarian cancers
Estimation of proliferative activity of the neoplasm has been reported to be a diagnostic and prognostic value with known clinicopathologic features in many tumors such as those of lymphatic system, lung, brain, breast, cervix, uterus, ovary, prostate, and in soft tissue sarcoma. There are many methods which can consider detecting the number of proliferating cells by proliferation index (PI). Recently Ki-67 tumor marker immunostaining is a promising objective for PI which required further methodological fine tuning. (Brown and Gatter, 2002; Mita et al., 2004; Gursan et al., 2009; Aune et al., 2011; Choudhury et al., 2011; Liu et al., 2012) Gerdeset al., demonstrated Ki-67 as nuclear non-histone protein. (Gerdes et al., 1991) The
Ki-67 immunoreactivity was of great value to identify as a tumor marker of cell proliferation because of its complete expression in proliferating tissues and the absence in quiescent cells. The Ki-67 gene is present on the long arm of human chromosome 10 (10q25). Ki-67 is also frequently measured both as a fixed tumor marker for proliferation activity and with many measurements during treatment, as a potential active intermediate or alternative tumor marker of treatment efficacy. (Gerdes et al., 1991; Scholzen and Gerdes, 2000) MIB-1 antibody has now been considered as a reference monoclonal mouse antibody for the detection of PI in formalin-fixed, paraffin embedded tissues for Ki-67 antigen. It reacts with the Ki-67 tumor marker nuclear protein which is present in two isoforms of 345 and 395 KDa. The Ki-67 antigen/ MIB-1 immunostaining are exist during all active stages of each cell's cycle (G1, S, G2 and M-phase) but absent in resting cells (G0 phase, quiescent state of the cell. (Scholzen and Gerdes, 2000; Mita et al., 2004; Kritpracha et al., 2005; Giurgea et al., 2012) Ki-67 is an easily available, inexpensive, rapid, applied on formalin-fixed paraffin embedded sections and a more reproducible biomarker available in developing countries, compared with other biomarkers such as proliferating cell nuclear antigen and bromodeoxy uridine. Ki-67 immunostaining needs only small tissue samples, allowing it to be used even in case of patients who are candidates for Neo Adjuvant Chemotherapy (NACT). (Scholzen and Gerdes, 2000; Brown and Gatter, 2002; Kritpracha et al., 2005; Giurgea et al., 2012) Ki-67 immunostaining is evaluated in most positively stained areas and all identifiable nuclear staining is interpreted as positive immunoreactivity regardless of intensity. Immunostaining is usually confined to the nucleus and cytoplasmic positivity is observed only during mitosis. Many studies have correlated this tumor marker in SEOC with other
prognostic markers such as histologic subtype, tumor grade, FIGO stage, chemotherapy response and also with survival rates. (Gerdes et al., 1991; Mita et al., 2004; Munstedt et al., 2004; Kobel et al., 2008; Liu et al., 2012; Marinas et al., 2012; Giurgea et al., 2012; Heeran et al., 2013; Khandakar et al., 2014) The SEOC expresses high Ki-67 L1 than benign and borderline tumors. (Min and Park, 2007; Khouja et al., 2007; Choudhury et al., 2011) Sylvia et al., studied 60 respective cases of epithelial ovarian neoplasms and found that; Ki-67 L1 was highest in cancer cases (Mean PI-48.6 ± 26.76) followed by borderline and lowest in benign. (Sylvia et al., 2012) In malignant group, serous had high index followed by endometroid and lowest in mucinous. They also documented that; CA-125 levels did not have a significant correlation with PI. Similar results were observed by Asha et al., they concluded that; the level of Ki-67 L1 was high in serous group and no correlation was found between CA-125 levels with L1. (Asha et al., 2017) Several studies have reported difference in Ki-67 expression in different histological subtype and have shown various distribution of immunostaining in serous with its subtype, mucinous, endometroid and clear cell. (Mita et al., 2004; Aune et al., 2011; Sylvia et al., 2012; Giurgea et al., 2012; Heeran et al., 2013)

In a retrospective study of 500 ovarian tumors by Kobel et al., Ki-67 immunohistochemical expression along with other biomarkers was assessed and it was concluded that; there is marked significance variation in PI between different subtypes but is not of prognostic significance within any subtype. (Kobel et al., 2008) Asha et al., study also concluded that; no significant association was found between the subtypes and also serous versus nonserous histology. (Asha et al., 2017)
Heenanet al., observed that; Ki-67 expression elevated with histological grade (p > 0001), (Heenanet al., 2013) and many authors have documented the same findings. (Jemal et al., 2007, Min and Park, 2007, Gursan et al., 2009, Aune et al., 2011; Patil et al., 2011; Asha et al., 2017) It has been documented in the serous carcinoma subtypes; the Ki-67 L1 was higher in the HGSC than the LGSC. (Henriksen et al., 1994; Rohke et al., 1997; Kobel et al., 2008; Prat, 2012) The study by Giurgea et al., noted the expression of both Ki-67 and p53 in 125 diagnosed cases of epithelial ovarian neoplasms and found serous to be the most common histological subtype (92.3%) and demonstrated high level of Ki-67 L1 in HGSC. (Giurgea et al., 2012) Similar results were obtained by Asha et al., they concluded that; there was a significant difference (p=0.001) in Ki-67 L1 expression between HGSC (65.34%) as compared to the LGSC. (37.96%) (Asha et al., 2017)

The Ki-67 immunostaining pattern has been detected as focal and heterogeneous in more number of low grade neoplasms as compared to diffuse pattern in higher grade. (Mita et al., 2004; Aune et al., 2011; Giurgea et al., 2012; Asha et al., 2017)

The FIGO system has been identified as independent prognostic factor with higher stage reflecting more aggressive state due to change in tumor biology. (Aune et al., 2011; Ezzati et al., 2014) Khouja et al., found a correlation between high Ki-67 expression with higher grade, poor differentiation, ascitis, the presence of residual disease after primary surgery and advanced FIGO stage. (Khouja et al., 2007) In Asha et al., study, high Ki-67 L1 was noted with advanced FIGO stage (stage III-70.6%) which was statistically significant (p> 0.001). (Asha et al., 2017)
Munstedt et al., found a significant correlation between Ki-67 determined tumor growth fraction and incidence of tumor recurrence (p > 0.001) in early stage ovarian carcinoma. (Munstedt et al., 2004) Several studies which have examined the relation between Ki-67 antigen expression and long term survival have reported that; the PI is a good predictor of patient outcome in epithelial ovarian cancer. The median survival of patients whose carcinoma had a high Ki-67 expression was lower compared to patients whose tumors demonstrated low Ki-67 expression. (Garzetti et al., 1995, Kritpracha et al., 2005, Liu et al., 2012, Heeran et al., 2013)

The assessment of the PI by Ki-67 L1 determines the proliferative potential of SEOC in diagnosing the high grade, HGSC and advanced stage along with routine histopathological report as aggressive tumors. This helps in prognosis and therapy, need of tailoring chemotherapy and longer survival rate. Even patient with early stage ovarian carcinomas with high Ki-67 L1 are likely to benefit from adjuvant chemotherapy despite of the tumor grade and type. (Kaern et al., 2005; Khandakar et al., 2014) The exclusive study on clear cell adenocarcinomas indicates the survival rate of the patients with high Ki-67 antigen expression was significantly greater than for low Ki-67 antigen expression and suggested low proliferation activity may contribute to chemoresistance. (Itamochi et al., 2002)

Conventional treatment for SEOC consists of surgical removal of tumor, followed by Platinum/Taxane based chemotherapy. (Sassen et al., 2007) Currently, sandwich therapy is preferred for advanced stage disease of the FIGO staging system that is, NACT with interval debulking surgery and post-surgery chemotherapy. (Sassen et al., 2007; Miller et al., 2008) Studies by Miller et al., and Khandakar et al., found a decrease in Ki-67 L1 subsequent to NACT and significant differences in the tumor
histomorphology as compared to the indigenous neoplasms with better survival outcome. (Miller et al., 2008; Khandakar et al., 2014)

Recent studies suggest that; Ki-67 is potentially an attractive therapeutic target in cancer due to the ubiquitous expression in all proliferating cells. Inactivation of the proliferation marker Ki-67 will lead to cell death specifically in proliferating cells and thus could be a potential strategy for the treatment not only of ovarian cancer but also of numerous other malignancies. (Rahmaanzadeh et al., 2010; Li et al., 2015)

2.4. Epidemiology

According to the National Cancer Institute (NCI), in 2015 there were an estimated 21,290 new cases of ovarian tumor 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. (National Cancer Institute, 2015) In 2016, an estimated 1,685,210 new cases of cancer will be diagnosed in the United States and 595,690 people will die from the disease. The most common cancers in 2016 are projected to be breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, bladder cancer, melanoma of the skin, non-Hodgkin lymphoma, thyroid cancer, kidney and renal pelvis cancer, leukemia, endometrial cancer, and pancreatic cancer. (National Cancer Institute, 2016)
Like many other cancers, when ovarian cancer is found at an early stage (for example, localized to the ovary or fallopian tube) the survival at 5 years is very good (about 92%); most women at stage 1 will still be alive at 5 years. However, the 5-year survival for all women diagnosed with ovarian cancer is only 45%. This is because it is often found at an advanced stage in which the disease has already spread within the abdomen. Survival is also dependent on the type of care the patient receives. Women suspected of having ovarian cancer should be referred to a gynecologic oncologist. These are physicians with special training in gynecologic (ovarian, uterine, cervical, vulvar, and vaginal) cancers. If a woman does not involve a doctor with this specialized training in her care, then studies show that her survival is significantly worse, often by many years. For this reason, every woman with this disease ideally will obtain a referral to a gynecologic oncologist before she starts any treatment or has any surgery. (American Cancer Society, 2015)

A total of 224,747 new cases of ovarian cancer were reported worldwide in 2008, with 99,521 cases being diagnosed in more developed regions, and 125,226 being diagnosed in less developed regions. Ovarian cancer was the seventh most common cancer diagnosis among women in the world overall, and fifth most common cancer diagnosis among women in more developed regions. The world rate is estimated to be 6.3 per 100,000, and is higher in developed countries and regions (9.3) compared to others. (Ferlay et al., 2010) Incidence rates for selected region. Rates range from 3.8 in the Southern and Western African regions to 11.8 in the region of Northern Europe. Continental rates are highest in Europe (10.1), followed by North America (8.7), Australia (including New Zealand, 7.8), South America (6.2), Asia (5.1), and Africa (4.2). (Ferlay et al., 2010)
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. (National Cancer Institute, 2105) Koper et al. reported a rate of 14.9 in the Netherlands, similar to that found in the United States. (Koper et al., 1996) In Alexandria, Egypt, the rate was 3.16. (Mahdy et al., 1999) 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 artefactual due to the long-standing high-quality data available for the United States. A Japanese analysis based on data from several regional cancer registries reported a 1.5 fold increase in ovarian cancer rates from 1975 to 1993. (Tamakoshi et al., 2001) 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) studies in 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 A et al., 2011)

2.5. Ovarian tumors 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., 2000–2006). 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., 2009–2010) ...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. (Saeed et al., 2014)
2.6. Previous studies

Several studies which have examined the relation between Ki-67 antigen expression and long term survival have reported that; the PI is a good predictor of patient outcome in epithelial ovarian cancer. The median survival of patients whose carcinoma had a high Ki-67 expression was lower compared to patients whose tumors demonstrated low Ki-67 expression. (Garzetti et al., 1995; Kritpracha et al., 2005; Liu et al., 2012; Heeran et al., 2013) The assessment of the PI by Ki-67 L1 determines the proliferative potential of Squamous Epithelial Ovarian Cance in diagnosing the high grade, HGSC and advanced stage along with routine histopathological report as aggressive tumors. This helps in prognosis and therapy, need of tailoring chemotherapy and longer survival rate. Even patient with early stage ovarian carcinoms with high Ki-67 L1 are likely to benefit from aduvant chemotherapy despite of the tumor grade and type. (Kaernet et al., 2005; Khandakar et al., 2014)

The exclusive study on clear cell adenocarcinomas indicates the survival rate of the patients with high Ki-67 antigen expression was significantly greater than for low Ki-67 antigen expression and suggested low proliferation activity may contribute to chemoresistance. (Itamochi et al., 2002)

Recent studies suggest that; Ki-67 is potentially an attractive therapeutic target in cancer due to the ubiquitous expression in all proliferating cells. Inactivation of the proliferation marker Ki-67 will lead to cell death specifically in proliferating cells and thus could be a potential strategy for the treatment not only of ovarian cancer but also of numerous other malignancies. (Rahmaanzadeh et al., 2010; Li et al., 2015)
2.7. Immunohistochemistry

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)
CHAPTER THREE
MATERIALS
&
METHODS
3.1. Study design
This was descriptive cross-sectional conducted to study the immunohistochemical expression of Ki-67 in ovarian tumors among Sudanese patients.

3.2. Study duration
This study was carried out during the period from September 2017 to May 2018.

3.3. Study area
The current study was conducted at Elrahma Medical Centre- Khartoum north- Sudan.

3.4. Study populations, samples and samples size
Populations subjected in this study were Formalin Fixed Paraffin Embedded (FFPE) tissue blocks previously diagnosed with ovarian tumors as case group. Sample size recruited in this study was forty samples with ovarian tumors.

3.5. Sampling technique
Convenient sampling technique was used to collect samples in this study.

3.6. Data collection tools and variables
Master sheets were used to record all patients and samples data. Master sheets were also used to record all IHC results. Ki-67 was detected using IHC method.
3.7. Quality control
Positive and negative control sections were used to evaluate the working solutions and to evaluate the testing slides. All precautions and quality approaches were issued as manufacture instructions.

3.8. Sample processing
One section from each block measured four microns was cut using Leica microtome (Leica Micro systems, RM 2125 RT, serial NO. 8843/04-2005-China) and then stained in Haematoxylin & Eosin (H & E) to confirm diagnosis of each block and to select the tumor area for preceding tissue microarray (TMA). All FFPE tissues were assembled in one TMA block using conventional mechanical pencil tips; a hollow needle was used to remove tissue cores as small as 1mm in diameter from regions of interest in FFPE tissue samples. These tissue cores were then inserted in a recipient paraffin block in a precisely spaced array pattern (1 mm), then the array block was covered by microscopic glass slide, after that introduced into dry oven at 37 °C until the block was warmed, then the slide was rotated over the cores, then let to cool at room temperature (RT), then incubated in the refrigerator freezer for complete solidification, then the TMA block was ready for sectioning.

One section from TMA block measured four microns was cut using the same type of microtome and then floated consecutively in 70% ethanol and water bath (Electrothermal ser NO.18861434-China) at 40°C. The floated section was mounted on positive charge immune slide (Thermo Scientific- Italy) to detect immune expression of Ki-67. Then the slide contained section was dried in dry oven (WTC binder 7200 TUTTLINGEN, B28, NO.88485-USA) at 60°C for 30 minutes.
3.9. Methods of detection

3.9.1. H & E staining method

Section of 4 micron thickness was stained using haematoxylin and eosin (Mayer's technique). Each section was dewaxed in hot plate oven and cleared in two changes of xylene for two minutes, then hydrated through ethanol (100%, 90%, 70%, 50%) and water two minutes for each, then stained in Mayer's haematoxylin for 7 minutes, then washed and blued in running tap water for ten minutes, then stained in eosin for three minutes, then washed in distilled water (D.W)... and hydrated through ascending grades of ethanol, cleared in xylene and mounted in Disterene, a plasticizer (polystyrene) and xylene (DPX).

3.9.2 Method of immunohistochemistry (IHC) technique

The immunohistochemical procedure was done as followed; following deparaffinization in xylene, slide was rehydrated through a graded series of alcohol and placed in running water. Then slide was steamed for antigen retrieval for Ki-67 using water bath in coplin jar containing sodium citrate buffer (pH 9.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol for 10 minutes, and then slide was incubated with 100-200 µl of primary monoclonal antibody for Ki-67 for 20 min at RT, then rinse in phosphate buffer saline (PBS) for 3 min. Then binding of antibody site was detected by incubating slide for 20 minutes with dextran labeled polymer. Finally, the sections was washed in three changes of PBS, followed by applying (DAB) as a chromogen to produce the characteristic brown stain for up to 5 min. Slide was then counter stained with haematoxylin for 5 minutes.
3.10 Interpretation of immunostaining slides

Negative staining achieved when no crisp brown staining were observed in the nucleus and/or cytoplasm of the target malignant cells (score is 0 and over expression assessment is negative). Observed nucleus and/or cytoplasm less than 10% of tumor cells regarded also as negative (score is 0 and over expression assessment is negative). A faint/barely perceptible nucleus and/or cytoplasm staining is detected in more than 10% of tumor cells and incomplete staining regarded also as negative (score is 1+ and over expression assessment is negative). A weak to moderate complete nucleus and/or cytoplasm staining is observed in more than 10% of tumor cells regarded as weakly positive (score is 2+ and over expression is weakly positive). A strong complete nucleus and/or cytoplasm staining is observed in more than 10% of tumor cells regarded as positive (score is 3+ and over expression assessment is positive). (Rajeev et al., 2011)

3.11 Data analysis and presentation

All obtained results were analyzed by Statistical Package for the Social Sciences (SPSS) version 22.0, with Pearson’s chi-square test used to assess intergroup significance. Other variables, frequencies, mean values were calculated and presented in form of figures and tables. The p value equal or <0.05 was considered statically significant.

3.12 Ethical consideration

This study was approved by the board of Medical Laboratory Sciences (MLS) at Shendi University. A written agreement was a signed prior to sample collection with each hospital and laboratory administration. Also permission for this study was obtained from the local authorities in the area
of the study. The aims and the benefits of this study were explained well with assurance on confidentiality.
CHAPTER FOUR
RESULTS
4. 1Results

A total of 40 cases (patients with histopathologically confirmed ovarian tumors) were included in this study. The age of patients was ranged from 32-65 years with average mean of 49.9 years. The ages were divided into two age groups; the first category of less than or equal to 45 years old (younger adult), this age group was represented 13 cases (32.5%) the second age group was older than 45 years old (menopausal), and this age group was represented 27 cases (67.5%) as indicated in figure 4.1.

Figure 4.2 summarizes the frequency of types of tumors among patients with ovarian tumors; adenocarcinoma was most frequent type was found in 29/40 cases (72.5%), followed by granulosa cell tumor 8/40 (20%), followed by immature teratoma, transitional cell carcinoma, mesenchymal cell tumor 1/40 (2.5%) respectively.

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

Figure 4.4 demonstrates the frequency of marker expression. Of the 40 cases 30 cases (75%) were positive and 10 cases (25%) were negative.

Figure 4.5 demonstrates the frequency of marker score expression. Of 40 cases, score I (mild expression) was recorded 24/40 (60%), followed by score zero (negative expression) 10/40 (25%), followed by score II (moderate expression) 4/40 (10%) and score III (high expression) 2/40 (5 %) respectively.

Table 4.1 illustrates the association of tumor types with marker positive expression. Of the 30 positive samples for Ki-67, adenocarinoma was the most commn type that showed positive expression 23/30 cases (76.7%), followed by granulosa cell tumors 5/30 (16.7%), followed by imaturte
teratoma 1/30 (3.3%), transitional cell cancer 1/30 (3.3%) and mesenchymal cell tumor 0.00/40 (0.00%), the p value was 0.327.

Table 4.2 illustrates the comparison between tumor grade and Ki-67 positive expression. Grade II was most positive grade expression 17/30 (56.7%) followed by grade I 10/30 (33.3%) and grade III 3/30 (10%), the p value was 0.579.

Table 4.3 illustrates the correlation of patients age with Ki-67 expression. Out of 40 cases, the age group up to 45 years (13 cases), 11/40 (27.5%) samples were showed positive expression, the remainder 2 samples (5%) were negative. Regarding the other age group of above than 45 years old (27 cases), 19/40 (47.5%), the remainder 8 samples (20%) were negative, the p value was 0.330.

Table 4.4 shows the association between Ki-67 and tumor grade. Out of 40 samples, score I was present in 24 (60%) samples as followed;
Eight samples out of 14 samples of grade I [8/14=57.1%], 13 samples out of 21 samples grade II [13/21=61.9%], 3 samples out of 5 samples of grade III [3/5=60%].
Score II was present in 4 (10%) out of 40 samples as followed;
Only one sample out of 14 samples of grade I [1/14=7.1%], 4 samples out of 21 samples grade II [4/21= 19%], zero- sample out of 5 samples of grade III [0.000/5=0.00%].
Score III was present in 2 (5%) out of 40 samples as followed;
Only one sample out of 14 samples of grade I [1/14=7.1%], 1 sample out of 21 samples of grade II [1/21=4.8%], zero sample out of 5 samples of grade III [0.00/5=0.00%].
Score zero was present in 10 samples (25%) out of 40 samples as followed;
Four samples out of 14 samples of grade I [4/14=28.6%], 3 samples out of 21 samples of grade II [3/21=14.3%], 2 samples out of 5 samples of grade III [2/5=40%], the p value was 0.728.
Figure (4.1): Shows frequency of age group among study populations.
Figure (4.2): Shows frequency of tumor types among study samples.
Figure 3: Shows frequency of tumor grades among study samples.
Figure 4: Shows frequency of Ki-67 expression among study samples.
Figure 5: Shows frequency of Ki-67 expression per scores among study samples.
Table 4.1: Correlation of Ki-67 expression with tumor types.

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>Ki-67 expression</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>Granulose cell tumor</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Immature teratoma</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Transitional cell carcinoma</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Malignant mesenchymal tumor</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

N=40
Table 4.2: Correlation of Ki-67 expression with tumor grades.

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>Ki-67 expression</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Grade I</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Grade II</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Grade III</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

N=40
Table 4.3: Correlation of Ki-67 expression with patient’s age.

<table>
<thead>
<tr>
<th>Patient age</th>
<th>Marker expression</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Up to 45 years</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Above 45 years</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>`10</td>
</tr>
</tbody>
</table>

\[N=40\]
Table 4.4: Correlation of Ki-67 expression score with tumor grades.

<table>
<thead>
<tr>
<th>Ki-67 expression score</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Total</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score I</td>
<td>8</td>
<td>13</td>
<td>3</td>
<td>24</td>
<td>0.728</td>
</tr>
<tr>
<td>Score II</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Score III</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Score zero</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>21</td>
<td>5</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

N=40
CHAPTER FIVE
DISCUSSION, CONCLUSION
&
RECOMMENDATIONS
5.1. Discussion

Ovarian cancer is the third most common malignancy among women accounting for 5.5% of all cancers (Gupta et al., 2003). About 80% of all ovarian tumors are benign and occur mostly in young women between 25 and 40 years of age (Crum, 2004). In the present study, the age of patients with ovarian cancers was between 32-65 years old; this finding was similar to that study of the Romanian authors (Luminta et al., 2012). 40 cases of ovarian tumors were distributed into 2 groups; the first age group was included samples from patients with years old up to 45 years old, the another age group contained samples from patients above 45 years old (this age division was done depending on the WHO menopausal ages (pre and post-menopausal respectively).

Regarding association between incidence of ovarian cancer and the age of patients at presentation; our study summarized that; the ovarian tumors occurs more frequently in females above than 45 years old, this result is consistent with the outcome of the study conducted by (Pooja, 2015), which concluded that; the peak incidence for benign tumors was in the third decade of life while for malignant tumors it was the fifth and sixth decades.

In this study, the Ki-67 positive immunexpression was predominant over negative expression, this result is near to the outcome of that study conducted by (Luminta et al., 2015), which concluded that; the Ki-67 was positive expression in 61.5% in ovarian tumors.

In the present study there was no statistically significant different between ki-67 expression and subtypes of ovarian tumor, as the p value was above 0.05, there was no published data that correlated Ki-67 with the subtypes of ovarian tumor, the outcome of the Indian study conducted by (Kumudini, 2018), concluded that; the present of significant different of ki-67 positivity
was observed between malignant, borderline and benign ovarian tumors, the
difference between our study and Indian study in that; our study
concentrated only on malignant ovarian tissues.
In the present study there was no statistically significant different between
ki-67 positivity with tumor grade, as the p value was above 0.05, this result
is inconsistent with the outcome of the study conducted by (Asha, 2017),
which concluded that; the present of significant different of ki67 positivity
and tumor grade (p. value 0.001), this difference in the results may be due to
variability in the grade of samples as the commonest grade in the current
study samples was grade II, followed by grade I and III respectively, also
may be due to variability in the sample number of each grade, also small
sample size in our study may play a role in this variability.
In the present study we found there was no relation between score of Ki-67
expression and tumor grade, this results do not agree with the results in
Indian study obtained by Monisha et al., in 2011, which found that; the Ki-
67 index was higher in advanced stage tumors; and also concluded that; a
higher Ki-67 index points toward the aggressive clinical behavior. This
difference in the results may be due to variability in the grade of samples,
also may be due to variability in the sample number of each grade, also
small sample size in our study may play a role in this variability.
5.2. Conclusion

On the base of the obtained results we concluded that;

❖ The incidence rate of ovarian tumors was more present in the post-menopausal women.
❖ Adenocarcinoma was the most frequent type of ovarian tumors.
❖ Ki-67 expression invalid to differentiate between different types of ovarian tumors.
❖ Ki-67 not reliable marker in grading ovarian tumors.
❖ Ki-67 expression score improper way to grade ovarian tumors.
References

5.3. Recommendations

On the base of the obtained results we recommended that;

- Further study should be conducted in ovarian tumors using larger sample size.
- For future study, advance techniques like polymerase chain reaction (PCR) and In situ hybridization (ISH) should be conducted to detect the exact role of Ki-67 in ovarian cancer.
- Further study should include normal, benign lesions and malignant lesions of ovary.
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Appendix

Preparation of Mayer's haematoxylin
Haematoxylin ................................................................. 1 g
Distilled water............................................................... 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
Distilled water .......................................................... 100 ml
Glacial acetic acid ........................................................ 0.05 ml
Crystal thymol ............................................................ small amount
Picture 1: Shows positive Ki-67 immunohistochemical expression.
Picture 2: Shows negative Ki-67 immunohistochemical expression.
Shendi University

Faculty of Graduate Studies and Scientific Research

Questionnaire sheet

Immunohistochemical Expression of Ki-67 among Sudanese Females with Ovarian Tumours in Khartoum State

<table>
<thead>
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<th>NO.</th>
<th>Age</th>
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<th>Grade</th>
<th>Ki-67 result</th>
<th>Expression score</th>
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