12

Potential Tumor Biomarkers for Ovarian Cancer

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1. Introduction

In the United States, invasive ovarian cancer is the 5th most deadly malignancy in females, accounting for an estimated 13,850 deaths in 2010 (Ahmad, 2011; American Cancer Society, 2010). The risk of dying from ovarian cancer depends on staging and varies greatly. Ovarian cancer patients diagnosed at the localized stage exhibit a 5 year survival rate of 94%. This rate is 73% when diagnosed at the regional stage following local dissemination and drops to 28% when a patient is diagnosed at the distant stage with metastasis to organs outside the pelvis. Overall, the combined 5 year survival rate for all ovarian cancer patients is an unmanageable 46% (American Cancer Society, 2010).

Upon histological evaluation, most ovarian cancers are found to be epithelial in nature and are collectively referred to as ovarian epithelial cancers (OEC). The most common OEC subtypes include, in decreasing order of frequency, serous adenocarcinomas, followed by endometrioid, and smaller subsets of mucinous, clear cell, transitional, and undifferentiated carcinomas (Tavassoli and Devilee, 2003).

The typical progression of invasive ovarian cancer is dissemination from the primary site into the peritoneal mesothelium. The close proximity of the ovary to the mesothelium explains the high incidence of peritoneal dissemination observed in nearly all cases of ovarian cancer. Tumors are thought to arise either from implanted cells from the fringe of the fallopian tube (Jarboe et al, 2008) or from dysplastic inclusion cysts which develop out of the mesothelial-like ovarian surface epithelium (OSE). As the tumor progresses, cells shed into the peritoneal fluid, escape apoptotic mechanisms, and begin to attach to their surrounding mesothelium via integrin-mediated interactions with extracellular matrix components (Ahmed et al, 2005; Cannistra et al., 1995; Yokoyama et al., 2007). Unlike most malignancies, ovarian cancers rarely metastasize through the hematogenous route until the advanced stages (Rose et al., 1989). Approximately 62% of cases of ovarian cancer are diagnosed at the distant stage (American Cancer Society, 2010) and the clinical prognosis for such patients is poor.

The high mortality associated with ovarian cancer stems, in part, from late detection and underpins the exigent need to identify predictive and early stage diagnostic biomarkers. The task is not an easy one. Difficulty in the validation of current screening tests is mainly attributed to the lack of uniformity in clinical presentation of the disease, which varies with epithelial cell morphology, depending on whether the carcinoma is of a serous, clear cell, mucinous, or endometrioid type. To the present date, blood concentration measurements of CA125 (mucin-16), in conjunction with ultrasonography, have been used to screen for ovarian cancer. However, it has been found that detection of serum CA125 alone is

inadequate for reliably detecting ovarian cancer for a number of reasons. These include a lack of specificity, questionable prognostic ability, frequent false positive readings, liver clearance of circulating antigen, and elevation associated more often with progression in late rather than early stages (Helzlsouer et al., 1993; Jacobs and Bast, 1989; Maggino et al, 1994). These confounds have hindered the diagnostic potential of CA125 for detecting OEC in stage I or II, when the disease shows much a much higher cure rate. Moreover, annual screening with CA125 and ultrasonography fails to reduce mortality and deleterious complications may arise from surgical interventions in women exhibiting false positive results (Buys et al, 2011). Clearly, efforts to identify better diagnostic markers are warranted.

2. Carbohydrate biomarkers

A major benefit of using carbohydrates as tumor biomarkers comes as a result of both their abundance and importance in shaping and maintaining the tumor microenvironment (Fukuda, 1996). Cellular communication, adhesion, and trafficking are all major functions of carbohydrate polymers, or glycans, which constitute a significant portion of glycoconjugates (Li et al., 2010a; Ohtsubo and Marth, 2006; Varki et al., 2009). Glycosylation, the formation of a linkage of a glycan with a protein, a lipid, or other organic molecule, greatly increases the complexity of those latter molecules and, resultantly, the potential for information storage. Altered protein glycosylation is believed to be an early event in tumorigenesis which contributes to invasion and metastasis of tumor cells (Chiang et al., 2010; Dall'Olio and Chiricolo, 2001; Hakomori, 1996; Hakomori, 2002; Varki et al, 1969; Kuzmanov et al., 2009; Meezan et al., 1969; Saldova et al, 2008; Yogeeswaran and Salk, 1981). Many prominent proteins in OEC pathophysiology, such as integrins and the receptor for epidermal growth factor (EGFR), are found to be heavily glycosylated (Stroop et al, 2000; Gu and Taniguchi, 2004).

Some important factors in cancer are the percentage of glycosylated proteins, the degree of branching versus linear polymers, the preponderance of specific chemical groups added to glycans, and the type, or "signature," of glycosylation observed. Proteins in cancer are generally highly glycosylated compared to non-malignant phenotypes, particularly on the cell surface and on proteins with a secretory function (Hakomori, 2002), suggesting an active role in establishing the extracellular tumor microenvironment. Varying conformations associated with differential glycan arrangements serve as molecular switches which can alter protein functions. Frequent protein modifications observed in cancer cells include alterations in core and terminal fucosylation, changes in sulfation and sialylation patterns, increased glycan branching, and alterations in Lewis isotopes (Varki et al, 2009). Lewis isotopes are glycoprotein antigens confined to red blood cells and epithelial secretions that belong to the Lewis blood group system.

Growing knowledge of the changing glycosylation patterns of small, soluble glycoproteins specific for certain cancers have made the possibility for developing novel diagnostic serum and even urine tests using protein markers with aberrantly expressed glycosylation patterns an endeavor worthy of pursuit. Some of the changes in glycosylation observed in OEC are described below, with potential tumor markers revealed.

2.1 Fucosylation

Addition of the five carbon sugar, fucose, to glycans reduces flexibility around glycosidic linkages of branching point antennae to enhance selectivity for ligands and increase

molecular stability of the glycoconjugate. Unlike most other carbohydrate moieties, fucose contains only one free hydroxyl group available for hydrogen bonding. This feature restricts rotational freedom and enhances stability. The presence of bulky terminal fucose groups in a glycoconjugate restricts access to galactose residues. These moieties are normally recognized by asialoglycoproteins that target the molecules for degradation. This inevitably leads to lifespan extension of the modified glycoconjugate. Changes in structure/function mechanics are attributed to core fucosylation as well. Core fucosylation of the innermost residue in the chain greatly affects ligand specificity of glycoproteins by providing an extended conformation with altered binding affinity (Stubbs et al., 1996).

A glycoprotein that has thus far become a legitimate candidate as a potential biomarker is thrombospondin 1 (THBS-1). THBS-1 is released by platelets to negatively regulate angiogenesis by disrupting vascular endothelial growth factor (VEGF) signaling (Zaslavsky et al, 2010). A four-fold increase in expression levels of this protein has been observed in serum of ovarian cancer patients, and shows considerable core fucosylation not seen in healthy patients as determined by reactivity with the Aleuria aurantia lectin (AAL), which preferentially binds most strongly with core fucose-containing glycoconjugates (Abbott et al, 2010, Yamashita et al, 1985). Abbott et al (2010) also identified a second marker, periostin (POSTN), that exhibited altered glycosylation in the form of increased bisecting N-acetylglucosamine (GlcNAc) in serum from ovarian cancer compared to sera from healthy controls (Abbott et al, 2010). These modifications were examined only in endometrioid OEC cases (Abbott et al, 2010). THBS-1 and POSTN are known to be highly expressed in other subtypes (Kodama et al, 2001, Zhu et al, 2010); though POSTN is associated more with late stages (Zhu et al, 2010). Core fucosylation patterns have not yet been described for ovarian cancers displaying non-endometrioid histology. If tumor-specific alterations in fucosylation patterns are replicated in other types of ovarian cancers, particularly in the prevalent serous phenotype, this glycan-modified cancer marker may be a useful diagnostic indicator in the future.

Fucosylation affects OEC physiology in additional ways. For instance, the difucosylated oligosaccharide, Le^Y (CD174), is often highly expressed on mucins 1 and 16 (Yin et al, 1996). Mucins are large glycoproteins that are widely expressed in a number of carcinomas, including OEC. Their ability to contribute to disease pathogenesis by a variety of mechanisms is well-documented (Bafna et al, 2010; Thériault et al, 2011). Increased Le^y antigens have been correlated with a number of tumorigenic effects, such as enhanced binding to mesothelial CD44, stimulation of $\alpha_5\beta_1$ signaling, increased expression of MMP-2/9, and down-regulation of inhibitory TIMP-1/2 (Gao et al, 2011; Li et al, 2010b; Yan et al, 2010). A positive effect on growth factor activation has additionally been observed, as Le^Y participates in EGFR signaling and aids in the secretion of the angiogenic factors VEGF and basic FGF (Basu et al, 1987; Li et al, 2010b).

Le^Y displays specificity for epithelium-derived cancers, and is present in 70-90% of malignancies with this provenance (Chhieng et al, 2003). This Lewis antigen is most frequently active during embryonic development, and its expression in adults is limited solely to epithelial cells and granulocytes (Li et al, 2010b). Specificity is somewhat diminished by its appearance in certain non-malignant conditions, such as in ovarian cysts (Yang et al, 2009). The Le^Y antigen is not expressed in normal OSE, however, and is expressed in only 25% of benign tumors compared to 81% of malignant and 52% of borderline tumors (Wang et al, 2011). Based on these data, Le^Y is a promising potential biomarker for OEC and its value in the recognition of specific cancer stages awaits further studies.

2.2 Sulfation

Enhanced adhesion of heparin-binding epidermal growth factor–like growth factor (HB-EGF) to heparan sulfate proteoglycans (HSPGs), and subsequent activation in OEC, is attributed to changes in sulfation patterns of these cell surface molecules (Shipp and Hsieh-Wilson, 2007). As HSPG sulfation increases, potential for interaction with HB-EGF also rises (Lai et al, 2003). Increased sulfation of glycosaminoglycan chains on HSPGs is a common feature in many epithelial cancers, including breast, kidney, hepatocellular, and ovarian cancers (Bret et al, 2011; Lai et al., 2003). A major mechanism by which this is achieved is through the down-regulation of HSulf-1. This enzyme is an arylsulfatase that degrades heparin preferentially at the C-6 position of glucosamine within specific subregions of the heparin molecule (Morimoto-Tomita et al, 2002). It is expressed ubiquitously in nonlymphoid tissue but significantly reduced in many epithelial cancers, including ovarian cancer.

Loss of HSulf-1 leads to increased sulfation of cell surface HSPGs and an expansion in the number of binding sites with HB-EGF. HB-EGF promotes transcoelomic metastasis in ovarian cancer through its involvement in epithelial-mesenchymal transition (EMT) (Yagi et al., 2008). Re-expression of the enzyme *in vitro* has been shown to diminish downstream signaling of HB-EGF, as demonstrated by reduction of ERK activity and abrogation of EGFR phosphorylation (Lai et al, 2003). Loss of HSulf-1 and the resultant hypersulfated state also modulates angiogenesis via binding of a VEGF isoform through its heparin-binding domain (Narita et al, 2006). Evidence suggests HSulf-1 down-regulation is an early event in tumorigenesis, as total inactivation of this enzyme was observed in fibrocystic breast cells with a normal phenotype while 80% of stage I/II ovarian cancer tumors exhibited barely detectable mRNA levels (Lai et al, 2003). Interestingly, the same study reported that >75% of ovarian tumors lacked HSulf-1 expression (Lai et al., 2003). Taken together, these observations suggest that loss of HSulf-1 could serve as an early biomarker for upcoming EMT events.

Down-regulation of HSulf-1 is a finding consistent with many epithelium-derived malignancies, and thus might not be highly effective as a stand-alone diagnostic marker for ovarian cancer. Its presence in serum may prove to be indicative of a cancerous state when concomitantly evaluated alongside additional markers. Further assessment of effects on specific HSPG substrates, such as the highly expressed proteoglycan, syndecan-1 (SDC1), may raise the value of HSulf-1 in OEC tumor diagnosis. The combination of HSulf-1 inhibition and SDC1 expression may be more specific for OEC than abrogation of HSulf-1 alone. SDC1 is a type of HSPG that is not expressed in normal OSE but quotidian to ovarian tumor tissue (Davies et al, 2004). In contrast, other HSPGs studied are found to be ubiquitously expressed in normal and diseased ovaries (Davies et al, 2004). Furthermore, the presence of SDC1 in serum as circulating CD138 antigen makes it relatively simple to detect in a noninvasive manner.

2.3 Sialylation

Over 50 types of neuraminic acid-derived monosaccharides have been described and are collectively referred to as sialic acids (Varki and Schauer, 2009). Sialic acids have been found to exhibit numerous cellular functions, examples of which include the stabilization of molecules and cell membranes, the enhancement of mucin viscosity, the protection of

molecules and cells from degradation and the modulation of cellular interactions with the microenvironment (Varki and Schauer, 2009). The contribution of sialylation per se to increased tumorigenesis rests in its ability to allow permissive regulation of cellular interactions. The strong negative charge resulting from the low acid-base dissociation constant of sialic acids produces a charge repulsion effect. This, in addition to prominent hydration and conformational instability give heavily sialylated glycans a slippery effect to substantially reduce cell-cell interactions (Dall'Olio and Chiricolo, 2001; Schauer et al, 2011). As a result, adhesion and differentiation effects are not favored, and metastatic characteristics of migration and invasion become exacerbated when sialylation becomes enhanced by constituents of the microenvironment. The presence of sialic acids can also mask antigenic sites and, in this regard, thwart the activity of immune cells (Schauer, 2000). Finally, through their ability to avoid recognition by immune cells, highly sialylated cancer cells can efficiently evade tumor surveillance mechanisms, further promoting the malignant phenotype (Schauer et al, 2011).

The most abundant sialic acid in human cells is Neu5Ac, in which the C-5 is substituted with an N-acetyl group. Other mammals mainly produce Neu5Gc, in which the substituted N-acetyl group is hydroxylated to form an N-glycol substituent. This latter modification of neuraminic acid can be acquired by humans through diet and, following absorption, can generate an antigenic inflammatory response (Hedlund et al, 2008; Tangvoranuntakul et al., 2003). While tumor tissue and serum samples have been found to contain secreted Neu5Gc (Higashi et al, 1984; Siskos and Spyridaki, 1999), the potential association between Neu5Gc intake from the diet and ovarian cancer risk requires further study. Evidence supporting a major role for Neu5Gc in OEC was discovered in the ovarian cancer cell line JHOC-5, where secreted sialoglycoproteins, and especially mucin-like proteins, exhibited up to 40% representation of total sialic acid content by this exogenous carbohydrate moiety (Inoue et al, 2010). These data suggest a possible role for Neu5Gc as a predictive biomarker for ovarian cancer.

Under usual circumstances, sialic acid is attached to substrates such as glycosphingolipids and N- or O-linked glycans as single molecules or short oligomers. The attachment of longer chains of sialic acids, known as polysialic acids, to substrates is less common. Sialic acids are normally removed from substrates through the activity of another class of enzymes known as the sialidases. The neuronal cell adhesion molecule (NCAM, CD56) is, however, a noteworthy exception which is modified post-translationally via polysialylation, particularly during embryonic development and then downregulated shortly thereafter (Rutishauser and Landmesser, 1996). The reappearance of polysialylated NCAM has been observed in some forms of cancer, such as malignant neuroblastomas and rhabdomyosarcomas (Daniel et al., 2001; Fukuda, 1996; Gluer et al., 1998a; Gluer et al, 1998b; Jensen and Berthold, 2007) and correlates with increased metastatic potential and poor clinical outcome (Seidenfaden et al., 2003). Recently, NCAM expression has been studied in serous ovarian tumors and found to correlate with greater peritoneal dissemination and larger tumor volume following surgical debulking (Zueva et al, 2010). Sialylation status of NCAM in ovarian cancer is yet to be deciphered.

Glycan branching in cancers inhibits molecular clustering and cell adhesion while increasing the number of available sialylation sites and facilitating migratory potential (André et al, 2009), so it is not surprising that ovarian cancer is associated with increased α2,6 branched sialyl expression and decreased α2,3 linear sialylation (Wang et al, 2005). A major

sialyltransferase responsible for branched sialylation of glycans is ST6Gal-I, which is abundantly expressed in OEC (Christie et al, 2008; Wang et al, 2005). Elevated ST3Gal-I and reduced levels of ST3Gal-III, ST3Gal-IV, and ST3Gal-VI have also been observed (Wang et al, 2005). A major function of ST6Gal-I in ovarian cancer is the sialylation of β_1 integrins (Wang et al, 2005). Sialylation enhances integrin-mediated signaling in cancers, leading to increased migration and invasiveness in the extracellular matrix (ECM) (Chiang et al, 2010). ST6Gal-I responds to a variety of genetic, inflammatory, and hormonal signals. Some triggers of ST6Gal-I overexpression that may be relevant to OEC are high IL-6 activity, Ras signaling (from either mutations or overexpression), and ER-α mutations (Hanasaki et al, 1994; Lau et al, 1999; Seales et al, 2003). The presence of serum cancer-specific markers synthesized by ST6Gal-I may adumbrate tumorigenic events if detected sufficiently early. Due to the documented high ST6Gal-I activity in OEC, it would be expected that β_1 integrins are hypersialylated. Determining alterations in sialylation patterns compared to controls may be useful in the quest for biomarker identification as these abundantly expressed integrins so crucial to early epithelial-to-mesenchymal transition (EMT) events are detectable in serum (Liu et al, 2005).

The presence of only one glycosylation site makes a candidate marker more amenable to testing than glycoconjugates with more convoluted patterns due to ease of identification with less confounding variables. Cancer-specific aberrations in the glycosylation signature of a macromolecule with a lone glycan moiety would improve sensitivity and specificity of a candidate biomarker. A tumor marker that has garnered much attention in ovarian cancer diagnosis is kallikrein-like peptidase-6 (KLK6) (Bast et al, 2005; El Sherbini et al, 2011; White et al, 2009). This protein is a trypsin-like serine protease consisting of a single N-glycosylation site. When juxtaposed against the same protein derived from a non-malignant site, only KLK6 taken from ovarian cancer ascitic fluid displayed α2,6 branched sialylation (Kuzmanov et al, 2009). KLK6 is also a serum marker and these results may translate to this less invasive approach. Recognition of this specific isoform can only improve the status of KLK6 as a marker for ovarian cancer. KLK6 is up-regulated in most ovarian cancer tumors (Shan et al, 2007). Sensitivity of the marker for early detection does not exceed that of CA125 (El Sherbini et al, 2011), although the combination was shown to improve sensitivity by 10-30% (Diamandis et al, 2003). Screening for the robust sialylated isoform of KLK6 in OEC tumors may possibly improve accuracy of detection.

There are several other abnormally sialylated molecules that may serve as molecular markers for ovarian cancer. Sialylated Lewis x (sLe^x) is a terminal glycan epitope that is positioned on the surface of cells attached to glycoconjugates and is preferentially recognized by endothelial selectins to promote cell migration. The sLe^x epitope of the Lewis blood group is composed of Neu5Ac in an α2,3 linkage to a galactose sugar. Following sialylation of Le^x, fucosylation occurs via the action of α(1,3/1,4) fucosyltransferases (Aubert et al, 2000). SLe^x has been identified in ovarian cancer on the surface of the acute phase proteins α_1-acid glycoprotein (AGP), α_1-antichymotrypsin, and haptoglobin (Hp) β-chain (Saldova et al, 2007) (See Table 1). The acute phase response is initiated during times of trauma, inflammation, and infection, and provides an environment to keep cells alive during these crisis situations. The combination of sialylation and fucosylation on acute phase proteins has been shown to prolong half-life and reduce apoptosis (Saldova et al, 2008). Sialylation is sometimes combined with sulfation as well. Ovarian cancers of mucinous, papillary serous, and clear cell subtypes often present with increased levels of N-acetylglucosamine 6-O-sulfotransferase 2 (GlcNAc6ST-2), which catalyzes formation of a 6-sulfo-sLe^x group on L-

selectin ligands (Kanoh et al, 2006). 6-sulfo-sLe^x (CD15su) is readily detectable in serum and thus may be conducive to analysis as a potential ovarian cancer biomarker.

Glycoprotein	Type of Modification	Modified Group
AGP	sialylation	sLe(x)
Hp β-chain	sialylation	sLe(x)
α_1-antichymotrypsin	sialylation	sLe(x)
CA15-3 (Muc1)	sialylation oligosaccharide replacement	sTn Tn
CA15-3	asialylation oligosaccharide replacement	 TF
CA15-3	fucosylation	Le(y)
CA125	fucosylation	Le(y)
THBS1	fucosylation	core fucose
CD138 (SDC1)	hypersulfation	GAG chain
β_1 integrin	sialylation	α2,6 sialic acid
L-selectin ligands	sialylation sulfation	6-sulfo-sLe(x)
POSTN	β1,4 branching	bisecting GlcNAc
KLK6	sialylation	α2,6 sialic acid

Table 1. Carbohydrate Modifications as Potential Biomarkers for Ovarian Cancer.

2.4 Altered glycosylation of epithelial mucins

O-glycans which are covalently α-linked via an N-acetylgalactosamine (GalNAc) moiety to the -OH of serine or threonine by an O-glycosidic bond are designated mucin O-glycans or, for short, mucins (Brockhausen et al., 2009). It is common to find the GalNAc further extended with galactose, N-acetylglucosamine, fucose, or sialic acid; alterations which give rise to different core structures (Brockhausen et al., 2009). These core mucin structures can be modified further with carbohydrate substituents, and can also be branched (Brockhausen et al., 2009). Due to the nature and complexity of their respective structures, mucins tend to be high molecular weight glycoproteins that are heterogeneous and heavily glycosylated. Mucins are synthesized by epithelial cells in various tissues, including the genitourinary epithelium. Mucin-1 (MUC-1) was the first mucin gene to have been identified and, to date, there are about 19 others known to exist (Brockhausen et al., 2009; Spurr-Michaud et al., 2007).

Two general categories of mucins include those which are secreted, to protect epithelial surfaces against damage and infection by pathogens, and those which span the plasma membrane and are involved in cell adhesion (van Klinken et al., 1995; Fukuda, 2002) or cell signaling (Hartel-Schenk et al., 2001). Transmembrane mucins are positioned for mediation of communication between the extracellular milieu and the interior of cells. It has recently

been proposed that the mucin covering of epithelia may be compromised when exposed to certain triggers, such as during processes involving elevated stress or remodeling (Kufe, 2009; Zhao et al, 2009), providing greater invasive potential. In this manner, a chronic inflammatory condition can theoretically turn the effects of transmembrane mucins against the cells they normally protect. Evidence supporting this assertion can be observed in a number of adenocarcinomas, where specific transmembrane mucins are often overexpressed (Jonckheere and Van Seuningen, 2010). The usual protective effects of mucins in epithelial cells with normal physiologic adhesion patterns become reversed in cancers by a perturbed glycosylation signature.

2.5 The role of hypoglycosylated mucins in cancer

The serum test for MUC1, also known as carcinoma antigen 15-3 (CA 15-3), has been validated for breast cancer diagnosis. High levels of the splice variant muc-1C have been associated with enhanced growth receptor signaling and activation of NFκβ in breast carcinoma (Ahmad et al, 2009). MUC1 is a potential indicator for OEC as well, as its expression soared from 5% to 90% in a comparison between paraffin-embedded sections of tissue from normal ovarian epithelia and cancerous lesions (Wang et al, 2007). In contrast, muc-16 (CA125), the only antigen FDA approved for diagnosis of ovarian cancer, is expressed in 80% of OEC tissue (Bast et al, 1981). MUC1 is detectable in ascitic fluid and serum in addition to tissue (Tuzun et al, 2009). In a study evaluating 49 biomarkers for ovarian cancer, MUC1 was ranked among the top five best candidates in terms of specificity and sensitivity (Cramer et al, 2011). It has been assessed as an early biomarker for stage I ovarian cancer and has demonstrated improved accuracy in tumor diagnosis as part of a four-marker composite test (Zhang et al, 2007).

A prime feature of mucins is the presence of 10-81 amino acid-comprised tandem repeats of proline-threonine-serine (PTS) in which O-glycosylation occurs at a high rate (Fontenot et al, 1993). In ovarian cancer, and possibly other cancers derived from a glandular origin, there is aberrant hypoglycosylation of mucins evinced by high levels of splice variants lacking the tandem repeat sites. This loss of structural integrity of these towering glycoproteins leading to exposure of the protein core is likely a reason for compromised protection. Smaller hypoglycosylated variants appear to provide better access to epithelium for a number of diverse molecules that would otherwise be thwarted from engaging in cell surface interactions (Zhao et al, 2009). MUC1 additionally promotes EGFR activation by inhibiting its degradation (Pochampalli et al, 2007). High expression of MUC1/Y, MUC1/Z, and, to a lesser extent, MUC1/X, have been demonstrated in ovarian cancer (Obermair et al, 2002). These three variants all lack the signature tandem repeat domain. Elevation of the former two variants has also been shown in prostate cancer, another glandular carcinoma (Schut et al, 2003). Aberrantly glycosylated muc-1 variants identified by glycoforms exhibiting short Tn/sTn oligosaccharides in place of the complex O-glycans that form on PTS repeat sites showed a strong correlation with all forms of ovarian cancer, being exhibited in 84% and 85% of primary tumors and metastatic lesions, respectively (Van Elssen et al, 2010).

The Thomsen-Friedenreich (TF) antigen is an additional short oligosaccharide presenting on a large number of hypoglycosylated epithelial cells. Sialylated TF is commonly found in hematopoietic and somatic cells, but the oligosaccharide is rarely observed in normal cells lacking sialyl groups (Schauer et al., 2011). In its desialylated form, this antigen is thought to be involved in triggering metastasis by stimulating interaction with galactoside-binding galectin-3 and exposing endothelial binding sites to cancer cells (Zhao et al, 2009).

2.6 Mucin-16 as a biomarker: Strengths and weaknesses

Mucin-16 (muc-16, CA125) is another type of transmembrane mucin that mediates adhesive interactions in ovarian cancer. Adhesion of ovarian cancer cells to the peritoneum is in part facilitated by the binding of cleaved cell surface MUC16 to mesothelin (Rump et al, 2004). Muc-16 additionally dampens immune response by binding to the inhibitory siglec-9 receptor on a wide range of cells involved in both innate and adaptive immunity, allowing growing tumors to evade immune system surveillance (Belisle et al, 2010).

The CA125 assay displays a superior sensitivity of 95% in tumors positive for the cell surface antigen in human serum (Cramer et al, 2011). Muc-16 becomes increasingly elevated with progression of OEC, and is expressed in approximately 80% of patients. Stage I tumors have much lower concentrations of muc-16, expressing the mucin at only a 58% rate (Jacobs and Bast, 1989). In addition, sonography is inefficient for detecting tumors that have not yet developed into a large mass. The CA125 assay has very low specificity, as this mucin is often expressed in additional cancers or inflammatory diseases. As a result, the current diagnosis strategy is highly inadequate for early tumor detection.

Despite inefficiency in early detection, the mainstay of ovarian cancer diagnosis continues to be muc-16 detection combined with ultrasonography. Recently, this has been challenged as a prospective study monitoring over 78,000 women showed that mortality was not decreased in women annually screened via this combination (Buys et al, 2011). In addition, surgical follow-up for false positive readings occurred unnecessarily in 1080 women, with 15% experiencing one or more serious complications (Buys et al, 2011).

Because CA125 alone is insufficient for early tumor detection, the focus of much research is the improvement of assay sensitivity by combining this mucin marker with one or more additional indicators. This practice has not yet led to the validation of a composite assay for ovarian cancer diagnosis, mainly because of the tradeoff in specificity encountered when increasing sensitivity via use of multiple agents (Florkowski, 2008). Ideally, a powerful diagnostic assay should consist of the minimum number of test agents possible for this reason. Combining CA125 with a marker that is not only highly expressed in OEC but is replete with a unique glycosylation signature specific for the disease is one viable option for optimizing sensitivity and specificity. The combination of CA125 with other protein markers occasionally yields productive data as well. Improved sensitivity in detecting early ovarian cancer has been observed when CA125 measurement was combined with mesothelin detection (McIntosh et al, 2004). In addition, combination of CA125 with the T-cell expressed B7-H4 protein was demonstrated to improve early detection by 13% over CA125 alone (Simon et al, 2006). Although current guidelines recommend CA125 measurement as the sole biomarker criterion for ovarian cancer diagnosis, it is likely that more powerful assays will develop from its use in combination with one or more highly specific agents. Novel discoveries from the increased use of proteomic and glycomic approaches will assuredly allow for the search for quality biomarkers to continue unabated.

3. Epigenetic modifications as tumor markers

Epigenetics is a branch of science which has for its purpose the study of heritable changes in gene function that do not occur as the result of changes in DNA sequence (Wu and Morris, 2001). In addition, chromatin architecture is affected by epigenetic mechanisms (Zaina et al., 2010). An “epigenetic pathway” involving three components has recently been proposed. In this pathway, a signal is received from the external environment, after which an epigenetic initiator determines the precise chromatin location to be affected, called the mark, and an

epigenetic maintainer works to sustain the changed chromatin environment (Berger et al., 2009). Whereas epigenetic initiators include DNA binding factors and non-coding RNAs, epigenetic maintainers include modifiers of histone proteins and histone variants and DNA modifiers, such as DNA methyltransferases (DNMTs) (Berger et al., 2009). The role of RNAs in epigenetic initiation, particularly with respect to marking targeted regions and silencing them via RNA-associated silencing, is also an area of intense study (Malecova and Morris, 2010; Zhou et al., 2010).

There are many examples of epigenetic deregulation in ovarian cancer, which include alterations in patterns of DNA methylation (Makarla et al, 2005; Rathi et al, 2002), histone modifications (Caslini et al, 2006), and microRNA (miRNA) expression (Li et al, 2010c; Wyman et al, 2009). Changes in histone modifications currently have little diagnostic value, due to low sensitivity and the need for obtaining tissue samples. Detection of hyper- and hypomethylation patterns of DNA proffers several advantages in the quest for quality biomarkers for early OEC diagnosis. Testing would be minimally invasive since DNA is easily accessible from the bloodstream and peritoneal fluid that is not quantitatively different from DNA in cells directly extracted from tumors (Asadollahi et al, 2010; Maradeo and Cairns, 2011). The regions of the genomes of cells in serum analyzed are often confined to specific locations, such as the CpG islands of promoter regions of specific genes. Once isolated, the DNA can then be amplified readily using methylation-specific PCR, ensuring high sensitivity (Cairns, 2007). Other advantages include stability of the portent indicators, via their resistance to degradation, and cost-effectiveness. A major limitation, however, is that different phenotypes lead to disparate methylation profiles because of the heterogeneous presentation of ovarian cancer. There is hope that, in time, selection of a combination of aberrantly methylated genes may serve as a composite marker specific for a general OEC phenotype, with certain markers serving as red flags for aggressive forms of cancer. As promoter methylation is a frequent early event in cancers, the ability to detect and analyze patterns consistent with malignancy in ovarian tumors may provide an opportunity for more accurate early detection.

3.1 Altered DNA methylation profiles

Cells from invasive tumors have widespread hypomethylation of repetitive elements with frequent hypermethylation of CpG dinucleotide-containing promoter regions of genes with tumor suppressive function (Balch et al, 2009). DNA methylation patterns reflect the stage and degree of tumor progression in ovarian cancer (Shih et al, 2010; Yang et al, 2006). Invasive tumors display a much larger set of genes whose methylation patterns are affected, with mean methylation index increasing threefold or higher compared with low malignancy tumors (Makarla et al, 2005). These differences reflect the category of tumor; whether the disease results from an accruement of gradual changes (low grade) or a sudden and more invasive phenotype from widespread chromosomal instability (CIN) (high grade). The latter is more prevalent, occurring in the majority of cases, including approximately 75% of serous carcinoma cases (Shih and Kurman, 2004). These tumors have recently been classified as CpG island methylator phenotype (CIMP) cancers, and are characterized by a rapid inactivation of a large number of genes, often by hypermethylation due to alterations in expression of DNMTs. Inactivation of *TP53* by mutation is a frequent result of CIN and accounted for in 96% of high grade serous ovarian carcinomas (Bell et al, 2011). In addition to the critical effects p53 maintains in cell cycle regulation, its inhibition is thought to play a role in the large scale hypermethylation of tumor suppressor genes, as its abrogation is

associated with activation of DNMT1 via PI3K/Akt signaling (Cheng et al, 2011). Upregulated expression of DNMTs occurs frequently in ovarian cancer (Ahluwalia et al, 2001). Complete inactivation of *TP53* by mutation is the most common mutational event in aggressive high grade OEC (Singer et al, 2005, Bell et al, 2011). In contrast, low-grade OEC is characterized instead by mutations in *KRAS/BRAF/ERBB2*. It is not associated with the sudden, highly invasive phenotype observed in high-grade disease but rather via a slow, indolent progression (Singer et al, 2005). Methylation patterns in low-grade tumors are closer to those of the benign cystadenomas that may develop into them, although more pronounced, reflecting their gradual progression (Shih et al, 2010).

The inhibitory effects of p53 on the cell adhesion protein, E-cadherin, are multifaceted. E-cadherin can either be transcriptionally repressed by the absence of p53 through Twist activation (Yang et al, 2004), or be silenced by promoter methylation by DNMT1 (Cheng et al, 2011). Benign adenomas exhibit promoter hypermethylation at a 13% rate. The percentage is increased to 17% in low malignancy tumors and 26% in invasive tumors, showing an increase with increasing malignancy potential (Makarla et al, 2005). The steady increase from benign to low-malignancy-potential adenomas appear to reflect the step-by-step progression observed in low grade ovarian tumors unrelated to *TP53* mutation, while widespread CIN coupled with *TP53* inactivation are believed to account for the higher percentage of methylation in high grade tumors. Because loss of E-cadherin is essential in precipitating EMT in certain subsets of OEC (Patel et al, 2003) , it may be speculated that p53 down-regulation as a result of CIN caused by aneuploidy from extensive remodeling of the ECM may have major effects on hypermethylation of tumor suppressor promoters from a fairly early stage (Cheng et al, 2011). In contrast, despite the hereditary involvement of BRCA1/2, aneuploidy and CIN are involved in all cases of serous OEC studied, regardless of BRCA status (Pradhan et al, 2010). In sporadic but not hereditary OEC, *BRCA1* is highly methylated (Bol et al, 2010). Therefore, inactivation of the *BRCA1* gene through either mutation, loss of heterozygosity or promoter hypermethylation may be implicated in maintaining the tumor promoting environment, while *TP53* inactivation may affect gene expression in a more direct manner through its effect on DNMT1 as well as its other effects on cell cycle regulation and DNA damage repair.

While thousands of genes may have their methylation patterns altered, several common genes repressed by promoter methylation in ovarian cancer that may be useful as part of a methylation biomarker panel are listed in Table 2. These include a number of genes involved in tumor suppression, apoptosis, and cell adhesion. Although these genes are frequently silenced by epigenetic dysregulation, a number of them can also be inactivated through other mechanisms, such as loss of heterozygosity, imprinting, mutation, or transcriptional downregulation. Most hypermethylated genes observed in ovarian tumor tissue are detectable in blood via methylation-specific PCR analysis, and various combinations can be tested for utility as composite serum markers for diagnostic screening with high sensitivity (Melnikov et al, 2009).

Global hypomethylation of genes and repetitive elements is also a frequent finding in OEC, with extent correlating with increasing invasiveness (Shih et al, 2010). Repetitive elements have lost function over the course of evolution, so sudden loss of methylation on DNA components silenced for thousands or millions of years may be a critical factor in the disruption of chromosomal integrity observed in invasive carcinomas (Eden et al, 2003). Hypomethylation of LINE1 transposons and Sat2/Satα repeats commonly occurs in ovarian cancer (Widschwendter et al, 2004). LINE1 elements contain many splice sites that, when

activated, could cause hybrid splicing events with closely positioned genes to alter their translational products in cancer (Belancio et al, 2006). Satellite repeats in heterochromatic regions of chromosome 1 have been observed to lose methylation status in proportion to

Gene	Functions	UniProt/Swiss-Prot ID
APC	Cell adhesion, Wnt inhibition, pro-apoptotic	P25054
ARHI	P21/p27 induction, STAT3 inhibition, pro-autophagic	O95661
BRCA1	DNA damage response, transcription regulation	P38398
DAPK	Pro-apoptotic	P53355
E-cadherin	Contact inhibition, p27 expression	P12830
GSTP1	Xenobiotic detoxification	P09211
H-cadherin	Contact inhibition, p21 expression	P55290
HIC1	Cooperative role with p53 via SIRT1 inhibition	Q14526
HSulf1	Inhibition of GF binding to HSPGs	Q8IWU6
ICAM1	Cell/matrix adhesion in immune/endothelial cells	P05362
IGFBP3	Inhibition of proliferation and invasion, pro-apoptotic	P17936
MCJ	MDR transporter inhibition	Q9Y5T4
MGMT	O6-MeG removal from damaged DNA	P16455
MLH1	DNA mismatch repair	P40692
OPCML	Cell adhesion, Ras inhibition	Q14982
PALB2	Colocalization with BRCA2 in damage response	Q86YC2
PAX5	B-cell differentiation	Q02548
PEG3	Re-localization of pro-apoptotic agents to favor apoptosis	Q9GZU2
PGR	Progesterone binding and signaling	P06401
PLAGL1	Inhibition of proliferation, pro-apoptotic	Q9UM63
PTEN	Inhibition of PI3K/Akt signaling, cell polarity establishment	P60484
P16	Cell cycle regulator, maintenance of senescence	Q8N726
RASSF1A	Ras inhibition, cyclin D1 inhibition, microtubule stabilization	Q9NS23
SPARC	LPA inhibition, prevention of GF-receptor binding, repression of VEGF-integrin-MMP axis	P09486
TCEAL7	NFκβ inhibition, apoptotic regulation	Q9BRU2
THBS1	Cell/matrix adhesion, platelet aggregation	P07996
14-3-3 sigma	Stabilization of p53	P31947

Table 2. Common Genes Repressed by Promoter Methylation in Ovarian Cancer.

tumor grade, and may help to differentiate between ovarian cancers of varying malignant potential as a result (Qu et al, 1999). Several oncogene promoters are hypomethylated as well in OEC, including synuclein-γ(*SNCG*), claudin-4 (*CLDN4*) and insulin-like growth factor-2 (*IGF2*), further contributing to the tumorigenic phenotype (Balch et al, 2009). These compounds have all been investigated as ovarian cancer biomarkers (Hibbs et al, 2004; Palmer et al, 2008), so identification of those genes displaying diminished methylation status may enhance their specificity for the disease.

3.2 miRNAs in ovarian cancer

miRNA signatures are 22-23 nucleotides in length once processed from precursor transcripts, and are being actively pursued as composite diagnostic markers for OEC. They can be analyzed in body fluids and show greater stability than mRNAs due to their greater resistance to RNase (Mitchell et al, 2008). Several miRNAs have been shown to be up-regulated in repeated experiments, and many have oncogenic potential by either inhibiting translation of tumor suppressors when up-regulated or facilitating unimpeded expression of oncogenes when down-regulated (Calin and Croce, 2006).

Common miRNAs frequently overexpressed in ovarian cancer include miR-93, miR-106b, miR-155, miR-200a/b/c, miR-221/222, and miR-372/373; underexpressed miRNAs include miR-15/16, miR-34b*/c, miR-125b1, miR-140, miR-145, and let-7i (Balch et al, 2009; Maradeo and Cairns, 2011). Increased neovascularization has been associated with high expression of miR-93, which may serve as an early indicator of tumor growth and angiogenesis (Fang et al, 2011). Other miRNAs, such as miR-106b and miR-221, target cell cycle inhibitors p21 and p27, respectively (le Sage et al, 2007; Li et al, 2011). Down-regulation of miR-34b*/c has been correlated with progression to advanced disease (Corney et al, 2010).

Some miRNAs may be up- or downregulated in the same tumor based on differentiation status of cells constituting the mass. Under the regulatory command of Twist, decreased miR-214 and miR-199a were observed in CD44+ OEC cells that were greatly dedifferentiated, while their normally differentiated CD44- counterparts exhibited higher concentrations of these non-coding RNAs (Yin et al, 2010). Low expression levels of these miRNAs, which silence PTEN and IKKβ/NF-κβ pathways, respectively, may have prognostic value, as the CD44+ cells studied displayed stem-like qualities and constitutively active inflammatory signaling (Chen et al, 2007). Additional clues for OEC characterization and prognosis will be provided as more miRNA markers are revealed and their functions elucidated. Along with evaluation of methylation signatures, miRNA signature analysis offers a promising non-invasive technique in the diagnosis and characterization of ovarian cancer adjuvant to traditional methods.

To recapitulate, epigenetic markers are gaining favor as diagnostic biomarkers for ovarian cancer because of their expression early in disease pathogenesis and the fact that most are amenable to the use of serum as a source. The types of methylation profiles vary based on malignancy potential and tumor source, so a panel of commonly expressed methylation markers could essentially help to differentiate between the multitudes of forms characterized by this heterogeneous cancer. Although it is far from an exhaustive list, Table 2 lists some of the more frequently hypermethylated genes frequently observed in ovarian cancer after over a decade of detailed analysis. Concomitant ongoing studies on miRNA profiles in ovarian cancer provide an alternate epigenetic approach for early detection. As patterns of epigenetic alterations are better clarified, panels consisting of the most sensitive

and specific of these markers identified will likely be developed for further testing and possible validation.

4. HE4 as a potential early marker

A promising protein marker receiving much attention for its potential role in the early diagnosis of ovarian cancer is human epididymis secretory protein 4 (HE4). This protein is a member of the whey acidic four-disulfide core (WFDC) family, which includes secretory leukocyte protease inhibitor (SLPI) and elafin. Its function has not yet been elucidated, although it does not appear to exhibit protease inhibitor activity like most other members of the WFDC family. HE4 was first identified in human epididymis epithelium (Kirchhoff et al, 1991). Since its discovery, HE4 has been found in some other tissues as well, including the respiratory tract and nasopharynx. It is a frequently expressed selective early marker for this disease. While normal OSE does not express HE4, the protein can be detected in sera of patients diagnosed with the most prevalent forms of OEC, and is detectable even in inclusion cysts that may precede tumor formation (Drapkin et al, 2005).

Finding a protein biomarker to rival CA125 in sensitivity and specificity has posed a major challenge. Despite the failure of CA125 to accurately predict early disease, this marker has alone displayed the greatest overall diagnostic ability in repeated studies (Canney et al, 1984; Cramer et al, 2011; Medeiros et al, 2009). However, detection of HE4 holds some advantages over CA125, and its use in combination with the mucin marker is currently being evaluated. Overall specificity for HE4 is comparable to CA125 with greater discriminatory ability for the detection of early disease in patients with a pelvic mass (Hellstrom and Hellstrom, 2011; Montagnana et al, 2009; Nolen et al, 2010). Detection of HE4 has displayed a better ability to differentiate between benign and malignant disease, as the sensitivity was 56.7% for HE4 compared to 10.8% with CA125 at high specificity (Hellstrom et al, 2003). Receiver operator characteristic (ROC) curves, which plot changes in sensitivity in relation to specificity, were used to ascertain information on the usefulness of both markers in a head-to-head comparison. The AUC values of ROC curves generated for both HE4 and CA125 showed comparable rates for early detection, with HE4 exhibiting slightly higher values. Comparison of ROC curves for all cases yielded superior detection rates for CA125. Similar results from ROC-AUC analyses were reproduced elsewhere (Anastasi et al, 2010; Montagnana et al, 2009). However, ROC curves are not used as diagnostic criteria for ovarian cancer detection. The major benefit of serum HE4 testing observed in the study by Hellstrom et al (2003) was that there were significantly less false positive readings than with CA125.

In a retrospective study comparing CA125 and two different HE4 assays, the HE4 assays showed better sensitivity (Ruggeri et al, 2011). At 95% specificity, sensitivity was 83.3% and 84.4% for HE4 compared to 76% for CA125, and as the specificity increased to 99%, the difference increased further, with a 79.2% sensitivity for both HE4 assays and a 59.4% sensitivity for the CA125 assay.

An additional benefit of HE4 lies in its ability to be quantified in not only serum and ascitic fluid but urine as well. Specificity and sensitivity rates for urine samples were demonstrated to be comparable to serum concentrations, displaying results of 94.4% and 86.6%, respectively, for stage I/II cancers (Hellstrom et al, 2010). These data allow for the possibility of a noninvasive urine test adjuvant to other diagnostic criteria for ovarian cancer if this can be reproduced in larger studies. Measurement of serum HE4 is also effective for

predicting early recurrence of ovarian cancer, as expression of HE4 increases an average of 5-8 months prior to a rise in CA125 in relapsing tumors (Anastasi et al, 2010).

Whereas CA125 is a better biomarker for overall ovarian cancer detection than HE4 based on multiple comparative studies (Cramer et al, 2011; Medeiros et al, 2009; Van Gorp et al, 2011), a composite assay measuring concentrations of both proteins may be ideal for enabling early detection. This could potentially translate into higher survival rates as the differences in mortality between early and late stage ovarian cancer are considerable. For this reason, combinatory testing has been explored in several prospective and retrospective studies (Andersen et al, 2010; Jacob et al, 2011; Shah et al, 2009; Van Gorp et al, 2011). The results thus far have been mixed, with naysayers arguing that the benefit of testing for HE4 in addition to CA125 is not sufficient to warrant clinical use.

In a prospective study of 389 patients with a pelvic mass of ovarian origin, ROC-AUC values showed only a slight advantage for HE4 testing in premenopausal patients compared to CA125 (Van Gorp et al, 2011). The CA125 assay was superior for postmenopausal patients, although a Risk of Ovarian Malignancy Algorithm (ROMA) based on a logarithmic formula of HE4 concentrations with menopausal status did improve detection ability in post-menopausal women. Unlike previous studies, sensitivity and specificity for HE4 were poor. Sensitivity was 74.5% at a specificity of 83.3%. In contrast, a case control study that included a large number of early stage patients demonstrated 77% sensitivity for HE4 detection at 94.9% specificity (Andersen et al, 2010). Overall sensitivity was slightly higher for CA125 (81%), but combining the two markers led to a significant increase in sensitivity without a major tradeoff in specificity. HE4 better detected early disease, and high risk patients were identified at 100% sensitivity compared to only 78.6% for CA125 at 95% specificity (Andersen et al, 2010). Shah and colleagues (2009) showed a benefit of HE4 over CA125 in discriminating between risk-matched healthy controls and cases in high risk groups. At a specificity of 95%, sensitivity in these cases was 87.8% for HE4 versus 82.9% for CA125. A cohort study of 160 subjects with mixed phenotypes (18% OEC) reproduced beneficial results for HE4 in early stage cancer detection, as well as a greater propensity for discriminating between borderline and malignant tumors (Jacob et al, 2011). High cost of HE4 screening caused the authors to caution against using the combination, however, as the overall benefits were minimal. Finally, a four marker panel consisting of HE4 and CA125 along with two additional markers (VCAM-1 and CEA) observed a 86% sensitivity at a high specificity of 98% (Yurkovetsky et al, 2010).

Although the advantages of combination testing with CA125 and HE4 biomarkers have been below expectations, the ability of HE4 to effectively diagnose early disease, identify disease in high risk patients for which screening is essential, and differentiate between borderline and malignant disease have increased its value as a diagnostic indicator. While data from older, post-menopausal women are subpar (Van Gorp et al, 2011), composite testing of CA125 and HE4 may be valuable for certain groups with further investigation, such as premenopausal women at high risk for disease.

5. Inherited mutations as biomarkers for ovarian cancer

There are several hereditary syndromes which increase the likelihood of ovarian cancer in a patient. Examples of such include hereditary breast and ovarian cancer (HBOC), hereditary nonpolyposis colorectal cancer (HNPCC), site-specific ovarian cancer (SSOC), Gorlin's syndrome, and Peutz-Jeghers syndrome (Russo et al., 2009). Of these, HBOC, HNPCC and

SSOC comprise about 99% of hereditary ovarian cancers. However, it is important to note that 10-13% of all ovarian cancer cases can be classified as hereditary and linked to the inherited mutations described below (Pal et al., 2005; Risch, 2001; Stratton JF, 1999; Sowter and Ashworth, 2005). In sporadic cancers, the mutational activation of oncogenes, coupled with non-mutational inactivation of tumor suppressor genes, is often observed (Kenemans et al., 2004). In hereditary cancers, germline mutations in a single allele confer an elevated risk for cancer development (Radice, 2002). Therefore, while genetic screening to identify at risk individuals is highly desirable in patients with a family history of breast, ovarian or colon cancer, the potential biomarkers described below may or may not be applicable for the detection of sporadic ovarian cancers.

5.1 Human MutS homolog 2 (*hMSH2*) and Human MutL homolog 1 (*hMLH1*)

Ovarian carcinomas in patients from HNPCC families typically present as early-onset, non-serous epithelial tumors (Ketabi et al., 2011). *hMSH2* and *hMLH1* are the two most frequently mutated genes in this syndrome and confer a 9-12% lifetime risk of ovarian cancer (Aarnio et al., 1995; Brown et al., 2001; Kasprzak et al., 1999; Russo et al., 2009). The hMSH2 and hMLH1 proteins are the fundamental components of DNA mismatch repair (MMR) (Kolodner et al., 1994) and defects in these genes significantly increase the rate of mutation, which is believed to contribute to cancer development (Loeb, 2011; Valeri et al., 2010). In particular, microsatellite instability (MSI) has been observed in tumors from HNPCC patients (Dietmaier et al., 1997) and stems, at least in part, from a mutation or inherited epigenetic inactivation of hMLH1 (Gazzoli et al., 2002; Goecke et al., 2006; Hitchins et al, 2007; Kane et al., 1997). Interestingly, Valeri and colleagues (2010) reported that a non-coding miRNA designated as miR-155 is significantly overexpressed in human colorectal cancers and that an inverse correlation exists between the expression of miR-155 and the expression of hMLH1 or hMSH2 proteins in these tissues. miR-155 has been detected in blood samples derived from patients with ovarian cancer, though the sensitivity is still too low to be used as a reliable and predictive indicator of disease progression (Hausler et al., 2010). miR-155 has been put forth as a potential biomarker for the detection of early pancreatic neoplasia (Habbe et al., 2009).

Screening for mutations in genes important to MMR, such as *hMSH2* and *hMLH1*, and for epigenetic changes relevant to MMR such as hMLH1 promoter methylation, should prove to be an effective strategy for identifying patients in HNPCC families who may also be at risk for developing ovarian cancer. Moreover, screening for the upregulation of the noncoding RNA miR-155 may also prove to be effective in this regard. Important questions concerning the latter remain to be answered; including whether miR-155 upregulation is involved with sporadic ovarian cancers and if this noncoding RNA can be used as an early diagnostic marker.

5.2 Breast Cancer Susceptibility Genes (*BRCA1* and *BRCA2*)

BRCA1 and BRCA2 are large nuclear proteins which act as tumor suppressors and contribute to genetic stability and DNA damage repair (Arai et al., 2004; Meindl et al., 2011; van der Groep et al., 2011). Whereas numerous biochemical and molecular functions have been described for both proteins (reviewed in Narod & Foulkes, 2004; Venkitaraman, 2002), they have both been implicated in the repair of double-strand breaks (DSBs) by homologous recombination (HR) (Badie et al., 2010; Boulton, 2006; Moynahan et al., 1999; Moynahan et al., 2001; Murphy and Moynahan, 2010; Shrivastav et al., 2008; Venkitaraman, 2003).

Approximately half of high grade serous carcinomas exhibit defects in HR, solidifying the importance of this process in its implications for disease pathology extending beyond the presence of germline mutations (Bell et al., 2011).

The risk of ovarian cancer is about 40% in carriers with BRCA1 mutations (Antoniou et al., 2003; Ford et al., 1994). BRCA1 is composed of 1863 amino acids and possesses a N-terminal RING domain and and two C-terminal BRCT domains, present in tandem, at its C-terminus. The RING domain is protein-protein interaction motif which mediates the binding of BRCA1 to its obligate partner BARD1 (Meza et al., 1999; Wu et al., 1996). The BRCA1:BARD1 complex possesses ubiquitin ligase activity (Starita et al., 2004) while the BRCT domains of BRCA1 serve as sites of numerous protein-protein interactions, regulate transcription, and possess the ability to bind to phosphopeptides (reviewed in Narod and Foulkes, 2004; Starita and Parvin, 2003; Manke et al., 2003). Numerous cancer-associated missense mutations which disrupt interactions with putative binding partners have been described in the RING and BRCT domains of BRCA1 (reviewed in Carvalho et al., 2007; Morris and Solomon, 2004; Szabo et al., 2004).

The risk of ovarian cancer is about 25% in patients with *BRCA2* mutations (Ford et al., 1998). BRCA2 is composed of 3418 amino acids and possesses two distinct classes of BRC repeats which interact with the RAD51 protein, the mammalian homolog of *Escherichia coli* RecA (Carreira and Kowalczykowski, 2011). In addition, the C-terminal region of BRCA2, TR2, interacts with RAD51 (van der Groep et al., 2011). A major mechanism by which RAD51 is recruited to damaged DNA is via its interaction with BRCA2 and, along with the latter, plays a critical role in homologous recombination (Badie et al., 2010; Davies et al., 2001; Jensen et al., 2010). Cancer associated point mutations on BRC repeats which disrupt interaction of BRCA2 with RAD51 have been reported (Venkitaraman, 2009). Based on the observation that BRC repeats bind distinct regions of RAD51 and are not equal in their mode of interaction, it was hypothesized that a mutation within even one of the eight BRC repeats in this region could be sufficient to affect the way that BRCA2 interacts with RAD51, and lead to an increased risk of cancer (Galkin et al., 2005). Interestingly, certain families exhibit *BRCA2* mutations which appear to predispose carriers to ovarian cancer and which are located within exon 11 (Gayther et al., 1997; Lubinski et al., 2004; Petrucelli et al., 2002; Thompson et al., 2001). While this area is generally referred to as the ovarian cancer cluster region, Al-Saffar and Foulkes (2002) proposed that this region of exon 11 be known as the diminished breast cancer risk region.

Ovarian tumors in women carrying mutations in *BRCA1* or *BRCA2* are generally serous carcinomas and tend to be of high grade when diagnosed (Sowter and Ashworth, 2005). High grade serous carcinomas associated with *BRCA* mutations are believed to arise from the distal fallopian tube (Crum, 2009; Piek et al., 2003) and are frequently accompanied by mutations in *TP53* (Ahmed et al., 2010; Milner et al, 1993; reviewed in Hall et al., 2004). A comprehensive model for the development of high grade serous ovarian cancer has been put forth by Bowtell (2010) in which the loss of p53 and BRCA disrupts the HR repair of damaged DNA and, in turn, leads to CIN and carcinogenesis. A link between ovarian inclusion cysts and serous carcinomas has been proposed (Sowter and Ashworth, 2005) and may be explained by a mechanism in which cells from the fimbria travel to inclusion cysts and there become transformed and malignant via endometriosis or a series of mitogenic events and malignant (Crum, 2009). Alternatively, high grade serous carcinomas may be derived from stem-like ovarian cancer cells which have been dysregulated due, at least in part, to *BRCA* inactivation (Foulkes, 2004; Yin et al., 2010). Other hypotheses to explain the tissue specific cancers observed in mutant *BRCA* carriers have also been reviewed elsewhere

(Billack and Monteiro, 2005). It is interesting to note that while epigenetic silencing of *BRCA1* in high grade tumors has been reported (Wilson et al., 1999), somatic mutations in *BRCA1* and *BRCA2* are rare in sporadic breast and ovarian cancers (Futreal et al., 1994; Lancaster et al., 1996).

While DNA testing for *BRCA* mutations is becoming more common, not all women will obtain a clear cut result. One possible outcome of *BRCA* genetic testing is the finding that the patient possesses a *BRCA* variant of uncertain significance for which there is no clinical information regarding its cancer association. Methods have been developed to assess the cancer risk of unclassified *BRCA* variants which involve the use of functional assays (Carvalho et al., 2007; Lee et al., 2010) and structure-based supervised learning computation models (Karchin et al., 2007). One example of how functional assays and computational models can be used to characterize rare *BRCA* alleles was recently described in a collaborative study involving our lab (Carvalho et al., 2009). In that study, a Swedish kindred L1383 revealed a proband with ovarian cancer at age 59 (Figure 1A, arrow). The proband's mother also had ovarian cancer while the proband's grandmother died from rectal cancer. Upon analysis it was found that this patient had a rare variant of *BRCA1* denoted as 5673insC which codes for an insertion of a cytosine at nt5673 in exon 24. The cytosine insertion produces a frameshift in which the last 12 amino acids of the protein are changed to a modified 15-amino acid segment. Functional growth assays utilizing a reporter gene driven by LexA were carried out to examine the effect of this insertion. Yeast transformed with fusion constructs coding for either wildtype (W) or mutated BRCA1 (5673insC) fused to a LexA DNA binding domain revealed that the mutant failed to activate the reporter gene, resulting in a significantly reduced growth compared to yeast expressing the wildtype construct (Figure 1B). Use of computational structural modeling suggested that the insertion could generate a novel 13-residue α-helix that might modify the binding of phosphopeptide to the BRCT binding pocket (Figure 1C, golden helix). Taken together, the functional data and the structure prediction suggest that the insertion leads to an impact on protein function. Despite the wealth of information generated via these functional and computational approaches, clinical validation is difficult to obtain due to the rarity of most uncharacterized *BRCA* variants. Moreover, the complexity of this approach makes high throughput analyses cumbersome. Nevertheless, the more information available for genetic counseling purposes the better.

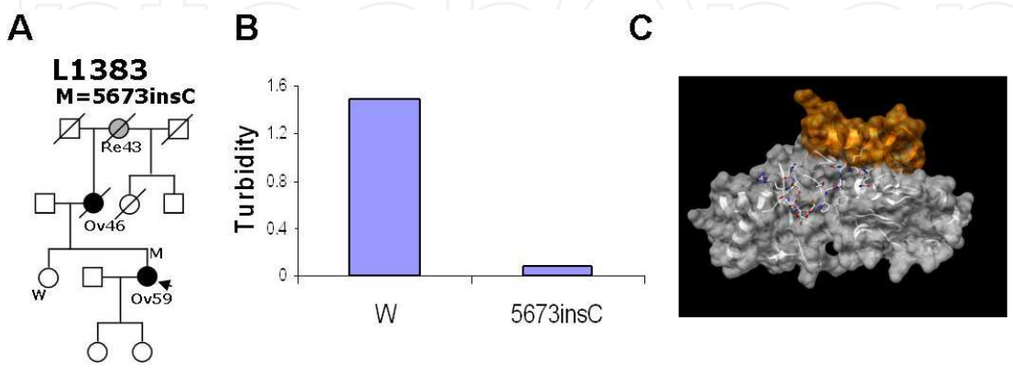

Fig. 1. Use of family history (Panel A), functional analysis (Panel B) and structure-based supervised learning computation models (Panel C) to assess uncharacterized variants of BRCA1. Reprinted from Carvalho et al., 2009, with permission from Elsevier.

There are medical options for a woman with a strong family history of cancer and altered *BRCA* status. In particular, a woman with a highly penetrant cancer-associated *BRCA* mutation who undergoes a prophylactic bilateral salpingo-oophorectomy decreases her risk of ovarian cancer by 80% (Brown and Parker, 2011; Finch et al., 2006). A decreased risk of ovarian cancer has been observed in carriers of *BRCA* mutations who undergo tubal ligation, though it should be noted that this procedure is not as effective as removal of the ovaries (Brown and Parker, 2011). About 2-5% of patients who undergo the prophylactic oophorectomy procedure exhibit an occult cancer of the ovaries upon histological examination (Lu et al., 2000; Schrag et al., 1997). Based on these observations, prophylactic oophorectomy for BRCA1 or BRCA2 mutation carriers appears to be the more effective method of reducing cancer risk, particularly if reproduction and child rearing has occurred (Olopade and Artioli, 2004; Salhab et al., 2010). It is also worthy to note that ovarian cancer patients with traditional *BRCA* mutations have been found to show better survival rates than those with hypermethylation silencing (Bell et al., 2011). It is therefore imperative to identify at risk patients harboring cancer predisposing and inherited mutations in *BRCA1* and *BRCA2*.

6. Sporadic ovarian cancer and new genetic markers

PCR-based technologies have the potential to allow for the rapid identification of patients who exhibit genetic variations within gene sequences, introns, promoters and other important regions of DNA, such as cancer susceptibility loci. Genetic variations associated with the androgen receptor have been observed to increase the risk of sporadic ovarian cancer in both Caucasian (Ludwig, 2009) and African-American (Schilkdkraut, 2007) populations. Furthermore, single nucleotide polymorphisms (SNPs) have been identified in several genes which are likely or very likely to associate with ovarian cancer including *CCND1* (Quaye et al., 2009), *MRPL23* (Quaye et al., 2009), *CDKN1B* (Goode et al., 2009), *CDKN2A/2B* (Goode et al., 2009) and *RB1* (Song et al., 2006; Braem et al., 2011). Aside from these SNPs in specific genes, several ovarian cancer susceptibility loci have been identified and analyzed using genome wide association studies. These studies have been reviewed by Braem and colleagues (2011), who conclude that there is strong evidence to establish a correlation between ovarian cancer and SNPs on chromosomes 9p22.2, 2q31, 8q24, and 3q25. Taken together, these studies point to several genes and susceptibility loci which may be amenable to high throughput screening and may help to identify ovarian cancer before it begins or in early stages, when survival is highest.

7. Summary and future directions

Understanding of the landscape of ovarian cancer pathogenesis has evolved over recent years, and with it, strategies for patient care. Early detection continues to be a top priority to diagnose this pernicious disease when it is still highly responsive to treatment. Novel discoveries in genomics, epigenetics, proteomics, and functional glycomics have rapidly expanded the number of potential tumor markers available. To make better sense of which candidate markers have the greatest significance, several strategies have been employed. Identification of cancer-specific alterations in glycosylation signatures and development of composite epigenetic serum panels are two minimally invasive approaches that may, in time, allow for more accurate early detection of ovarian cancer.

Aside from CA125, which currently remains the sole validated ovarian cancer biomarker, other serum markers may be comparable or superior for early detection. Among these, HE4 appears especially promising, and the use of CA125 testing with HE4 or other emerging markers may prove to be clinically useful. In addition, better identification of women with greatest genetic risk may help to isolate a small subset of the population that requires the closest monitoring. By employing strategies such as those described above, it is hopeful that ovarian cancer mortality rates, which have remained intractably high over the past several decades, will finally begin to decline.

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9. Dedication

This chapter is dedicated to all women who are living with and those who have died from ovarian cancer.

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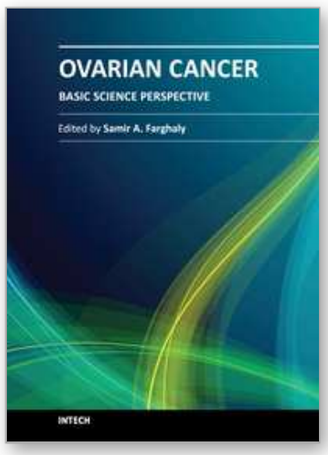

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Worldwide, Ovarian carcinoma continues to be responsible for more deaths than all other gynecologic malignancies combined. International leaders in the field address the critical biologic and basic science issues relevant to the disease. The book details the molecular biological aspects of ovarian cancer. It provides molecular biology techniques of understanding this cancer. The techniques are designed to determine tumor genetics, expression, and protein function, and to elucidate the genetic mechanisms by which gene and immunotherapies may be perfected. It provides an analysis of current research into aspects of malignant transformation, growth control, and metastasis. A comprehensive spectrum of topics is covered providing up to date information on scientific discoveries and management considerations.

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