Chapter 16

Ovarian Cancer *in vitro* Diagnostics: New Approaches to Earlier Detection

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1. Introduction

The overall mortality of ovarian cancer has remained unchanged despite new chemotherapeutic agents that have improved 5-year survival rates. In the United States, ovarian cancer is among the most lethal malignant gynaecological pathology. Each year, more than 230,000 new cases of ovarian cancer are diagnosed. More than 90% of these cases occur in women without clearly identifiable risk factors. In the majority of cases, ovarian cancer is first diagnosed as disseminated disease that has a five-year survival rate of less than 30%. Ovarian cancer, thus, remains a significant health care challenge and the most lethal of women's reproductive tract cancers.

Although ovarian cancer is often considered to be a single disease, it is composed of several related but distinct tumour categories, including: surface epithelial tumours, sex-cord stromal tumours, germ cell tumours, and metastatic tumours. The most frequent are the epithelial tumours that are also divided according to their histologic types: serous, mucinous, endometrioid, clear cell, and transitional. Epithelial tumours may be classified into two further groups, according to their clinical behaviour: either low malignant potential (LMP) or high malignant potential (HMP). In addition, HMP epithelial tumours are also divided into type 1 and 2 depending upon whether or not there is a pre-malignant lesion. Considering this new classification, specific mutations have been isolated depending on the type of tumour. Furthermore, the primary origin of serous epithelial ovarian cancer has been questioned. Crum *et al*. (2007), proposed that the majority of ovarian carcinomas originate outside the ovary and are derived from fallopian tube epithelial cells. The identification of

cells with a molecular phenotype similar to Type 2 ovarian cancer within the fimbria is consistent with the hypothesis that ovarian cancer may indeed originate from intraepithelial carcinomas of the fallopian tubule.

Despite significant advances in the development of mathematical modelling and validation of *in vitro* diagnostics, to date none have achieved the level of diagnostic performance required for implementation as a screening test for asymptomatic women in the general population. In the absence of a screening test, however, it is important for women presenting to primary care to be diagnosed in the most effective and timely way to ensure that they are directed to the most appropriate clinical treatment available.

Ongoing studies continue to search for the presence of other biomarkers (in addition to CA125 and ultrasound imaging) to detect ovarian cancer in its initial stages. Of recent note has been the identification of tumour-specific exosomes in the blood of women with ovarian cancer. Other novel diagnosis techniques have been described: intra-fallopian tubule sampling, uterine washing sampling and the sampling of cervicovaginal swabs. While these approaches afford some promise of increasing diagnostic performance for asymptomatic populations, they await clinical validation.

2. Reclassification of disease type

According to the classification of the World Health Organisation in 2003, from an histopathological point of view epithelial ovarian tumour are classified in serous (60%), endometrioid (10-20%), clear cell (<10%), transitional (6%), mucinous (5%), and undifferentiated (<1%) [1,2]. Furthermore, ovarian tumours are also classified, according to behaviour, into low malignant potential (LMP) and high malignant potential (HMP) depending on the grade of invasion.[3] High serous malignant tumours are divided into type I and type II [4].

Type I tumours originate from the progressive transformation of low malignant potential ovarian tumours, whose behaviour is considered to be relatively benign. This group include: mucinous carcinoma; endometrioid carcinoma; Brenner tumours; and clear cell carcinoma. Type II tumours, however, do not display a defined pre-malignant lesion, and their behaviour is aggressive, and rapidly progressive, metastising in early stages of diagnosis. Serous carcinomas, sarcocarcinomas and undifferentiated carcinomas belong in this group. Preliminary studies report that epithelial LMP tumours (mucinous, endometrioid, and clear cell carcinoma) could evolve from low to mildle undifferentiated tumours becoming HMP ovarian tumours [5].

The development of a new classification of epithelial ovarian tumours (*i.e.* type 1 and type 2) has led to the identification of specific molecular phenotypes previously unidentified because of the confounding effects of multiple histopathological types of tumours. Type I and type II tumours display different characteristics and activation of molecular pathways. Type I tumours are associated with mutations in the Ras pathway (BRAF, KRAS, ErbB2) while, type II tumours are frequently associated with mutations in the

TP53 pathway, although there is little information relating to other molecular mutations. [6,7]. When stratified by type (*i.e.* high-grade and low-grade serous carcinoma), it became evident that the mutation TP53 is present in almost 100% of type II high-grade serous carcinomas [8]. Taking into account that TP53 mutation is precocious and ubiquitous (at least in advance stages) it remains to be proven whether or not this mutation plays an aetiological role in the development of this phenotype.

Women with mutations in BRCA 1-2 genes have around 30-70% probability of developing ovarian cancer before reaching old age, in most cases, type II HMP tumours.[9] The BRCA 1-2 genes are crucial components in the DNA repair pathway of homologous recombinant required to resolve errors in the double-stranded DNA [10]. It is likely that inherited mutations in BRCA 1-2 genes predispose the epithelial ovarian surface to neoplastic transformation secondary to genetic instability [5]. The loss of function in the BRCA 1-2 genes is often lethal to the cell because of the associated apoptotic response with p53.[11] Since the loss of BRCA gene function is very common in high-grade serous carcinomas, secondary mutations are expected to be present to ensure the survival of the cells involved [3].

There are undoubtedly many mutations involved in the survival and adaptation of epithelial ovarian carcinomas that have yet to be studied. Currently, the processes that occur between an initial carcinoma and its progression to widely disseminated disease remain unknown. It is presumed that there are multiple mutations in the tumourigenesis pathways that allow the tumour to overcome hypoxia, cytokines, the detachment from the basal membrane and the metabolic demands of many rapidly dividing cells. [7]

3. Fallopian tubule involvement in ovarian cancer

Within the advances in histopathological and genetic investigations, recent dogma regarding the origin of serous ovarian cancer involving pre-cancerous lesions from the ovarian surface epithelium or intra–ovarian inclusion cysts has been questioned. In women with BRCA-1 and BRCA-2 germline mutations, tubal intra-epithelial carcinoma in the fimbria has been identified as a very probable precursor of advanced high-grade serous ovarian cancer (particularly in Type 2 ovarian cancer) [12]-[14]. This is also validated by the coexistence of identical TP53 mutations in tubal intra-epithelial carcinoma and in those tumours classified as ovarian in origin [15].

This evidence is consistent with the idea that the fallopian tube (especially its distal portion: the fimbria) is an important site for the initiation of high-grade serous ovarian cancer [16]. Crum et al. (2007), further, proposed that most ovarian carcinomas originate outside the ovary and are derived from fallopian tube epithelial cells. They suggest that fimbrial epithelial cells detach and implant on the deluded, damaged surface of the ovary resulting in the formation of inclusion cysts that subsequently give rise to what until now was known as "ovarian" cancer. The identification of cells with a molecular phenotype similar to Type 2 ovarian cancer within the fimbria is consistent with the hypothesis that ovarian cancer may indeed originate from intraepithelial carcinomas of the fallopian tubule [17].

Even though the genesis of this pathology remains unclear, there are some groups that support the idea of "endosalpingiosis" as the preliminary event. This means that even when the primary tumour seems to originate in the ovary, it is possible that the fallopian tube epithelium provides the originating cell through earlier entrapment in the ovary [16].

These studies potentially have significant impact on clinical practice and raise important questions, including:

- Should the complete removal of the fallopian tube during hysterectomy and/or oophorectomy be a general practice? Bowtell et al. and Dietl et al. consider this approach essential in reducing the risk of high-grade serous cancer [16, 18].
- Is the removal of fallopian tubes a good idea when practicing a prophylactic hysterectomy in women with BRCA mutations? According to Dietl and Wishhusen, a salpingectomy-only for women at increased risk of ovarian cancer would be a proper prophylactic option [18].

Future research should be oriented towards answering these and many other questions related to the development of new surgical and medical techniques in the treatment and prevention of ovarian cancer.

4. Recent advances in the development of IVDs

Early detection and accurate diagnosis of ovarian cancer is a pending issue in gynaecologic oncology. Tools such as physical examination, transvaginal ultrasound and serum markers (*e.g.* Ca125) have limited sensitivity. Moreover, genetic counselling is warranted only in high-risk patients, such as those with a family history of BRCA-1, BRCA-2 or Lynch syndrome [19].

Considering the high mortality of this type of cancer, it is necessary to develop new and more efficient diagnostic strategies. One recent approach to improve diagnostic efficiency has been the development of multivariate index assays (IVDMIA). IVDMIAs were defined by FDA guidelines in 2007 as a tool that: 1. Combines multiple variables using a performance function to obtain a specific result for a specific patient; and 2. Provides a result whose derivation is non-transparent and cannot be independently derived or verified by the end user.[20] The purpose of the multivariate analysis is to integrate different biomarkers into a single test, to optimise the sensitivity and specificity of the diagnostic through non-lineal functions.[21]

To date, such tests are not methods of screening, but diagnostic tools in the evaluation of women with pelvic tumours. They help to determine the likelihood of malignancy and thus the categorisation of urgency at the time of referral to a gynaecological oncologist. [19]

OVA1 (Vermillion, Inc., Austin, TX) is the first ovarian cancer IVDMIA approved by the FDA, and combines five tests: CA125 II, prealbumin, apolipoprotein A-1, β2-microglobulin, and transferrin, obtaining a score of 0-10, in which 10 is the highest risk of malignancy. The

cut-off values to define high probability of malignancy in premenopausal women are 5.0 and 4.4 in postmenopausal women. These tests optimise sensitivity compared to physical examination in both nongynecologic oncologists (72% to 92%) as in gynecologic oncologists (78% to 99%), even at 100% stage II in both pre- and postmenopausal women [22]. In addition to its association with physical examination, it has a sensitivity of 96%, while physical examination and CA125 alone have a sensitivity of 75% and 77%, respectively [21].

Even though OVA1 has a high sensitivity, its specificity is low in both nongynecologic oncologists and gynecologic oncologists (being 42% and 26% respectively). Other IVDMIAs have shown greater specificity, for example OvaSure, a 6 IVDMIA analysing protein biomarker, has a sensitivity of 95.3% and a specificity of 99.4% [23], however, OvaSure has yet to be approved by the FDA.

In a recent study, Autelitano et al. analysed a unique multianalyte test that integrates CA125, C-reactive protein, amyloid-A, plasma interleukin-6 and interleukin-8. This test has a high specificity (92.3%) and a moderate sensitivity (76.4%) for the diagnosis of ovarian cancer in symptomatic women. The panel performs significantly better than CA125 alone, as measured by the area under the receiver operator characteristic curve (88.4% and 84.3%, respectively, $p < 0.001$) [24].

The development of IVDMIAs for ovarian cancer based on known candidate biomarkers offers promise for improving diagnostic efficiency of not only adrenal masses but also the earlier detection of ovarian cancer and prognosis.

Optimising preoperative diagnosis and opportune referral to specialists, would not only assist in the development of a specific management strategy for individual patients, but would also allow for more accurate determination of perioperative morbidity and chance of survival. Further studies, however, are needed to validate not only a comparison with classical clinical or serological parameters, but also between different IVDMIAs, to determine which one is the better diagnostic tool.

5. Novel approaches to the diagnosis of ovarian cancer

Ovarian cancer is generally diagnosed in its advanced stages due to the lack of overt symptoms of disease (70% of the cases approximately), resulting in a poor prognosis (rate survival around 30%) [25]. Only a small number of ovarian cancers are detected early and these are the ones that can generally be treated.

The reason ovarian cancer is difficult to diagnose in its initial stage is due to the lack of specific and appropriately sensitive serum biomarkers associated with the unspecific symptoms. The most utilised serum biomarker in the diagnosis of ovarian cancer is CA125, but unfortunately its ability to detect ovarian cancer in a general population is quite low [26, 27].

As a response to this difficult scenario, current investigations include the search for other serum biomarkers that would improve our ability to detect ovarian cancer in its initial

stages, possibly in combination with CA125 and ultrasound imaging. The recent identification of tumour-specific nanoparticle (exosomes) in the blood of patients with various diseases/complications, including ovarian cancer, affords an alternative approach to the identification of more effective biomarkers.

Exosomes are small (40-100 nm) membrane vesicles that are released following the exocytotic fusion of multi-vesicular bodies with the cell membrane. They are characterised by: a cup-shaped form; a buoyant density of 1,13-1,19 g ⁄ ml [28,29] endosomal origin; and the enrichment of late endosomal membrane markers, including Tsg101, CD63, CD9 and CD81 [30-32]. Exosomes have been identified in plasma under both normal and pathological conditions, and their concentration has been reported to increase in association with disease severity and/or progression. While, the process(es) of exosome formation remains to be fully elucidated, available data support an endosomal origin and formation by the inward budding of multi-vesicular bodies [33].

Tumor cells release exosomes into peripheral circulation [34], indeed the first vesicular structures described in plasma were observed in women with ovarian cancer [35]. In ovarian cancer, the concentration of exosomes (measured as exosomal protein in peripheral blood) increases with disease stage and are associated with tumour-specific microRNA [36]. These results suggest that microRNA profiling of circulating tumor exosomes could potentially be used as surrogate diagnostic markers and may be of utility for screening asymptomatic populations. Recent data further suggests that the release of exosomes from cells may represent a normal mechanism for cell-to-cell communication [37] their role in the pathogenesis of ovarian cancer, however, remain to be established.

Other novel diagnosis techniques have been described: intra-fallopian tubule sampling, the sampling of uterine washings and the sampling of cervicovaginal swabs. While these approaches represent a very promising alternatives for the diagnosis of ovarian cancer, there is a paucity of data and clinical validation to support their implementation as viable alternatives to CA125 and ultrasound imaging.

6. Concluding comments

The alignment of metastatic and molecular phenotypes of ovarian cancer is affording new insights into the aetiology and treatment of this disease cluster.

Recent evidence supports a tubal origin of epithelial ovarian cancer, including the coexistance of similar gene mutations in the tubal intraepithelial carcinoma and those classified as ovarian origin (*e.g.* TP53 gene mutation). On the basis of these data, some have proposed "endosalpingiosis" as the initial event in ovarian cancer, suggesting that the epithelial cells of the tube migrate to the surface of the ovary constituting ovarian cancer genesis. If proven to be correct, new opportunities for the management of ovarian cancer may be realised, particularly for those patients carrying BRCA-1 and -2 mutations that require prophylactic surgery.

Within the field of gynaecologic oncology, an aspect that has been particularly disappointing is the development of early detection tests for ovarian cancer. Classical methods based on physical examination, images and some serum markers such as CA125, have not resulted in significant advances in early detection rates. Tests, such as OvaSure and OVA 1, have integrated various clinical and serum markers for the diagnosis of cancer with different sensitivities and specificities, but are aimed at defining malignancy in patients with ovarian tumours, rather than providing either an earlier diagnosis or a screening test.

A possible answer to the problem is seen with the recognition of specific membrane particles in ovarian tumors (exosomes), as well as other tissue surfaces. These particles are tissue-specific and may allow the identification of specific cell types in preclinical stages of the disease. The potential detection of these specific exosomes in biofluids also offers new perspectives in research on the early detection of ovarian cancer. Such ovarian cancer–specific non-particles may be present in fallopian tubule fluid, uterine washings or even cervicovaginal fluids. Further research is needed in this area to assess the utility of such approaches in order to develop simple and safe methods of detecting ovarian cancer in its early stage.

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