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REVIEW ARTICLE

Circulating and non-circulating proteins and nucleic acids as biomarkers and therapeutic molecules in ovarian cancer

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Abstract Ovarian cancer is the second most fatal gynecological cancer. For the last decade or so significant use of non-circulating and circulating biomarkers has been highlighted. However, the study of such biomarkers at nanovesicle technology such as exosomes, proteomic and genomics studies could further contribute to better identification of anomalous protein and networks which could act as potential targets for biomarker and immunotherapy development. This review provides an overview of the circulating and non-circulating biomarkers with the aim of addressing the current challenges and potential biomarkers that could lead to early ovarian cancer diagnosis and better management. By means of this review we also lay a hypothesis that characterization of exosomal protein, nucleic acid content from body fluids (serum, plasma, urine, etc.) can decode the secret of disease and potentially improve diagnostic sensitivity which could further lead to more effective screening and early detection of the disease.

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Introduction

Ovarian cancer (OC) is the leading cause of death from gynaecologic malignancy. In 2021, 21,410 women were diagnosed with OC in USA. The staging of OC is demonstrated (Fig. 1). The rate of survival corresponds to the stage with survival at 90% in stage I and II, which drastically drops during the stages III and IV.[1] Stage I tumors are usually confined to the ovaries and have a favourable prognosis and lead to 10% of OC related deaths. In the early stage, patients are asymptomatic, thereby the diagnosis of OC becomes difficult. Pathologically, there are three main types of tumors based on the type of cells that the tumor originates from: epithelium, germ cell, or stroma. Epithelial ovarian tumors are subdivided into five histological subtypes: serous, mucinous, endometrioid, clear cell, and transitional, with epithelial serous carcinomas representing most common primary ovarian carcinomas. Ovarian germ cell tumors develop from the cells that produce the ova or eggs, whereas ovarian stromal tumors are a rare class of malignancies that develop from connective tissue and produce estrogen and progesterone. Germ cell and stromal tumors combined account for 5%—10% of all ovarian cancers. Over 70% of the patients are diagnosed at an advanced stage, when the disease is far beyond the pelvis. With the advent of many new technologies which have been based on molecular approaches, having a thorough knowledge about different genes and proteins that play a crucial role at various signaling events leading to OC and could be potential molecular targets for diagnosis.[2]

Various screening methods have been used for OC such as transvaginal ultrasonography, CA125 serum. OC is a heterogeneous type of malignant tumors which impacts and perturbs different locations of the peritoneal cavity.[3] It fulfils the criteria of the World Health Organization (WHO) screening.

The emerging field of proteomics has known to play a vital role in new directions and may provide a mechanism for early-stage diagnosis. Mass spectrometry (MS) is one such technique of proteomics that can provide an overview of the proteome in time and space. It can be used for protein isolation, identification, and characterization.[4] MS can be used for profiling of all peptides and proteins in the serum as well as characterization of single peptides of interest. The introduction of cancer has led many groups to use MS mixed with different interfaces such as surface enhanced laser desorption and ionisation (SELDI) or matrix assisted lase desorption and ionisation (MALDI) to obtain the serum proteome for diagnostic and prognostic purposes.[5]

There is also a need for the development of a predictive biomarkers. The most important question that governs the selection of such biomarkers is the specificity, sensitivity, and toxicity of the targeted molecule. As a result, clinical trials are needed to confirm the alteration of the expected target for the optimal use of such new molecular target. Serum proteomics provides a tool to identify the nature of cells in normal conditions as well as in disease states.[6] Apart from serum proteomics, tumor biomarkers also play a vital role in the detection and management of ovarian cancer. A biomarker, CA125, has played an important role

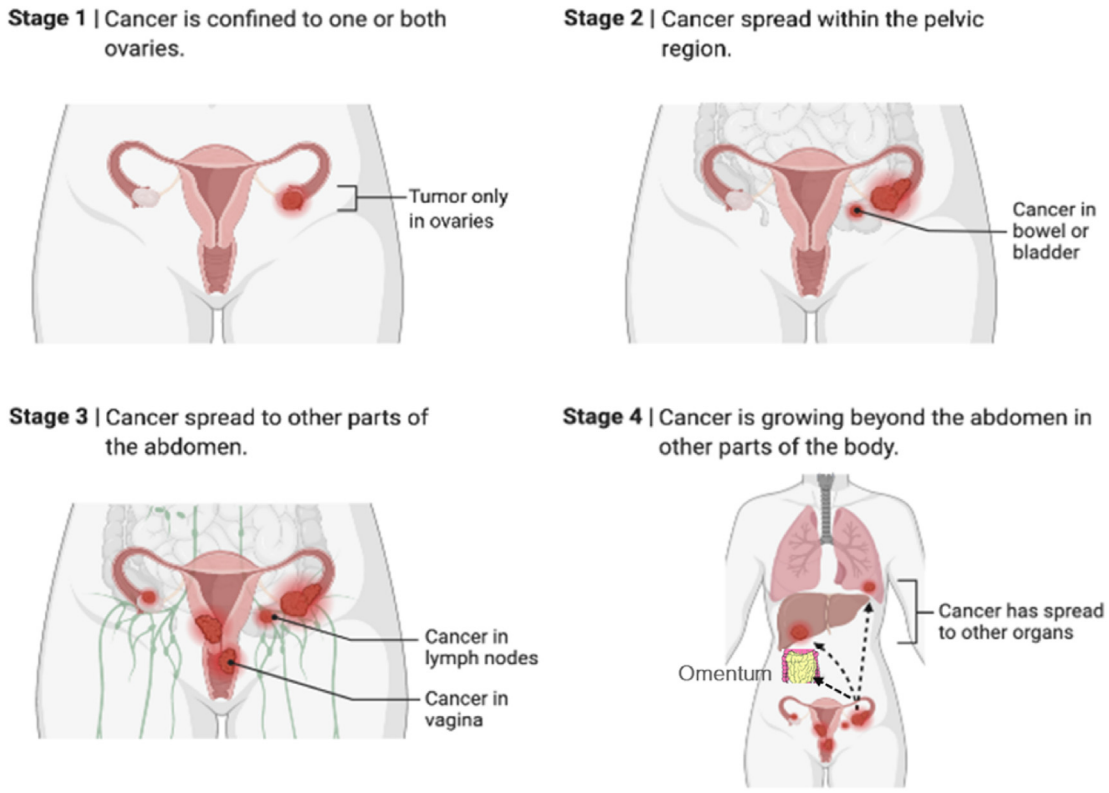

Figure 1 Ovarian cancer staging. Image created and adopted from BioRender (biorender.com).

in screening, detecting, and managing ovarian cancer for the last few decades.

CA-125 (cancer antigen-125) as a clinical biomarker

Structure of CA-125

CA-125, a membrane bound glycoprotein was discovered in 1981 by Bast and his colleagues during the development of an antibody (OC125).[7] CA-125 has been found in amniotic fluid, chorionic membrane (of the foetus in high abundance), epithelial cells of the airways, respiratory gland, secretory mammary gland, bronchial mucus, tissues derived from Mullerian and coelomic epithelia, endocervix, apocrine sweat gland, intestines, peritoneum, pleura, pericardium, endocervix, and endometrium.[8–12] CA125 is present as an epitope of the MUC16 gene of glycoprotein which is available in variable weight. The biochemical analysis of CA125 revealed that it contains O- and N-linked glycosylated fragments. The N-terminal has a high concentration of serine, threonine, and proline.[13] MUC16 includes several domains, including 60 tandem repeats of 156 amino acids, the amino terminus, a transmembrane domain, a 32 amino acid cytoplasmic tail and 56 sea-urchin, enterokinase, and agrin (SEA) domains.[14] These SEA domains differ from those found in mucins with a single SEA domain because they are not cleaved.[15] CA125 contains two antigenic domains that bind OC125-like (group A) and M11-like (group B) antibodies separately[16] as schematically demonstrated (Fig. 2, 3).

Function

The role of CA125 in health and disease is under analysis. There is some evidence that the oligosaccharide feature is linked to CA125 in cell-mediated immune response. It can inhibit the cytotoxic responses of human natural killer cells (NK), and its inhibition correlates with the reduction in CD16 expression on the NK cell surface, through which the cancer cell is not recognised.[17] The MUC16 gene is one of the three most frequently mutated genes in different cancer types. MUC16 interacts with other biomolecules like Galectin-1 and 3, mesothelin, E and P-selectins, and siglec-9.[18] Mesothelin and CA125 are both involved in ovarian adenocarcinomas and in metastasis by adhesion to the peritoneal mesothelium.[19] The MUC16's cytoplasmic tail plays an important role in tumour proliferation, invasiveness, and cell motility.[20]

CA-125 as a biomarker

Ovarian cancer is the second most lethal gynaecological malignancy in the world. Numerous cancer biomarkers have been discovered and among these biomarkers, CA125 plays an important role in screening, detecting, and management of the disease. CA125 is the only serum marker that has been studied as both a risk marker and a potential early detection marker.[21–24] The concentration of soluble CA125 is measured by a second-generation assay based on double-determinant ELISA tests that use two monoclonal antibodies, M11 and OC125. Early detection of this marker could reduce mortality from OC, but due to low prevalence of OC and the ideal screening test must have a sensitivity above 75% and a specificity of at least 99.6%, it is a challenge to find a screening test. The only way to overcome this is to change the cut-off points from 0 to 35 μ/mL, thereby affecting the profile of the test. The nonoverlapping epitope domains of CA 125 are recognised with the help of anti-CA125 antibodies, which are divided into three groups: OC125- like (group A), M11-like (group B), and Ov197-like. The three antibodies can recognise either denatured or native CA125 antibodies of group A and group B, which are used to detect denatured CA125 antibodies on the membrane.[25] Recent research has shown us ways to improve screening methods by adding known biomarker to the panel that include CA125. The other novel markers are CA72-4, M-CSF, B7—H4, HE4, kallikreins 6, 10, and 11 and mesothelin.[26] These biomarkers are discussed below.

Human epididymis-specific protein 4 (HE4)

A new potential biomarker HE4 has been identified in the epithelium of the distal epididymis.[27] These are identified using Northern blot analysis and transcript hybridisation.[28] HE4 is also known as WFDC-2 (whey acidic protein four-disulfide core domain protein 2) because it is made up of two whey acid protein (WAP) domains and a four-disulphide bond core comprising eight cysteine residues.[29] Some members of the four disulphide core family proteins are protease inhibitors, like secretory leucocyte protease inhibitor, which have the potential to inhibit chemo trypsin, trypsin, elastase, and cathepsin G and elastin, except for the HE4 protein.[30] Expression of the HE4 gene in normal tissue is highly inhibited because its protease is an inhibitor of sperm production but is present in a limited amount in the reproductive tract's epithelium and respiratory airways.[31] HE4 is absent in the normal ovarian surface epithelium (OSE).[32,33] Human cells expressing HE4 include the telomerase immortalised OSE cell line T29, the clear cell carcinoma cell line ES-2, and the ovarian serous carcinoma cell line SKOV-3.[34] Overexpression of HE4 promotes cell apoptosis and adhesion and contributes to the inhibition of cell proliferation, migration, and tumour formation.

The level of HE4 is determined by using the enzyme linked immunoassays (ELISA) and immunoradiometric assay (IRMA). IRMA consists of a non-competitive sandwich type double determinant immunoassay using two monoclonal antibodies, 2H5 and 3D8, which present a signal at 160-pg level.[35] According to studies on the serum of postmenopausal patients with ovarian carcinoma, HE4 has the same specificity and sensitivity as CA125 in ELISA-based diagnosis.

The sensitivity of HE4 is high in the early stages of ovarian cancer as compared to CA125.[36] HE4 is a potential biomarker for various solid tumours like ovarian cancer, pulmonary adenocarcinoma, mesothelioma, endometrial cancer, and breast cancer. Due to limited presence, HE4 is used for all three monitoring processes, which is comprised of early monitoring, recurrence, and progression.[37]

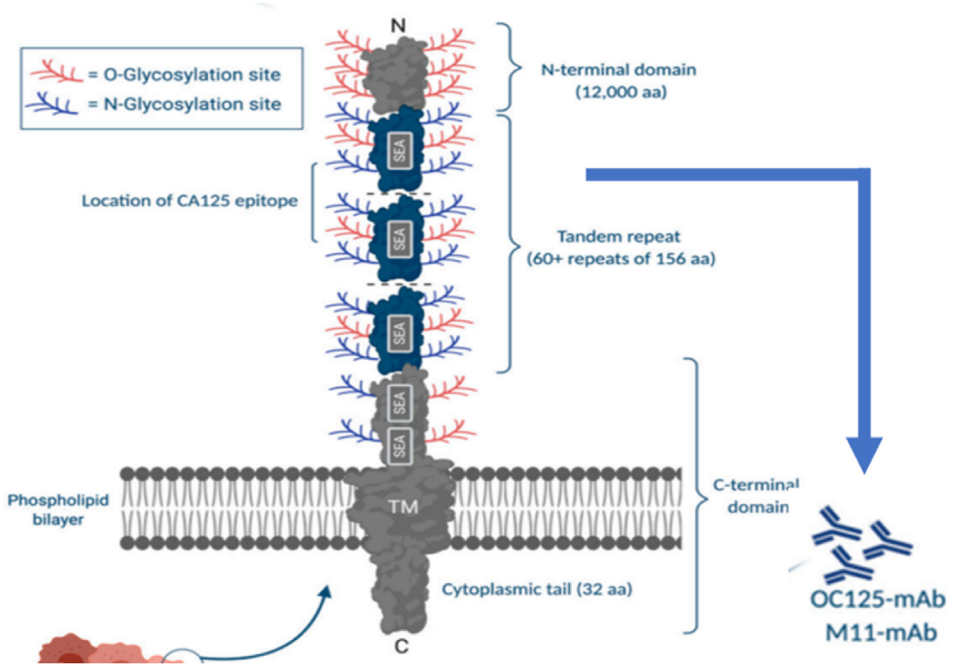

Figure 2 Detailed structure of MUC16.

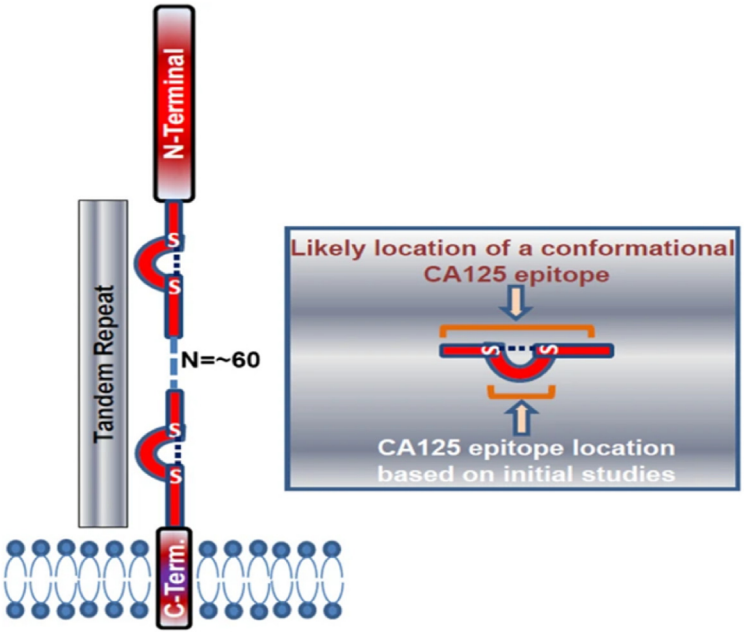

Figure 3 CA125 as an epitope of MUC16.

Extracellular vesicles (EVs) as circulating biomarkers

Exosomes

Exosomes are small extracellular vesicles (EVs) produced by almost all types of cells.[38–40] Exosomes are multivesicular structures endosomal in origin and heterogenous in nature due to the way they reflect the phenotypic state of the cell from which they were generated.[41] They serve as microRNA carrying vehicles and are utilized as delivery system for miR-181a in leukaemia cell proliferation.

Compared to a healthy individual, cancer patients have shown to have twice or higher levels of blood exosomes due to heterogenous nature of the pathological conditions.[42] Exosome expressing CD19 CAR (chimeric antigen receptor) have shown to exhibit anti-leukemic nano-immunotherapeutic potential.[43] The proteome present in exosomes includes endosomal, plasma, cytosolic and nuclear proteins.[44] The proteins inside the exosome are associated with exosome biogenesis, transport, and fusion. The exosomes

contain GTPase, annexins, heat shock protein, integrins, tetraspanins, MHC class II protein, epithelium cell adhesion molecule (EpCAM), and the membrane of the human epidermal receptors. After the release of these exosomes from the cell surface, protein present on the exosome engages the cell surface receptor of the recipient cell, which fuses with the plasma membrane and induces signal transduction. Exosomes also contain protein, lipid, DNA, RNA, and their different forms.[45,46] Due to abundant presence of exosomes and development of proteomics, genetic profiling can provide better diagnosis and monitoring of ovarian cancer. From recent discoveries, exosomes have shown to play a role in tumorigenesis by regulating angiogenesis, immunity, and metastasis. Circulating exosomes are potential biomarkers for early detection, diagnosis, prognosis, and treatment of cancer patients. Three molecules inside the exosome that have been identified as potentially biomarkers for ovarian cancer. CD24 (tetraspanins), microRNA, and epithelium cell adhesion molecule (EpCAM). EpCAM are detected in OC exosomes. EpCAM is a glycoprotein found in the pseudo-stratified transitional epithelia which is associated with the homo-typical adhesion of cells and identified as an invesicular protein in exosomes. At stages I and II, EpCAM concentrations are very low, but in the later stages of ovarian cancer, EpCAM concentration increases many fold.[47,48] The concentration of EpCAM and CD24 is directly proportional to each other. CD24 is another glycoprotein found in the cytoplasm and released into the body fluid via exosomes, with bad prognosis. miRNA is a small non-coding RNA that plays an important role in controlling cellular processes like proliferation, cellular death, maturation, and differentiation.[49] In the early stages of ovarian carcinoma, miRNA is not detected, but in advanced stages, the tumour-derived exosome of ovarian cancer has a copy of the tumour miRNA. This suggests miRNA could also potentially serve as an early diagnostic marker.

Many more potential biomarkers exist in the exosome, but due to a lack of understanding about exosome physiological function, these markers are still under investigation.[47]

Serum based diagnosis by proteomic analysis

Serum proteomics is a study that is based on the proteomic profile obtained from patient's serum and is widely used in screening and identification of tumor markers as it has shown potential clinical application.[50] The information for the detection of OC using serum proteomics can be gathered from the peripheral venous blood leaving the tumor. This is because it is challenging to identify a population of normal ovarian epithelial cells and compare them with epithelial ovarian cancer cells as blood enters the tumor and adapts cellular products of secreted protein as well as debris from dead cells.[6] Several methods are available for the analysis of serum for diagnosis of OC, most widely accepted method is mass spectrometry.[51–53]

Mass spectrometry

Mass spectrometry is used to express protein and peptide information and contributed to significant development in proteomics analysis and has shown a great impact on biomedical science.[54] MS was first used in clinical proteomics for only the detection of proteins and peptides, but nowadays the improvement in technology allows for quantitative investigations as well. Different laser desorption techniques like matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI) connected with time of flight (TOF) detectors and electrospray ionisation (ESI) have been used in clinical diagnosis.[55,56] Research has shown that linking the MS technique with liquid chromatography or capillary electrophoresis can provide a high-resolution spectral proteomic pattern for the detection of OC.[52] In an experiment, a proteomic signature pattern for OC was derived and tested using SELDI-MS with a ciphergen biosystems mass spectrometer. A total sample of model signature pattern was tested against 116 masked samples, and that gave 100% sensitivity and 95% specificity, with 94% positive predictive value (PPV) in the test. Four models were generated using genetic algorithms and bioinformatics procedures, and each showed 100% sensitivity and specificity.[57] In one experiment, a set of 80 cancer patients and 91 healthy female patients were screened, using the SELDI-MS method to identify accepted biomarkers for OC.[58] The results identified a peak of approximately 11,700 kDa increased in cancer patients as compared to controls. This peak was identified due to the alpha chain of haptoglobin, but due to improper validation of the required marker before introduction to the clinical trials the results were not fully accepted by the scientific world. There were several reasons for lack of acceptance few mentioned here: 1) the lack of identification of key peaks[59]; 2) lack of cross validation of the identified protein and peptide peaks; 3) difficulty in reproducing results using different bioinformatics[58]; 4) lack of insertion of existing significant biomarkers. Hence, there is a need for new experimental setups to considerate the different alterations for proper standardization of these protocols.

Transvaginal ultrasonography

Transvaginal ultrasonography (TU) is one of the best methods to observe the morphology of ovaries and detect OC, and it could potentially show the extent of disease. Many studies have suggested transvaginal ultrasonography can be used as a method for screening early-stage OC. TU is usually the preliminary step in most of OC examinations, whether it is used as an initial screening or as a secondary test in women with an abnormal biomarker profile.

It is performed using a 5–7.5 MHz vaginal probe, to generate an accurate ovarian image which can be used for the detection of early changes in ovarian morphology. The volume is calculated using a prolate ellipsoid formula (length $\times$ width $\times$ height $\times$ 0.523). Both volume and morphology are involved in screening criteria for ovarian abnormalities. Standard deviations have been published with normal ovarian volumes of less than 20 cm^3 in premenopausal women and less than 10 cm^3 in postmenopausal women.[60] Septate ovarian cysts less than 10 cm in diameter that are identified by screening are no longer considered abnormal and do not require surgical removal.[61] It has the advantage of being a non-invasive, non-ionizing imaging

modality, however it is limited by the fact that it is operator dependent making it difficult to standardize and not good for screening purpose. Finally, laparoscopy can also be performed for ovarian tumors. This is an invasive examination, and it can't be used as a routine screening method.

Mesothelin

Mesothelin is a protein which is present on the cell surface of the normal mesothelial cell lining of the body cavities.[50] Mesothelin is expressed by several cancers such as mesotheliomas, ovarian cancer, pancreatic cancers, and some squamous cell carcinomas.[62] Human mesothelin is derived from 69 kDa polypeptide with a hydrophobic sequence at the carboxyl end which is removed and replaced by phosphatidylinositol. This glycosyl-phosphatidylinositol linkage attaches mesothelin to the cell membrane.[63] After glycosylation is complete at one or more of its four recognized points, it is probably spliced by the protease furin to yield a 32 kDa soluble protein called megakaryocyte potentiating factor (MPF) and a 40 kDa cell membrane bound protein called mesothelin.[50] But till now, the proteolytic splitting of mesothelin by furin has not been clearly shown for human tumors. MPF has been shown to increase megakaryocyte proliferation in mouse bone marrow cultures in the presence of interleukin-3.[64] A recent study has shown evidence that mesothelin binds with CA125 and may therefore play a role in the spread of OC in the peritoneal cavity.[19]

Cell bound mesothelin is a positive target for antibody-based treatment of cancers that overexpress mesothelin, and currently, clinical trials are under way for anti-mesothelin immunotoxin.[63] Mesothelin is an immunogenic protein, and anti-mesothelin antibodies are frequently found in patients with mesotheliomas and ovarian cancer.[65] Even if mesothelin is attached to the cell membrane, it could detach like many other proteins.[66]

However, the accurate relationship of small multidrug resistance protein to membrane bound mesothelin is still not clear and reactivity of the antibodies has not been shown, which is used to measure small multidrug resistance protein with mesothelin.[67] Mesothelin deficient mice have been used to develop anti-human mesothelin monoclonal antibodies (mAbs) which react with different epitopes of human mesothelin.[68] To detect mesothelin that is shed into the serum, a double determinant ELISA was developed using one of the newly generated anti-human mesothelin. Results showed that serum mesothelin levels are elevated in patients with mesothelioma and ovarian cancer compared to normal healthy volunteers and decrease after surgical therapy for mesothelioma. Double determinant ELISA can accurately measure serum mesothelin levels and it may be useful as a tumor marker for diagnosis and to follow response to treatment in patients who express mesothelin in cancer.[69]

Urine based diagnosis by proteomic analysis

The proteins and peptides of the urine are smaller in size with their posttranslational modification. Urine is thermodynamically more stable than serum and plasma, making it more suitable for biomarker research using mass spectrometry. Urine is the primary mode for biochemical excretion in human making it a great candidate for disease monitoring.

Eosinophil-derived neurotoxin (EDN)

EDN, is a major secretory product of eosinophils and is also a nuclease of pyrimidine-specific RNase, known as RNase2. Serum EDN and CA125[70,71] levels increase in OC patients due to an increase in RNase activity by 1000 folds.[72] The increased level of urinary EDN in serum is a reason for the eosinophilia or unknown tumour products. Eosinophilia count is increased in some types of cancer, such as lung, breast, and colorectal cancers.[73] The presence and degranulation of eosinophils in the tissue of OC is shown by Samoszuk's group.[57] Urine collected from patients who underwent pelvic surgery for the proteomic analysis, yielded two possible biomarkers for OC. Using two-dimensional Western blotting test and SELDI-TOF-MS test, two potential urine biomarkers were identified, the hyperglycosylated EDN and the cluster of COOH-terminal osteopontin fragments. Both are present in abundance in urine, and osteopontin protein concentration level ranges are in ng/ml.[74]

The N-linked glycosylation of EDN shows that it contains five potential asparagine residues of different molecular weights with two peaks in the mass spectroscopy profile. After treatment with either trifluoromethanesulfonic acid or glycanase, EDN showed decrease in molecular weights.[75] EDN has an inhibitory effect on oocyte formation in pregnant women.[76] EDN is multifunctional in women, like formation of conjugation with other hormones that mainly target the ovary like the gonadotropins, and other functions due to differential glycosylation.[57] These functions suggest that EDN presence in ovarian cancer patients is more likely to be glycosylated.[77]

Osteopontin

Osteopontin (OPN) is an integrin-binding matrix phosphorylated glycoprotein which is an arginine-glycine-aspartic acid, first reported by Sanger as a marker of epithelial cells in 1979.[78] OPN is expressed in various cells of the body, such as in osteoclasts, osteoblasts, epithelial cells of the breast, vascular smooth muscles and endothelial cells, T cells, NK cells, and macrophages. OPN induces growth in many parts of the body like bone cells, epidermal cells, macrophages. In tumor cells its associated in processes like bone resorption, inflammation, and ischemia-reperfusion, and progression.[79]

OPN takes part in various signalling pathways such as cellular adhesion and chemotaxis, suppression of macrophages preventing cells from apoptosis, stress-dependent angiogenesis and anchorage independent growth of tumour cells, proliferation, migration, and invasions.[80] A messenger RNA-osteopontin molecule was detected in CD68 macrophages.

OPN plays an important role in various physiological cellular functions like tumour proliferation and invasion in lung, ovarian, breast, and colon cancers. Serum OPN levels are useful in detecting ovarian cancer and can work as a

proteomics biomarker. The elevated OPN level in urine is also used as an early diagnosis as a non-invasiveness screening test. In ovarian serous carcinoma, OPN doesn't show any valid difference between high grade and low-grade carcinoma, but it shows significant differences in other types of cancer like breast, uterus, rectum, thyroid, and lungs.[81]

OPN is a substrate for the proteolytic cleavage by the protease thrombin and the matrix metalloproteinases.[82] This aids OPN fragments in lowering their molecular weight, allowing them to enter cells and perform both biological functions for adhesions and migration, but the OPN molecules are not observed as enacted. In a recent *in vivo* study, it was proposed that osteopontin cleaved fragments are a family of small integrin-binding ligands of N-linked glycoprotein (SIBLING) through which they interact with the specific matrix metalloproteinase of cancer cells that increases invasiveness.[57] Among the cleaved fragments, the COOH-terminal OPN fragments show the strongest invasiveness in ovarian cancer with high specificity and possibility of high sensitivity. When compared with benign and inflammatory diseases, it achieved 93% specificity and 72% sensitivity for early-stage ovarian cancer.[83] When glycosylated EDN and COOH-terminal OPN are combined, the false positive rate in the early detection of ovarian cancer is reduced.

Fibrinogen and collagen

In a recent study of urinary ovarian cancer biomarkers, the proteolytic fragments of the proteins fibrinogen and collagen were identified in large amounts. The roles of fibrinogen and collagen in cancer growth are well documented in the literature. Endogenous formation of fibrinogen through interaction with fibroblast growth factor (FGF-2) promotes lung and prostate cancer.[84] The most abundant protein in humans, type-I collagen, promotes blood vessel development through a process called angiogenesis. Angiogenesis is one of the key components of the pathogenic processes like tumour growth and metastasis.[85]

In recent years, various potential biomarkers have been detected in the serum, plasma, ascites, and urine that excites the interest of academic world working on ovarian cancer.[86] There are some other protein fragments detected that have biomarker potential, analysed through the same technology as transthyretin, apolipoprotein, and haemoglobin.[87] Compared to previous studies, equaliser beads and SELDI-TOF MS technology improve the detection method for more diluted urinary protein or protein fragments.[88] From the urine samples of pelvic mass patients, through the equaliser beads, SELDI-TOF MS and gel electrophoresis, we see three proteomics profiles as urine biomarkers for specific ovarian cancer, the fibrinogen alpha fragments, fibrinogen beta N-terminal fragments and collagen alpha-I (III) fragments are identified as potential biomarkers.[89]

The ROC-AUC of the three-biomarkers identified in the study, fibrinogen alpha fragments, fibrinogen beta N-terminal fragments, and the collagen alpha-I (III) fragments in combined form, have a 0.88 value, while the CA125 alone has a ROC-AUC value of 0.94, however, the ROC-AUC value of the combined CA125 and three protein fragments is 0.96.[90] This suggests the combination of these markers as potentially powerful biomarkers for detection of ovarian cancer.

Plasma based diagnosis by proteomic analysis

Blood is the primary source of every disease's early diagnosis. Due to the abundance of proteins in the blood fluid, including immunoglobin, albumin, and coagulation cascade, they mask the less abundant and oncologically novel and interesting proteins and peptides. The thermostability of plasma makes its fraction the best for proteomics marker detection.

Amyloid A1

Serum amyloid A (SAA) is also known as an acute phase reactant. SAA is a protein which is formed in liver.[91] In recent studies, for plasma protein detection, a protein chip using SELDI-TOF-MS has been developed with high efficiency for the cancer protein marker.[92] The SELDI-TOF-MS uses an artificial intelligence data analysis algorithm-based method for differentiating between women with ovarian cancer and women without any neoplastic disease with high accuracy.[57] Rise in two peaks detected by using cystine modification in the SELDI-TOF mass spectrometry. The first one is of 11.7 kDa molecular mass known as serum amyloid A1 (SAA) and the other one is glycosylated haptoglobin alpha-1's N-terminal arginine-truncated form having a molecular mass of 11.52 kDa.[93] Further research revealed that the molecular mass of the SAA is like that of CA125 and elevated in ovarian cancer. In lung cancer, the same molecular mass peak is predicted as a protein kinase C inhibitor.[94] Amyloid A1 concentrations increase primarily during inflammation (primarily irritation and swelling) and is considered as a sensitive biomarker for inflammation because it increases over 1000 folds in cancer patients.[95] As mentioned in the literature, chronic infection, and inflammation (proinflammatory cytokines) are associated with cancer symptoms.[96] Other types of cancer, such as lung cancer,[97] uterine cervical cancer,[98] gastric cancer,[99] renal cancer,[100] and others, have significantly higher SAA concentrations.

The sensitivity, specificity, and AUC value of SAA in OC were calculated, and they are as follows: 0.78, 0.86, and 0.89. The same values were calculated for CA-125 and they are as follows: 0.81, 0.92, and 0.87 respectively. SAA shows less sensitivity and specificity than CA125, but in combined form they show the highest sensitivity and specificity and can be used in early detection of ovarian cancer.

Matrix metalloproteinases

Matrix metalloproteinases (MMP) are a unique class of zinc-dependent proteinases that degrade diverse extracellular matrix (ECM) components and are involved in a variety of physiological processes, including tumour invasion and metastasis.[101–103] MMPs are known to play a key role in cancer cell invasion, metastasis and confer a poor prognosis

because of their ability to degrade extracellular protein. MMP2 and MMP9 were found to be linked with cervical cancer, endometrial carcinoma, and ovarian cancer. Overexpression of MMPs is linked to ovarian cancers' higher metastatic potential, which leads to a worse prognosis and shorter survival. MMPs could also be used as sensitive biomarkers to investigate ovarian cancer's biological characteristics. MMP-2 expression is a beneficial indicator in the advancement of ovarian cancer because tumour invasion and metastasis are mostly dependent on the destruction of ECM. MMP-2 expression is found to be increased in ovarian cancer cells with peritoneal implants and is linked to an increased risk of death.[104]

MMP3 is found to be overexpressed in cancerous cells, and it also regulates extracellular matrix and activates other MMPs. MMP7 is the smallest member of the MMP family and is also known as matrilysin, which activates the gelatin enzyme MMP7, and promotes metastasis in ovarian carcinoma.[105] MMP1-PAR1 activation causes ovarian cancer cells to secrete angiogenic factors such as interleukin 8 (IL-8) and growth-regulated oncogene-alpha, which acts in a paracrine way on endothelial CXCR1/2 receptors, causing endothelial cell proliferation, tube formation, and migration. MMP-9 levels were shown to be greater in OC than in normal ovarian tissues and benign ovarian tumours.

MMP-9 has been proposed as a possible serum marker for ovarian cancer diagnosis, and a high MMP-9 level in the blood could be a prediction of resistant tumours.[106] MMP10 is highly increased in ovarian cancer cells that developed resistance to platinum-based chemotherapy when compared to non-resistant cells during chemotherapeutic treatment.[107]

Post-translational modifications

The ability to adapt and survive in a changing environment is a trait shared by all living species. A living organism's proteins, or proteome, must alter to respond to environmental changes. One of the later phases in protein production, PTMs (post-translational modifications), refers to chemical changes in a polypeptide chain which occur after transcribing DNA into RNA and then translating into protein. Chemical changes to amino acid side chains range from enzymatic breaking of peptide bonds to covalent additions of specific chemical groups, lipids, carbohydrates, or even whole proteins. In the Uniport database, one of the most extensive PTM databases, with more than 80 experimentally validated reported changed sites, there are around 435 different types of PTM reported.[108] There are 24 significant PTMs, according to the dbPTM. They have an impact on many eukaryotic proteins and they can interact within or between them, regulating various activities or facilitating functional relationships between changed residues of the same or distinct PTM kinds. Many cellular processes rely on protein post-translational modifications, including cellular differentiation, protein degradation, signalling and regulatory processes, gene expression regulation, and protein—protein interactions.[109] By covalently adding functional groups or proteins, proteolytic cleavage of regulatory subunits, or destruction of complete proteins, protein post-translational modifications improve the functional diversity of the proteome. Two types of post-translational protein modification mechanisms can be distinguished. Proteolytic processes, which are mostly cleavages of peptide bonds that result in the elimination of some of the generated polypeptide fragments, fall under the first category. The mechanisms that change the side chains of amino acid residues and do not usually interfere with the polypeptide backbone make up the second group.[110] PTM might be reversible or irreversible.[111]

Covalent modifications are found in reversible processes, while proteolytic modifications are found in irreversible reactions that proceed in one direction.[112] PTMs can occur in a single amino acid or in a group of amino acids, and they cause changes in the chemical characteristics of changed sites.[113] Sensitive MS methods like MALDI-TOF or LC-ESI paired with dissociation procedures are necessary to identify post translational alterations and describe their structure. Phosphorylation, glycosylation, ubiquitination, methylation, nitrosylation, acetylation, proteolysis, and lipidation are just a few of the alterations that affect every aspect of normal cell life and disease.[110] PTM such as tyrosine phosphorylation and lysine acetylation appear to have increased expression and activity in cancer and play an important role in the development of ovarian cancer and its resistance to chemotherapy.[114,115]

PTM assay methods can be used to identify protein probes PTM on the human proteome array. 19 kinases in tyrosine phosphorylation were identified which were potentially responsible for the dysregulated signalling pathway observed in high grade serous ovarian carcinoma (HGSOC). When the autophosphorylation status of PTK2 (pY397) and PTK2B (pY402) as a proxy for kinase activity was tested in ovarian cancer cell lines, higher kinase activity was detected.[115] Transthyretin (TTR), apolipoprotein A1 (APOA1), and casein kinase 11 alpha 1 subunit isoform-a (CSNK2A1) are proteins linked to ovarian cancer, while destrin (DSTN), tumor rejection antigen (gp96) (HSP90B1), and EGF-containing fibulin like extracellular matrix protein (EFEMP1) are markers for drug resistance. TP53 gene, is mutated in almost all these malignancies. There is a low incidence of mutation in genes including BRCA1, BRCA2, CDK12, NF1, CSMD3, GABRA6, RBI, and FAT3. Recent research has discovered that numerous forms of PTMs, including Tyr phosphorylation, SUMOylation, ubiquitination and acetylation, are engaged in the development and progression of ovarian cancer, likely due to dysregulation of the related enzymes. Abbott et al looked at tumor-specific glycan alterations in tumour and normal ovarian tissue and found glycoprotein markers that showed tumor-specific glycosylation modifications.[116] Multiple signalling pathways involved in EOC pathogenesis have been identified, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF—B) pathway, the activator of transcription 3 (Jak-STAT3) pathway, the mitogen-activated protein kinase (MAPK) pathway, the proto-oncogene tyrosine protein kinase Src pathway, ER beta pathway, the Mullerian inhibitory substance receptor pathway. Cancer cell growth, metabolism, motility, and metastasis are all affected by these pathways.[117] Exploration of the effects of various PTMs on these proteins, as well as further deconstruction of their mechanisms of action, can aid in the identification of novel cancer biomarkers and therapeutic molecules.

Phosphorylation

Phosphorylation is one of the most common PTMs of proteins and is an important physiological regulation mechanism since numerous proteins, enzymes, and receptors are regulated by phosphorylation and dephosphorylation.[118] It is a reversible mechanism that governs a variety of cellular functions via protein kinases and phosphatases, including protein synthesis, cell division, signal transmission, cell growth, development, and ageing. Protein kinases, in particular, are responsible for cellular signal transduction, and their overexpression or failure can be detected in a variety of illnesses, most notably malignancies. Serine (Ser or S), threonine (Thr or T), and tyrosine (Tyr or Y) are the most changed amino acid residues for phosphorylation, and they play an important role in cancer growth.[119] Dysregulated tyrosine kinase activity is reported in different types of cancers. The cell cycle is dominated by several cellular signalling pathways, including tyrosine kinase, MAP kinase, the cadherin–catenin complex, and dysregulation in their phosphorylation–dephosphorylation cascade has been linked to several types of malignancies. In various malignancies, including breast, prostate, and non-small cell lung cancer, phosphorylation of Akt/protein kinase B (PKB), a serine/threonine kinase, modulates biological responses.

Ubiquitination

Ubiquitation is a post translational modification seen in many malignancies including OC. Activating enzymes (E1), conjugating enzymes (E2), and ligases are the three primary types of ubiquitination enzymes (E3). Uncontrolled production of these enzymes, including as DUBs (deubiquitinating enzymes) and other complexes involved in the ubiquitination pathway (SCF complex), contributes to oncogene signalling and cancer growth and metastasis.[120] The ubiquitin pathway plays an important role in the progression of ovarian cancer and its treatment. Ubiquitination also acts as a switch, allowing specific protein kinase activity to be turned on and off.[121] Damage to the tumour suppressor gene BRCA1 raises the risk of cancer. BRCA1 gene mutations have been linked to an increased risk of breast and ovarian cancer. Hayami[122] and colleagues discovered that cyclin dependent kinase 2 (CDK2) inhibits the ubiquitin activity of BRCA1-BARD1 and consequently plays a role in the advancement of ovarian cancer. According to Jensen et al,[123] Bap 1 could operate as a tumour suppressor in the BRCA-1-related cell growth regulation pathway. USP11, a deubiquitinating enzyme that forms complexes with BRCA2, may play a role in ovarian cancer by regulating BRCA2 expression. Also, constitutive activation of extracellular signal-regulated kinase has been linked to increased tumorigenicity and chemoresistance in ovarian cancer. Increased ubiquitination has been linked to MKP3 degradation, which leads to abnormal ERK1/2 activation and contributes to tumorigenicity and chemo resistance in human ovarian cancer. In ovarian cancer, cyclin E breakdown via phosphorylation has also been documented in cells with enhanced ubiquitination. In ovarian cancer cells, it is hypothesised that p53 regulates protein homeostasis by downregulating ubiquitin-proteasome system function in response to cellular stress.

Acetylation

Acetylation and deacetylation play important role for many important cellular processes; malfunctioning in this machinery can result in severe conditions including cancer. Cancer patients with acetylation-specific site mutations have a worse survival rate. According to a recent study, 6405 ubiquitination-related SNVs, 2106 acetylation-related single nucleotide variants, and 883 SNVs in shared sites of the two PTMs were discovered in the cancer samples of various types.[124] In this study, oncoproteins like TP53, AKT1, and IDHI were found to be modified for acetylation in cancer. Histone acetyltransferases (HAT) and histone deacetylases (HDACs) regulate histone acetylation.[125] The aetiology of ovarian cancer is aided by an imbalance between HAT and HDACs. In ovarian cancer, abnormal expression of immunological components such as HLA-class I and II (The human leukocyte antigen (HLA) system or complex is a gene complex encoding the major histocompatibility complex (MHC) proteins in humans) has been discovered. Through modulation of the expression of its target gene HCP5, hMOF may have a role in influencing tumour antigen-specific immune responses in ovarian cancer. Reduced hMOF levels have been linked to a lower overall patient survival rate. As a result, hMOF protein expression represents a separate risk factor for the prognosis of malignant ovarian tumours.[126] Therefore, hMOF could be used as an epigenetic biomarker for the diagnosis of OC.

Challenges in the detection of early ovarian cancer

Laparoscopy or laparotomy can be a positive screening test for ovarian cancer. The dire need of early detection, importance, and advantages of early detection of ovarian cancer is well described.[127] OC is a relatively uncommon disease, but it is an important cause of mortality. It is uncommon because of its death rate, which is no greater than 40 per 100,000 per year, even in postmenopausal women.[128] Clinical researchers agree that a screening strategy must achieve a minimum positive predictive value (PPV) of 10%, which is no more than nine false positives for each true positive for acceptance in the context of ovarian cancer. Specificity of 99.6% is needed to achieve the 10% PPV target on screening the overall population of postmenopausal women with the incidence of 40 per 100,000 per annum. This strategy demands very high specificity of screening for ovarian cancer. Most of the biomarkers have been developed and calculated using a sample of patients with clinically diagnosed cancer, which is often done in advanced stage cancer. A major challenge is to seek out biomarkers that not only detect clinically apparent OC but also early phases of the disease before symptoms appear. For detection of clinically diagnosed ovarian cancer, there are many markers with high sensitivity, but only a few of

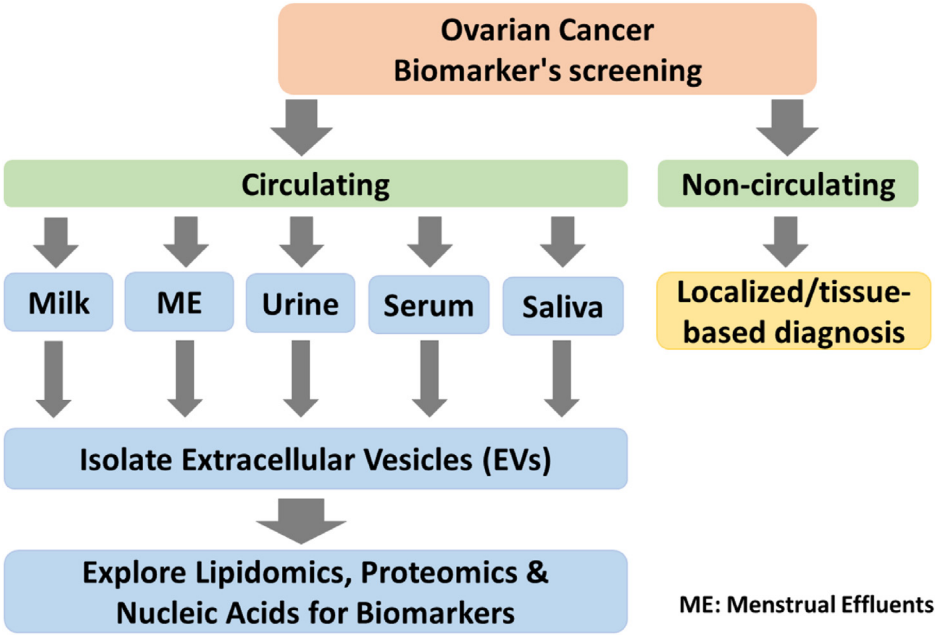

Figure 4 The proposed model shows approach towards screening of circulating biomarkers and developing non-invasive method.

them have high sensitivity for pre-clinical disease. Reports which are based on high sensitivity in samples from patients with clinically diagnosed disease can be highly misleading. The best way to alter the outcome of OC is by interfering early in the natural history of the disease. An important parameter is the duration of a positive marker in the pre-clinical phase. It is difficult to study the lead time required to successfully change the natural history of OC as there is a lack of suitable samples and a lack of information. So, before starting expensive trials, there should be evidence that potential tests have good sensitivity for preclinical disease.[129]

Another challenge is to define the most suitable target population for screening purpose. Patients with sporadic OC with risk are defined by post-menopausal status and age greater than or equal to 50, family history of ovarian malignancy and presence of BRCA1 and BRCA2 mutations. Most of the OC are sporadic and occur in the general populations. A few other factors that pose risks in the general population include menopause, years of oral contraceptive use, and pregnancy. A maximum of 5%—10% of OC are caused due to hereditary syndromes. Members of first-degree female relatives have a lifetime risk of developing ovarian cancer which is greater than 10%. And this risk is due to the mutations in BRCA1 and BRCA2 genes. The average risk of ovarian cancer by age 70 years is 39% and 11% in BRCA1 and BRCA2 gene mutation carriers respectively. In recent past, one of the most important study shows ovarian cancer population screening and its mortality rate after long term follow-up under UK collaborative trial of ovarian cancer screening (UKCTOCS). This study was executed in 13 different centers of National Health Services (NHS) in England, Wales, and Northern Ireland. More than 200 thousand women were included in the data analysis of this study, challenges associated with screening of OC within general population is also discussed.[130,131]

Conclusion

Despite advances in surgical treatment, chemotherapy, radiation therapy, bio-targeted therapy, and other technologies, OC remains a deadly disease with a poor long-term survival. Circulating as well as non-circulating markers are suggested as novel potential biomarkers for early diagnosis of OC. CA-125 has played a vital role in screening, treatment during different phases of ovarian cancer management. The sensitivity of CA-125 can be improved by combining it with other biomarkers such as serum-directed mass spectrometry, which appears to be promising. Most blood-based biomarkers seen in serum or plasma are acute phase proteins that are unrelated to any cancer making finding novel biomarkers difficult. Screening biomarkers for early diagnosis have received a lot of attention recently, but markers that predict response to treatment could be useful in identifying those who will benefit the most from therapy. As a result, if significant reductions in ovarian cancer mortality are to be achieved, present CA125 and exosomes-based screening will need to be revisited in combination with other biomarkers. The proposed model (Fig. 4) shows approach towards screening of multiple circulation biomarkers and developing non-invasive method.

Conflict of interests

The authors declare no competing interests.

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