INTRODUCTION

Ovarian cancer is the leading cause of gynaecologic cancer death, while constituting only 3% of all female cancers worldwide ***(Hennessy et al., 2009).*** The poor survival rate of ovarian cancer patients is due to non-specific symptoms and the lack of sensitive and specific methods for the detection of early-stage ovarian cancer ***(Zahedi et al., 2012).***

Epithelial ovarian cancer is the most common ovarian malignancy with substantial histopathological heterogeneity ***(Koukoura et al., 2014).*** Serous epithelial ovarian cancer is the most common subtype, with the majority of women presenting with advanced disease ***(Marsh et al., 2014).***

The widely used tumor biomarker for ovarian cancer is CA125, which is a high molecular weight glycoprotein ***(Sarojini et al., 2012).*** It is elevated in some benign conditions such as endometriosis and peritonitis and exhibit fluctuations associated with menstrual cycle and pregnancy ***(Moore et al., 2012).*** Despite of being used for current screening measures with trans-vaginal ultrasound, mortality rates remain high. As a result, no CA125 screening guidelines are recommended for the general population ***(Chu and Rubin, 2006; Sarojini et al., 2012).***

It is recognized that both genetic and epigenetic events - refer to the changes in gene expression, with no changes in the DNA sequence- play a role in the development of ovarian cancer, with epigenetic changes occurring early in the carcinogenic process ***(Wei et al., 2006; Ting et al., 2013).***

DNA methylation comprises the best-known epigenetic mechanism associated with gene expression ***(Herman and Baylin, 2003).*** DNA methylation occurs on the cytosine residues of cytosine guanine dinucleotides (CG), also designated as (cytosine phosphodinucleotides guanine) CpG. Enzymes known as DNA methyltransferases catalyse the addition of a methyl group to the cytosine ring to form methyl cytosine ***(Shaheen and Ahmed, 2013).***

The aberrant methylation of CpG island in gene promoters has been correlated with a loss of gene expression, and it appears that DNA methylation provides an alternative pathway to gene deletion or mutation for the loss of tumor suppressor gene function ***(Baylin and Chen, 2005).***

The Ras association domain family (RASSF) genes comprises 10 members, known as RASSF1 to RASSF10 ***(Akino et al., 2005).*** Ras proteins carry several characteristic domains and are important in biological processes, such as pro-apoptotic pathways, cell cycle and cytoskeleton regulation. They play a direct causal role in human cancer with activating mutations in Ras proteins occurring in about 30% of tumors ***(Weyden and Adams, 2007).*** RASSF genes are tumor suppressor genes, which encode RASSFs proteins that are negative Ras effectors proteins, which bind to active state of Ras proteins to inhibit cell growth and stimulate apoptosis ***(Fernandes et al., 2013).***

The RASSF2 gene, which is located on chromosome 20, encodes for three protein isoforms (RASSF2A, RASSF2B and RASSF2C) ***(Vos et al., 2003; Hesson and Latif, 2010).*** Several members of this family are inactivated by promoter DNA hypermethylation in a broad range of cancers ***(Baylin and Chen, 2005).*** RASSF2A is the only isoform of RASSF2 that contains CpG island in its promoter and it is reported to be inactivated by its promoter methylation in several human cancers ***(Zhang et al., 2006).***

Due to the high mortality rate associated with ovarian cancer, a number of studies have been carried out in an attempt to discover novel therapeutic approaches ***(Vaughan et al., 2011).***

Therefore, a study on the molecular mechanism underlying ovarian cancer progression, including a search for methylation status, is important for early diagnosis and effective therapy for ovarian cancer. There are limited data about the genetic cause in ovarian cancer ***(Wu et al., 2014).***

Aim of the Work

This study aims:

Investigate the association between methylation of RASSF2A and ovarian cancer and correlate the results with tumor marker CA125.

I. OVARIAN CANCER

A) Definition of Ovarian Cancer:

The term ovarian cancer (OC) refers to a diversity of malignant tumors that arise from ovaries with different histological subtypes, molecular features and clinicopathalogical characteristics ***(McCluggage, 2011).***

B) Epidemiology of Ovarian Cancer:

Worldwide, OC is the seventh most common malignancy in women after breast, colorectal, lung, uterine cervix, uterine corpus and stomach cancers ***(Ferlay et al., 2015).*** It is responsible for the most of associated gynecological cancer deaths worldwide ***(Grandi et al., 2015).***

According to National Cancer institute (Cairo University), there were 135 OC cases presented during the years 2003-2004. They accounted for 29.22% of the female genital tract malignancies and 1.37% of the total malignancies ***(Nassar et al., 2016).*** According to Egypt National Cancer Registry, 35 cases of OC were reported that represented 5.6% of all female cancer cases in Aswan ***(Ibrahim et al., 2014a).***

C) Classification of Ovarian Cancer:

According to the World Health Organization (WHO), OC is classified into: (1) Epithelial OC, (2) Sex cord–stromal tumors, (3) Gem cell tumors, (4) Others that include; gonadoblastoma and non-gonadoblastoma germ cell sex cord–stromal tumors, tumors

of rete ovarii, mesothelial tumors, gestational trophoblastic diseases, soft tissue tumors not specific to the ovary, malignant lymphomas, leukemias, plasmacytomas, secondary metastatic tumors and unclassified tumors of uncertain origin ***(Kurman, 2013; Tewari and Monk, 2015).***

A new classification for epithelial OC was published by the International Federation of Gynecology and Obstetrics (FIGO) in 2014. Epithelial OC is the most common type that comprises 98% of all ovarian cancers and is responsible for the most of deaths. Epithelial OC is classified into: (1) High-grade serous adenocarcinoma (HGSC) (70%), (2) Low-grade serous adenocarcinoma (LGSC), (3) Endometrioid carcinoma, (4) Clear-cell carcinoma, (5) Mucinous carcinoma. (6) Malignant mixed mesodermal tumors, (7) Transitional tumors ***(Mungenast and Thalhammer, 2014).***

D) Risk Factors of Epithelial Ovarian Cancer:

1) Age:

The incidence of OC is lower in women under the age of 40, but rises steeply after the fifth decade to reach a peak in the eighth decade ***(Doufekas and Olaitan, 2014).***

2) Hormonal and reproductive factors:

Long-term exposure to estrogen, such as in early menarche, late menopause, nulliparous women, endometriosis and hormonal replacement therapy, has a great effect on ovarian epithelium and increases the risk of OC ***(Al Bakir and Gabra, 2014).*** Estrogen constitutes a potent force for the

neoplastic transformation of normal ovarian surface epithelial cells through growth stimulation and inhibition of apoptosis ***(Salehi et al., 2008; Hunn and Rodriguez, 2012).***

The exposure of the ovarian surface epithelium to high dose of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) during the menstrual cycle promotes cell proliferation and tumor growth ***(Mungenast and Thalhammer, 2014).*** Progesterone hormone, which is increased during pregnancy and with oral contraceptive pills (OCPs) administration, promotes clearing of transformed cells from the ovarian surface epithelial layers so the long-term use of OCPs for more than ten years and high parity is associated with decreased risk of OC to half in comparison to women with no OCPs or nullipara ***(Beral et al., 2008).***

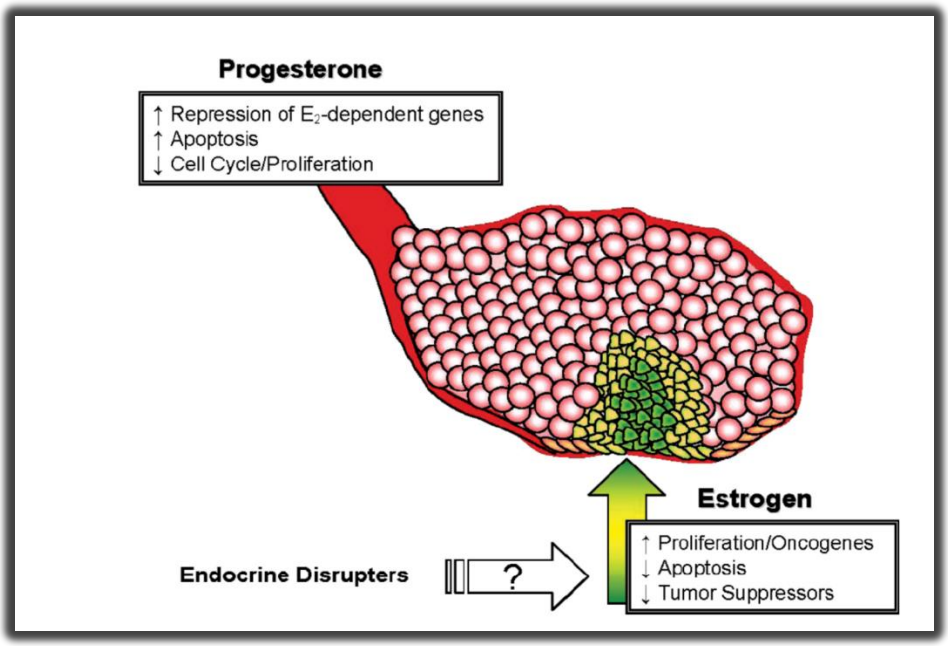

Figure (1): Hormonal factors in OC ***(Salehi et al., 2008)***

3) Obesity:

Obesity is a risk factor for OC because of its relationship to sex-steroid hormones as it increases adrenal secretion of androgens, enhance conversion of gonadal and adrenal androgens to biologically active estrogens and reduce sex hormone-binding globulin capacity, which increases the amount of free biologically active estradiol (E2) ***(Hunn and Rodriguez, 2012).*** On the other hand, moderate exercise has been suggested to be a protective factor ***(Cannioto and Moysich, 2015).***

4) Inflammatory factors:

Studies suggest that factors related to inflammation of the ovarian surface epithelium such as ovulation, endometriosis and pelvic inflammatory diseases are associated with an increased risk of epithelial OC through inflammatory mediators and several cytokines such as interleukins (ILs). Ovulation is associated with repetitive wounding and subsequent activation of tissue remodelling mechanisms through inflammatory mediators ***(Macciò and Madeddu, 2012).***

Endometriosis was significantly associated with increased risk of clear-cell and endometrioid OC through: (1) Oxidative stress that is enhanced by the high-level of heme and free iron from retrograde menstruation, (2) Chronic inflammation through secretion of growth factors and pro-

inflammatory cytokines (3) Hyper-estrogenism that causes genetic alterations in genetically susceptible women ***(Heidemann et al., 2014; Grandi et al., 2015).***

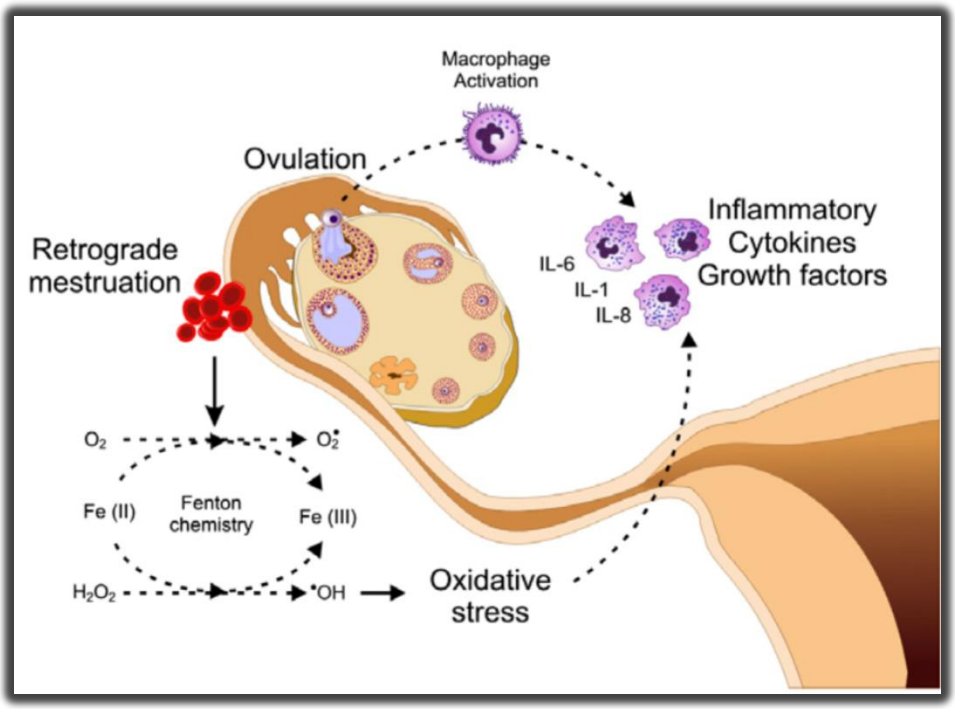

Figure (2): Inflammatory process in OC, IL: interleukin ***(Macciò and Madeddu, 2012).***

5) Genetic factors:

Cancers develop from accumulated genetic alterations that lead to abnormal cell functions such as uncontrolled cell growth, immune invasion and angiogenesis ***(Hanahan and Weinberg, 2011).*** Cancers develop through germline (inherited) mutations or a series of events that start with somatic (acquired) mutations in the tissue of origin. These mutations lead to inactivation of tumor suppressor genes (TSGs) that encode for

proteins that inhibit the tumor growth, or activation of oncogene that encode for proteins, which promote the tumor growth ***(Karlan et al., 2016; Levine et al., 2016).***

a) Inherited (germline) mutations in ovarian cancer:

Although about 90% of the cases are sporadic, 10% of OC cases are associated with autosomal dominant genetic inheritance. The risk of OC in first degree relatives of patients with OC is about three times greater than the risk in general population due to mutation in the deoxynucleic acid (DNA) repair genes ***(Lalwani et al., 2011; Jervis et al., 2014).***

Hereditary breast and ovarian cancer (HBOC) syndrome, presented with premenopausal breast cancer and/or ovarian cancer or both, is associated with mutations in breast cancer 1 (BRCA1) or breast cancer 2 (BRCA2) tumor suppressor genes in about 15% of all patients with HBOC, but also BRCA1 and BRCA2 somatic mutations are found in about 30% of HGSC ***(Karlan et al., 2016).***

Also Lynch syndrome, known as heritable non-polyposis colorectal cancer (HNPCC), is associated with an increased frequency of extra-colonic tumors including endometrial, ovarian, urogenital, brain, renal as well as gastric and biliary cancers in young age. HNPCC is associated with autosomal dominant mutation in mismatch repair (MMR) genes. MMR genes encode for proteins that recognize and correct short insertions and deletions as well as single base mismatches ***(Weissman et al., 2012; Al Bakir and Gabra, 2014).***

Mutated tumor protein 53 (TP53) gene is seen in some inherited OC cases, but commonly associated with somatic mutations ***(Karlan et al., 2016).***

b) Acquired (somatic) mutations in ovarian cancer:

Somatic mutations may occur in any cell division from the first cleavage of the fertilized ova to the cell divisions that replace cells in a senile individual and occasionally amplified by clonal expansions. Somatic mutations are frequently caused by: (1) Exogenous factors such as chemicals, ultraviolet light and ionizing radiation, (2) Endogenous factors such as reactive oxygen species or enzymes involved in DNA repair or genome editing ***(Martincorena and Campbell, 2015).***

These mutations lead to inactivation of TSGs that encode for proteins, which inhibit the tumor growth, or lead to activation of oncogenes that encode for proteins, which promote the tumor growth ***(Karlan et al., 2016).***

i. Tumor suppressor genes:

BRCA1 and BRCA2 somatic mutations are found in about 30% of HGSC. TP53 mutation is found in nearly all cases of HGSCs and around 50% of mucinous cases ***(Rechsteiner et al., 2013; Belanger et al., 2015; Karlan et al., 2016)***. Phosphatase and tensin homologue (PTEN), AT-rich interactive domain 1A (ARID1A) are two TSGs, which are mutated in endometroid and clear cell OC ***(Maniar et al., 2013).***

ii. Oncogenes:

Mutations of Kirsten rat sarcoma 2 viral oncogene homolog (KRAS), murine sarcoma viral oncogene homolog B1 (BRAF), phosphatidylinositol 3-kinase catalytic alpha (PIK3CA**),** cyclin-dependent kinase inhibitor 2A (CDKN2A) and erythroblastic leukemia viral oncogene homologue 2 (ERBB2) have been found in some OC cases ***(Maniar et al., 2013; Karlan et al., 2016).***

E) Pathogenesis of Epithelial Ovarian Cancer:

The aetiology of OC as well as cancer in general is not well established ***(Madsen et al., 2015).***

1) Incessant ovulation hypothesis:

This hypothesis attributes OC to repetitive wounding during ovulation and the subsequent activation of repair mechanisms that are characterized by the generation of an enormous amount of pro-inflammatory cytokines and chemokines ***(Macciò and Madeddu, 2012).*** These processes are associated with an increased number of mutations that are accumulated in epithelial cells that drive tumor formation and progression in genetically susceptible women ***(Mungenast and Thalhammer, 2014).***

Benign inclusion cysts, are less than 1 cm cysts in ovarian stroma and lined by ovarian epithelium, may result from invaginations of ovarian surface epithelium into the

ovarian stroma, which is either caused by ovulation or aging. Inclusion cysts have different microenvironment than normal ovarian surface epithelium and they undergo dysplasia and eventually turn to OC in genetically susceptible women ***(Auersperg et al., 2001).***

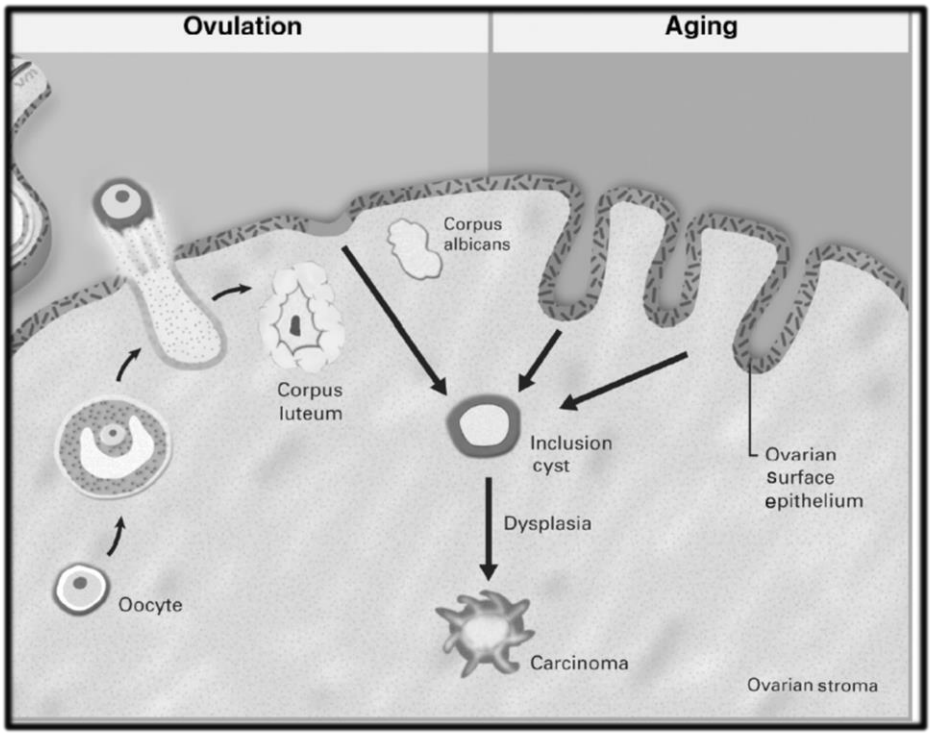

Figure (3): Incessant ovulation theory ***(Auersperg et al., 2001).***

2) Retrograde menstruation or inflammation hypothesis:

Retrograde menstruation occurs due to the retrograde flow of sloughed endometrial cells and blood cells via the fallopian tubes into the ovaries and pelvic cavity during menstruation. It is a physiologic event in most of menstruating women, as blood in the Douglas pouch was found in

laparoscopy of 90% of women with patent tubes ***(Vercellini et al., 2011; Sourial et al., 2014).*** Retrograde menstruation brings endogenous carcinogens (uterine growth factors) to fallopian tubes and ovaries. During retrograde menstruation, an inflammatory process takes place in the form of accumulating leucocytes, inflammatory cytokines with the release of nitric oxide that may lead to DNA damage ***(Fleming, 2006; Madsen et al., 2015).***

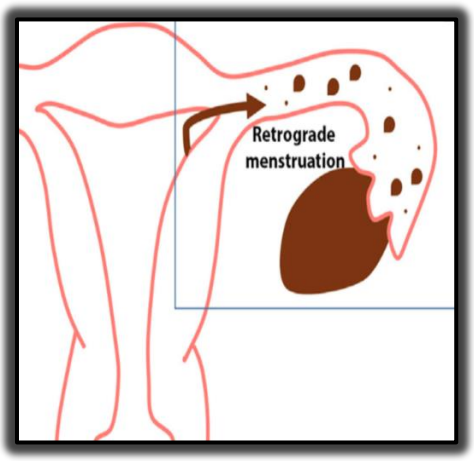

Figure (4): Retrograde menstruation theory ***(George et al., 2016).***

3) A non-ovarian origin theory:

There are no OC precursor lesions have been found in ovaries while precursor lesions from Fallopian tubes fimbria (serous tubal intraepithelial) carcinomas were found in patients who underwent prophylactic salpingo-oophrectomy due to elevated genetic predisposition to OC especially BRCA and TP53 mutations. Those patients had altered p53 expression (p53 signature) and were proposed as a potential precursor to HGSC ***(George and Shaw, 2014; Quartuccio et al., 2015).***

Refluxed blood is accumulated in Douglas pouch where the tubal fimbria lay so the epithelium of the fambria is chronically exposes to blood, heme and free iron. Over expression of p53 in fimbrial epithelium may constitute a physiologic response to oxidative stress while its mutation initiates the carcinogenic process ***(Vercellini et al., 2011).***

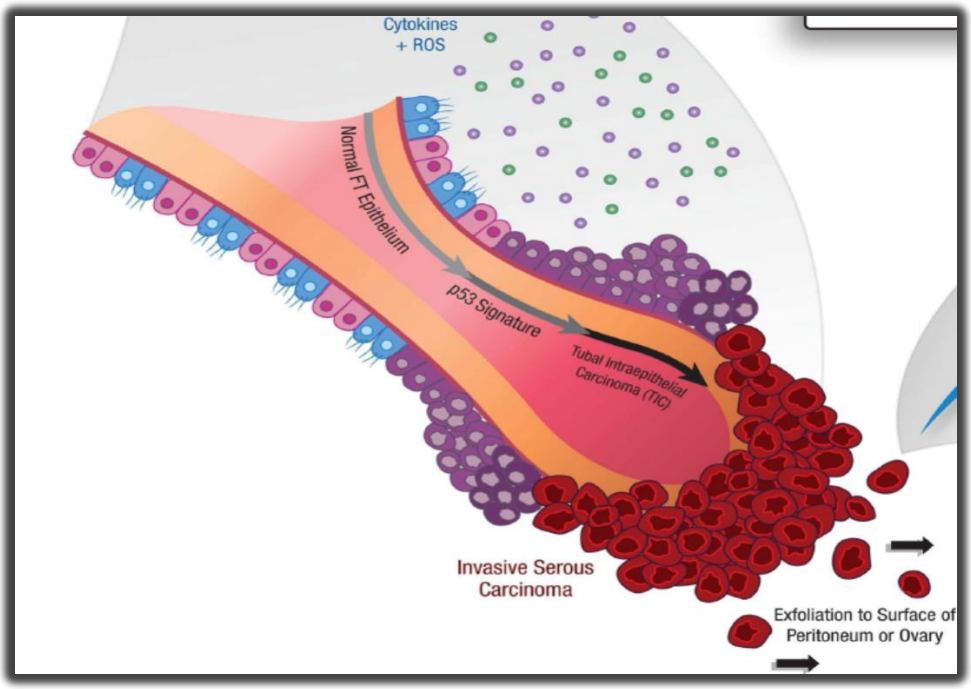

Figure (5): Early tumor progression within the fallopian tube and the resultant genetic profile, ROS; reactive oxygen species
(Jones and Drapkin, 2013).

The precursor of endometroid and clear cell subtypes are thought to be endometrial in origin as endometriosis was significantly associated with increased risk of clear cell and endometrioid OC ***(Heidemann et al., 2014).***