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Cervical, Ovarian and Endometrial Tumor Markers: Potential Clinical Value

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Tumors markers can be described as molecular products expressed by neoplasia tissues (immunohistochemistry), or metabolized and secreted by tumor and characterized biochemically in body fluids such as blood and urine. They may have utility as indicators of tumor stage and grade as well useful for monitoring responses to treatment and predicting recurrence, progression, development of metastases, or even patient survival. Unfortunately, in some cases they may have no identified clinical potential.

Several investigations have been carried out, especially in the last decade, using biotechnological methods, in order to identify new potential tumor markers. By translating these findings into clinical use one may facilitate accurate diagnosis and prognostic prediction, and contribute to individualized treatment.

The objective of this review is to describe some biomarkers with potential use in clinical settings of uterine cervix, ovary, and endometrium carcinomas.

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Introduction

Tumor markers supposedly encompass a plethora of molecular, clinical and/or pathological variables that reflect, partially or comprehensively, the biological behavior of malignant neoplasia of different regions of the human body. The search for biological parameters with substantial high degree of confidence to predict cancer behavior is constantly improved in medical literature and some are currently incorporated in routine medical care. In order to facilitate understanding of the most representative, and potentially inclusive tumor markers in low genital tract malignancies, we divided this review into 3 topics: endometrial, ovarian, and cervical cancers.

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Endometrial Cancer

Endometrial cancer (EC) is the most common gynecological malignant tumor in developed countries.[1] Two clinic pathological variants are described. Type I cancers (endometrioids) comprise most cases (70%-80%) and are known to be related to unopposed estrogen stimulation. Type 1 cancers are most frequently observed among women in the midlife perimenopause, and related to some risk factors of hyperestrogenism such as obesity, hypertension, and diabetes mellitus. Type II (nonendometrioid cancer) follows the non−estrogen-related pathway, which appears to be related to endometrial atrophy. Type II tumors are commonly diagnosed in older postmenopausal women, and are generally less differentiated, representing an unfavorable prognosis.[2]

The typical clinical presentation is a postmenopausal woman with vaginal bleeding. Despite being one of the most common gynecological neoplasms, routine screening is not recommended, and most patients present with early stage disease (stage I or II), having favorable prognosis and excellent survival (overall survival at 5 years 75%-90%).[2]

However, women encountering more advanced or recurrent disease will have an extremely poor clinical outcome. Thus, renewed research focus on better understanding the molecular changes associated with EC, which is mainly prompted by the dramatic increase in incidence observed in the recent years.[3]

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Although Papanicolaou (Pap) test, transvaginal ultrasound, and endometrial sampling are techniques under investigation for their ability to reveal EC at an early stage, there is still insufficient evidence to recommend any cost-effective screening method in women with average to high risk and without symptom presentation.[4]

Some biomarkers have been examined for EC of the respective subtype. Defects in DNA mismatch repair genes, microsatellite instability, and mutations in the PTEN and K-ras and/or B-catenin genes are mutated in high rates for type I, whereas alteration in the p53 suppressor gene with mutation of Her-2/neu are commonly observed in type II.[2]

In this article, we will present some promising biomarkers in endometrial cancer.

Human Epididymis Protein 4

Human epididymis protein 4 (HE4) have emerged as a promising biomarker in gynecological neoplasms. This protein is also known as Whey acidic protein (WFCD2); is located on the human chromosome 20q12-13.1 and is identified as 1 of 4 highly expressed cDNAs in the epididymis, trachea, lung, prostate, endometrium, and breast.[5]

Brennan et al. conducted a population-based cohort study to evaluate if serum HE4 can be used as a primary preoperative risk stratification tool for EC. Serum HE4 was an independent marker of poor prognosis in this study, and it was suggested to use HE4 serum test as a cost-effective approach to avoid unnecessary lymphadenectomy in patients with low-risk EC.[6]

Additionally, Li et al. studied the expression of HE4 in EC and its relations to clinic pathological parameters and prognosis of EC. The goal was to detect the expression rate of HE4, by means of immunohistochemical using streptavidin-peroxidase, in EC, endometrial atypical hyperplasia, and normal endometrial tissue samples, respectively. The results implied that the intensity of HE4 expression increased with degree of malignancy. Thus, the level of HE4 in EC was significantly higher than that of hyperplasia and normal endometrium. Furthermore, the investigation showed no relations of HE4 to the pathological subtype but rather strong relations to other factors like cancer stage, metastasis, myometrial invasion depth, recurrence, degree of differentiation, and the overall survival rate.[7]

Bian et al. have analyzed the serum level of HE4, CA125, CA724, and CA19-9 in single and combined, in order to evaluate their clinical value in EC, and the results showed that the level of tumor markers was obviously higher in the EC group than that in the healthy controls, mainly for HE4 and CA125. Positive rate of HE4 was significantly associated with advanced age at diagnosis, higher pathology grade, and positive adnexal involvement group and was irrelevant to FIGO stage, pathology subtypes, and histologic type. Positive rate of CA125 was noticeably associated with advanced age and higher FIGO stage but also not related to pathology subtypes and histologic type. Positive rate of CA19-9 was associated with higher FIGO stage and positive lymph nodes, and CA724 was associated with positive lymph nodes. Sensitivity of HE4 in patients with EC was 58%, much higher than sensitivity of CA125, CA724, and CA19-9 (35.4%, 11.3%, and 16.3%, respectively), and combined detection of the 4 tumor markers had the highest sensitivity (59.1%) but had little difference with combined detection of HE4, CA125, and CA724 (58.6%).[8]

Astrocyte Elevated Gene-1

Astrocyte elevated gene-1 (AEG-1) is located at chromosome 8q22 and is also known as metadherin (MTDH) and lysine-rich CEACAM1 coisolated (LYRIC).[9]

Song et al. conducted a study that evidenced AEG-1 as the centerpiece in the carcinogenesis and progression of endometrial cancer. The expression rate of AEG-1 was investigated in 35 normal endometrial tissue, 40 atypical hyperplasia, and 174 EC tissue (161 cases of endometrioid carcinoma) showing a positive and gravel relationship between expression and the transition from normality to cancerous tissue. Thus, AEG-1 was found to be significantly correlated with clinic pathological parameters including FIGO stage ($P < 0.001$), depth of myometrial invasion ($P = 0.015$), lymph node metastasis ($P = 0.005$), lymph vascular space invasion ($P < 0.001$), recurrence ($P < 0.001$), and Ki-67 expression ($P = 0.032$).[10]

Other authors have also suggested that an up-regulation of AEG-1 is correlated of malignant aggression, becoming an independent prognostic factor for unfavorable clinical outcomes.[11]

MicroRNAs

MicroRNAs (MiRNAs) are a family of small (21-22 nucleotides) non−protein-coding RNAs responsible for messenger-RNA (mRNA) stability and expression of proteins at a post-transcriptional level. MiRNAs have aroused wide attention because of their suggested role as important regulators of gene expression in a broad spectrum of diseases, including solid and hematologic malignancies. MiRNAs may either act as oncogenes or tumor suppressors presenting increased or decreased expression in tumor cells; these alterations in miRNA expression may be involved in the initiation, cancer progression, and metastatic process in different cancer types.[12,13]

Tsukamoto et al. suggested that miRNAs are involved in cell proliferation, differentiation, apoptosis, and carcinogenesis of endometrium. Using next-generation sequencing were identified 11 candidate Endometrioid endometrial carcinoma (EEC)-associated miRNAs, and quantitative reverse-transcriptase PCR identified 8 EEC-associated miRNAs in tissue (upregulated: miR-499, miR-135b, miR-205, downregulated: miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21). They also concluded that measurement of tissue and plasma EEC-associated miRNAs may be useful for early detection, diagnostic, and follow-up tests for EEC. Expression of hsa-miR-499 in Stage IA and Grade 1 (FIGO) was significantly lower than in others. Two miRNA signatures (miR135b/miR195 and miR135b/miR30a-3p) could distinguish between EEC and normal endometrial tissue samples with accuracy. Circulating levels of 3 EEC-associated miRNAs (miR-135b, miR-205, and miR-30a-3p) in plasma were significantly decreased after hysterectomy.[13]

Torres et al. collected tissue and plasma samples from 122 women (77 endometrioid endometrial cancers and 45 controls), to define miRNA signatures. The expression of a number of miRNA was associated with FIGO stage, grade, relapse, and nodal metastases. Two miRNA signatures: miR-92a/miR-410 and miR-92a/miR-205/miR-410 classified tumor tissues with higher accuracy in comparison to single miRNAs. miRNA signature composed of miR-205 and miR200a predicted relapse, and tissue miRNA signatures were independent prognostic markers of overall and progression-free survival.[14]

Boren et al. measured expression of 335 miRNAs in 61 fresh-frozen endometrial specimens, including 37 endometrial cancers, 20 normal endometria and 4 complex atypical hyperplasia samples, and described a total of 13 miRNAs that demonstrated a significance difference in level of expression. In the transition from normal endometrium through atypical hyperplasia to cancer, 5 miRNAs (miR-let 7i, miR-221, miR-193, miR-152, miR-30c) exhibited a decrease in expression, leaving the remaining 8 miRNAs (miR-185, miR-106a, miR-181a, miR-210, miR-423, miR-103, miR-107, miR-let 7c) with a relative increase in expression. Initially, there was no association between the miRNA expression and cancer stage or grade.[15]

Cervical Cancer

Cervical cancer is the second largest cause of cancer death among women in the world.[16-18] The highest incidence is seen in underdeveloped countries, especially in Africa, Latin America, parts of Asia, and in some Eastern European countries with incidences in excess of 100/100,000 women.[19] In contrast, in developed countries, the incidence may be below 10/100,000 women.[20] Another peculiarity: cancer in general is a more common disease among the older population, but cervical cancer mainly affects young women, with the majority diagnosed between the ages of 35 and 50, at which point women are actively involved with their careers and in the care of their families.[21]

Of all malignancies, cervical cancer is one of the most effectively screened. Detection of cytological abnormalities and subsequent treatment of high-grade lesions prevents the onset of cancer.[22] Organized screening every 3-5 years can reduce the incidence by more than 80%.[23]

In developed countries, the incidence and mortality from cervical cancer have dramatically decreased, probably as a consequence of screening.[24–26] However, cytology screening is not optimal because a considerable number of women are diagnosed with invasive carcinoma despite regular screening.[27,28] One of the main shortcomings of cytology is its low sensitivity, mainly related to collection and reading errors, which is not automated or only partially automated. In addition, low coverage in developing countries is another cause of poor performance, related, however, to tracking logistics rather than to the test itself.[29-31]

The use of biomarkers, both in cytology and histology, proved to be effective, with fewer false positives and negatives, increasing the positive predictive value of the tests and there are a number of possible applications, including early detection of cancer, increased reproducibility of histopathological diagnoses, surveillance of patients at higher risk, and follow-up after treatment.

MicroRNAs

As mentioned before, there is increasing evidence that miRNA expression is aberrant in human cancers. Moreover, miRNA expression signatures are associated with clinical outcomes of many diseases.[32,33]

Just as in the case of endometrial cancer, a meta-analysis showed that aberrant expression of 3 miRNAs, miR-125, −145 and −196 were independently associated with adverse overall survival of cervical cancer patients.[34]

Another meta-analysis identified that miR-29a and miR-21 are the most frequently down- and up-regulated in cervical cancer progression, respectively. Microarray-based studies show a small overlap, with miR-10a, miR-20b, miR-9, miR-16, and miR-106 found repeatedly deregulated. miR-34a, miR-125, and miR-375 were also found deregulated in cervical exfoliated cells in relation to cancer progression.[35]

Hou et al. showed that miR-196a directly targeted FOXO1 and p27Kip1, 2 key effectors of PI3K/Akt signaling; when overexpressed, miR-196a increased proliferation and G1/S-phase transition of cervical cancer cells whereas its suppression had the opposite effect.[36]

Fan et al. demonstrated that high miR-125a expression suppressed the growth, invasion and epithelial-mesenchymal transition of cervical cancer cells both in vivo and in vitro by reducing STAT3 expression; it also conferred G2/M cell cycle arrest by inhibiting several G2/M checkpoint proteins.[37]

p16

Overexpression of p16 is a direct consequence of deregulated expression of human papillomavirus (HPV) oncogenes.[38] Nondysplastic epithelium infected with high or low risk HPV does not exhibit diffuse expression of p16INK4a. Conversely, in resting cells with aberrant differentiation for HPV infection, the pathological expression of p16INK4a is indicated by a very strong diffuse staining pattern in the cells in basal and parabasal layer replication.[39]

Basically, all cervical carcinomas, CIN 3 lesions, and most CIN 2 lesions present a diffusely positive immunohistochemical pattern for p16INK4a. In contrast, only a small part of the NIC 1 lesions shows positivity for p16INK4a.[40,41]

Some studies show that low-grade lesions p16INK4a-positive have a higher risk of progressing to high-grade lesions compared to p16INK4a-negative lesions, suggesting that p16INK4a may be used as a marker to discriminate lesions with higher risk of progression.[42-44]

Based on the successful application of p16INK4a in immunohistochemistry, there are also studies evaluating its application in cytological samples. Several studies have shown that the use of p16INK4a in cytology can detect high-grade lesions with greater sensitivity than isolated cytology.[45-49]

To increase the specificity of the use of p16INK4a in cytology, a nuclear score was defined in order to facilitate the evaluation of p16INK4aINK4a positive cells. Using this score,

there is an increase in specificity in the diagnosis of relevant lesions, while sensitivity is not affected.[50]

In direct comparison with conventional cytology, cytology + p16INK4a identified 98% of high-grade squamous intraepithelial lesion, while only 1% of normal cases and 10% of low-grade squamous intraepithelial lesion (LSIL) presented positivity for p16INK4a. Based on these results, the immunocytology for p16INK4a can be used to highlight potentially abnormal cells in a panorama of normal, reactive or nonmalignant cells. The immunoassay for p16INK4a could be used for case screening of atypical squamous cells of undetermined significance (ASCUS-US) and LSIL.[51]

Ki-67

The increased proliferation of cervical epithelial cells induced by the deregulated expression of HPV oncogenes is reflected in the activation of proliferation markers as ki-67 (MIB-1). Ki-67 is a nuclear and nucleolus protein expressed during G1, S, G2 and M phases of the cell cycle, but not in resting cells (G0 phase), and can therefore indicate the fraction of cells in multiplication. While the Ki-67 function remains uncertain, its expression seems to be an absolute requirement for the progression of the cell division cycle.[52,53]

Ki-67 can be expressed in normal cells of the basal and parabasal layer which maintains proliferation capacity, but in dysplasia and carcinoma its expression extends beyond the basal third of the epithelium and the number of positive cells increases, with a positive relation with the severity of the injury.[54-56] It is possible that the presence of ki-67 cell clusters is a good criterion for differentiating low-grade lesions of the normal and/or reactive epithelium.[57]

The utility of Ki-67 is also demonstrated in immunocytochemistry, both in traditional Papanicolaou and in liquid-based cytology.[54,58-61]

In patients with ASC-US and LSIL, immunocytochemistry showed 96% sensitivity, 67% specificity, 49% predictive value, and 98% negative predictive value for CIN 2/3 detection.[58]

Sahebali et al. demonstrated an accuracy of 0.68, 0.72 and 0.86 for ASC-US, LSIL and high grade squamous intraepithelial lesion, respectively.[60]

Ovarian Cancer

Although uncommon, ovarian cancer remains the most fatal gynecologic malignancies worldwide, with an estimated incidence of 239,000 cases and 152,000 deaths each year.[62] For several factors, such as delayed diagnosis, high rates of tumor recurrence and resistance to chemotherapy, the 5-year survival rate of patients with advanced disease was approximately 30%.[63]; besides that, more than 60% of cases are diagnosed after the cancer has metastasized.[64]

Screening trials using CA-125 testing, transvaginal ultrasound, or both, have shown no effect on mortality and have documented harms; positive test results from screening asymptomatic women often reveal benign pelvic conditions or normal ovaries on surgical investigation, and cancer cases are often missed with screening.[65-67]

There are 2 broad categories of epithelial ovarian cancer (EOC) with shared clinical and histological features that represent distinct models of epithelial ovarian carcinogenesis.[68] Type I tumors are generally low-grade, indolent tumors, which are often associated with somatic mutations in a number of genes (eg, KRAS, BRAF, ERBB2) and develop from extraovarian lesions implanted on the ovary. Type II tumors are more likely to derive from the fallopian tube or ovarian surface epithelium. These cancers are generally high grade and are genetically unstable, including high rates of TP53 and BRCA mutations.[69] There is a current effort to clarify the distinctions between the molecular, pathological, and clinical features of ovarian cancer and a growing understanding that survival differences are more likely attributable to cancer type than stage at diagnosis, with the most common type II cancers being particularly lethal regardless of stage, owing to microscopic metastases.[69,70]

Ovarian cancer remains the most fatal gynecologic malignancy worldwide due to delayed diagnosis as well as recurrence and drug resistance−−there is an urgent need to explore new avenues for early diagnosis, prognosis and therapeutic targets for ovarian cancer.

Cancer-testis Antigens

Cancer-testis antigens (CTAs), of which approximately 250 have now been identified, are encoded by genes that are normally expressed only in the human germ line, but are also expressed in several types of tumor tissues.[71] The similarity between gametogenesis and tumorigenesis includes the following: immortalization of primordial germ cells and transformation of tumor cells; ploidy cycles in meiosis and aneuploidy in tumor cells; migration of primordial germ cells and metastasis of tumor cells. Emerging studies have suggested that aberrant expression of CTAs may drive soma-to-germline transformation and result in tumorigenesis and tumor progression.[72,73] CTAs are being evaluated for their role in oncogenesis—recapitulation of portions of the germline gene-expression program might contribute characteristic features to the neoplastic phenotype, including immortality, invasiveness, immune evasion, hypomethylation, and metastatic capacity.[74]

As one classical type of CT gene, the expression levels of MAGE genes in ovarian cancer have been widely investigated, for example, the frequency of MAGE-A1 mRNA expression was found to be 20.7% among 58 ovarian cancer tissues.[75] Daudi et al. studied MAGE expression in 400 EOC tissues by reverse transcription-polymerase chain reaction (PCR) and immunohistochemistry and found that at least one of 5 MAGE antigens (MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, and MAGE-C1) was expressed in approximately 78% of EOC patients.[76] Another study, conducted by Kawagoe et al. showed that MAGE-A4 serum levels were significantly increased in ovarian cancer patients compared with those with benign diseases, and the MAGE-A4 protein was expressed in 22% of primary ovarian cancer patients.[77] One interesting finding is that MAGE-A3/6 protein was found to be present on plasma-derived exosomes from all ovarian

cancer patients assessed but not on those from benign tumors or healthy controls.[78] Hofmann et al. detected BAGE, MAGE-A1, MAGE-A3, and GAGE1/−2 mRNAs in peritoneal fluid from ovarian cancer patients using multiplex reverse transcription-PCR analysis, with the combination of the 4 markers exhibiting increased diagnostic sensitivity of 94% compared with cytopathology alone.[79]

The expression of some MAGE CT genes is also correlated with grade, staging, and prognosis. MAGE-C1 expression are significantly correlated with the grade of endometrioid ovarian cancer and the histological subtype,[80] the expression of MAGE-A1 and MAGE-A3 correlates with tumor differentiation and clinical stage in ovarian cancer[81] and MAGE-A9 expression was also found to be significantly associated with high histological grade, International Federation of Gynecology and Obstetrics (FIGO) stage, CA-125 level and metastasis.[82] MAGE-A1 and MAGE-A10 expression is also significantly associated with poor progression-free survival in EOC,[76] patients with MAGE-A9 expression exhibited poor overall survival[82] and MAGE-A4 is a reliable prognostic factor for serous carcinoma patients.[83]

Drug resistance is another important aspect to be considered in ovarian cancer, and the MAGE expression also sounds to be correlated. One study demonstrated that MAGE gene families are overexpressed in some drug-resistant ovarian cell lines and that transfection of MAGE-A2 and MAGE-A6 into a sensitive cell line can facilitate cell growth and induce paclitaxel and doxorubicin resistance.[84] Another study indicated that overexpression of MAGE-A3 correlates with a doxorubicin-resistant phenotype.[85] Daudi et al. demonstrated that MAGE-C1 expression was associated with platinum-sensitive disease and clinical response.[76]

Other genes besides the MAGE type were associated with ovarian cancer, but with fewer descriptions in the literature. Although the function of CT genes in ovarian cancer remains unclear, it appears that these genes may exert oncogenic functions in tumorigenesis, progression, and drug resistance. The unique properties of CT genes, such as their inherent immunogenicity and heterogeneity in their expression patterns, indicate that they have great potential for clinical application.[86]

Circulating Tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) are emerging as "liquid biopsies" modalities. They represent noninvasive biomarkers and are viable, as they can be isolated from human plasma, serum and other body fluids.[87]

Cell-free DNA (cfDNA) circulates at high concentrations in peripheral blood of cancer patients and can be used for the detection of several molecular alterations related to cancer development. Circulating tumor DNA (ctDNA) represents a small percentage of cfDNA that is shed in circulation by tumor cells and carries all these molecular alterations including tumor specific mutations, microsatellite instability,[88] loss of heterozygosity (LOH)[89] and DNA methylation.[90] ctDNA is a very promising noninvasive diagnostic, prognostic and predictive tool, as it provides an easily accessible source of DNA derived directly from the tumor.[91]

Kuhlmann et al. isolated circulating cell-free fDNA (cfDNA) of 63 primary epithelial ovarian cancer patients before surgery and after chemotherapy. They used a PCR-based fluorescence microsatellite analysis in order to measure the LOH in 2 fractions of cfDNA, the high and low molecular-weight fraction (HMWF and LMWF, respectively). They reported that LOH at 2 markers can predict tumor grade ($P = 0.033$) and FIGO stage ($P = 0.004$) in the LMWF cfDNA. Remarkably, a LOH at another marker can significantly predict patients' OS (P=0.030) in both high molecular-weight fraction and LMWF.[89]

Harris et al. introduced an algorithm for the quantification of cfDNA using a PCR assay in order to predict relapse and treatment response. They identified aberrant chromosomal junctions in primary tumors of 10 ovarian cancer patients and detected them in plasma ctDNA of 8 patients before surgery. In 3 cases, ctDNA was also detected after surgery, indicating the presence of the disease, but in the remaining 5 cases, ctDNA was absent after surgery, suggesting absence of the disease.[92]

Cohen et al. applied a commercial and well-established noninvasive prenatal testing platform in cfDNA of 16 presurgery early and 16 advanced high-grade serous ovarian cancer (HGSC) patients. The obtained data were analyzed for the detection of subchromosomal changes and the determination of whole chromosome gains or losses. They detected 40.6% of all HGSC cases, and more specifically, 38% of early stages, indicating a potential utility for early HGSC screening in plasma cfDNA based on specific multiple segmental chromosome gains and losses.[93]

Vanderstichele et al. reported for the first time the potential of using cfDNA for primary HGSC diagnosis. They studied 68 patients with an adnexal mass: 57 diagnosed with invasive or borderline carcinoma and 11 with benign disease. They measured specific patterns of chromosomal instability in plasma cfDNA of all patients and reported a significantly higher quantitative measure of chromosomal instability in ovarian cancer patients compared to patients with benign disease or healthy individuals.[94]

Teschendorff et al. performed a methylation study in peripheral blood DNA of pre- and post-treatment ovarian cancer patients and they observed a significantly different methylation pattern in blood DNA of epithelial ovarian cancer patients compared to healthy controls.[95]

Similarly, Flanagan et al. investigated WBCs DNA methylation status in 880 epithelial ovarian cancer patients enrolled in a phase III clinical trial, using bisulfite pyrosequencing and reported a significant correlation between mean sucrose nonfermentable (SFN) methylation and progression-free survival ($P = 0.016$).[96] The same group also analyzed blood DNA methylation patterns in 247 ovarian cancer patients enrolled in the previous clinical trial. They identified specific CpGs alterations in blood DNA at relapse after platinum-based chemotherapy and found an independent significant association with survival ($P = 2.8 \times 10-4$).[97]

The application of cfDNA could be correlated with the prediction of response to targeted therapy, the identification of subpopulations/gene signatures of cfDNA, allowing diagnosis, stratification of therapy, and more accurate prognosis.[87]

Conclusions and Future Perspectives

Several markers have been investigated in gynecological malignancies. However, despite the high numbers of promising biomarkers proposed, there is a great challenge before the use in clinical practice. The development of a cancer biomarker and its implementation in the clinical routine requires a multistage procedure and constitutes the final result of multiannual and toilsome research approaches. However, multiple preanalytical, analytical and postanalytical issues should be overcome and studies on the assay validations with regard to repeatability and reproducibility are also necessary

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