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Review Article

Potential value of circulating tumor DNA in gynecological tumors

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Abstract: Though the survival of patients with gynecological tumors has been significantly prolonged by radiotherapy, chemotherapy, targeted therapy and other treatments, the way to improve the patients' life quality still needs investigation. Circulating tumor DNA (ctDNA), which contains tumor genetic information, has the potential in early diagnosis of malignancies due to its high consistency with tumor tissues. Using the key words including "digital PCR", "ctDNA", "technology of digital PCR", and "detection method", "gynecological tumor", we retrieved the original articles and reviews in PubMed and WEB OF SCI database published before May 10, 2019 and performed a thorough review of them. The analysis of ctDNA could provide a comprehensive description of tumor genome, overcome the heterogeneity of tissue biopsy, and supplement the missing mutations in tissue samples. Furthermore, ctDNA could be used as a target of liquid biopsy. Our study also showed that digital PCR technology has a good potential to detect ctDNA in gynecological tumors.

Keywords: Digital PCR, ctDNA, gynecological tumors, monitoring, prognosis evaluation

Overview of ctDNA

Circulating tumor DNA (ctDNA), the DNA fragment released into the blood by apoptotic or necrotic tumor cells, carries both the basic genetic information of tumor cells and the information after gene mutation. Therefore, ctDNA biopsies can provide more information than conventional biopsies. Due to the limited sampling range, the conventional biopsies could only show the tumor characteristics of the sampling site, while ctDNA, derived from both primary and metastatic tumor cells, could reveal more comprehensive information [1]. A study on NSCLC showed that the size of most ctDNA fragments was with in 60-160 bp. The half-life of ctDNA varies from 16 min to 2 h. In healthy people, the cfDNA content in blood is maintained at a low level due to the presence of macrophages and lysosomes [2]. However, ctDNA in the blood of cancer patients increases significantly due to the proliferation of cancer cells. Studies have shown that somatic changes in tumor origin, whose DNA can be detected in the plasma of tumor patients in the form of cell-free DNA [3]. Although an increasing number of studies have focused on ctDNA, there are still many technical difficulties in applying ctDNA as molecular markers in clinical practice due to the following unique biological properties of ctDNA: (I) Low content: only a few dozen copies of ctDNA could be found in per milliliter of plasma samples, and there are significant differences in ctDNA content among different cancerous individuals; (II) Coexistence with other wild-type DNAs: the amount of mutant ctDNA in the blood accounts only 1% of all wild-type DNAs or even less, and the wild-type DNA with only one different base can seriously interfere with the detection of mutant ctDNA [4]; (III)

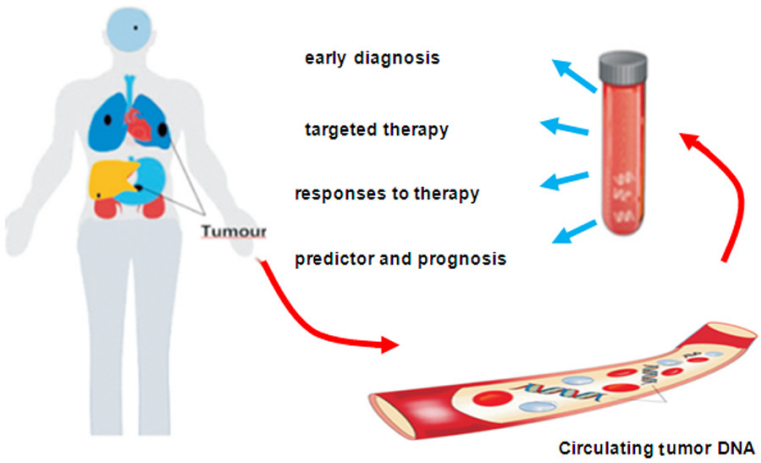

Figure 1. Potential applications of ctDNA.

High degree of fragmentation [6]; (IV) Short half-life: usually 2 h [5]; (V) Being in a dynamic and changing state: the ctDNA content changes dynamically with the tumor stage and the treatment the patient received [6]. These characteristics make the detection of ctDNA more difficult than that of somatic cells. Mutation detection with high sensitivity and specificity is required to amplify and detect trace mutant ctDNA from a large number of wild-type DNAs. The direct sequencing methods (including Sanger sequencing and pyrosequencing) commonly used in clinical practice can only detect 5% to 20% of the gene mutations, and thus are incapable of detecting ctDNA gene mutation in plasma [7]. At present, the methods that can detect ctDNA are Amplification Refractory Mutation System PCR (ARMS-PCR), digital-PCR and high-throughput sequencing. Among them, digital-PCR has the advantages in detecting trace DNA molecules and realizing absolute quantification of single-molecule DNA and quantitative analysis, and thus is reliable in monitoring tumor recurrence and minimal residual diseases [8].

The present study reviews the methods of ctDNA detection and the potential value of ctDNA in gynecologic tumors.

Clinical value of ctDNA detection and droplet digital PCR process

ctDNA can be detected in more than 75% of patients with advanced ovarian, breast and liver cancers. However, the detection rate of ctDNA in prostate cancer is less than 50%, while the relative detection rate in local pancreatic and breast cancer is 48% and 50% respectively, indicating the limitation of ctDNA detection in early and local tumors. Hence, at present, ctDNA is mainly used to detect advanced tumors. Clinical trials have shown that: 1) ctDNA concentration is proportional to tumor load, but the stage, location and size of the tumor cannot be determined by ctDNA detection; 2) Monitoring the level of ctDNA can detect specific mutations in the tissues and plasma of tumor patients, so as to accurately classify the tumor and guide the targeted therapy; 3) ctDNA testing, especially the detection of ctDNA mutations, can monitor tumor progression and prognosis, and ctDNA levels can be used as an independent indicator for the prognosis of certain gynecological tumors like ovarian and endometrial cancers; in addition, ctDNA detection can also indicate the recurrence, metastasis and minimal residual disease of the tumor; 4) ctDNA test can reflect the effectiveness of the anticancer treatment and drug resistance, so as to timely adjust the treatment regimen, reduce the cost of treatment, achieve personalized and precise treatment (**Figure 1**) [9-12].

ctDNA detection can be divided into four steps: (I) Blood sampling: due to the instability of ctDNA, blood samples are usually collected using specialized cfDNA vessels. Studies have shown that the best sample type is plasma, and the blood is better collected using either an anticoagulant tube containing a cell stabilizer or an EDTA anticoagulant tube. When using the EDTA anticoagulant tube, the plasma should be separated within 6 h after sampling. In this process, the dilution effect of cellular-free DNA produced by leukolysis on ctDNA should be minimized; (II) Obtaining plasma: The blood treated with anticoagulation is centrifuged twice at a low temperature to separate the plasma without cell components. For ctDNA analysis, the plasma samples are superior to the serum. The total amount of cfDNA in the serum was 3-22 times higher than in the plasma, probably caused by the contamination of the

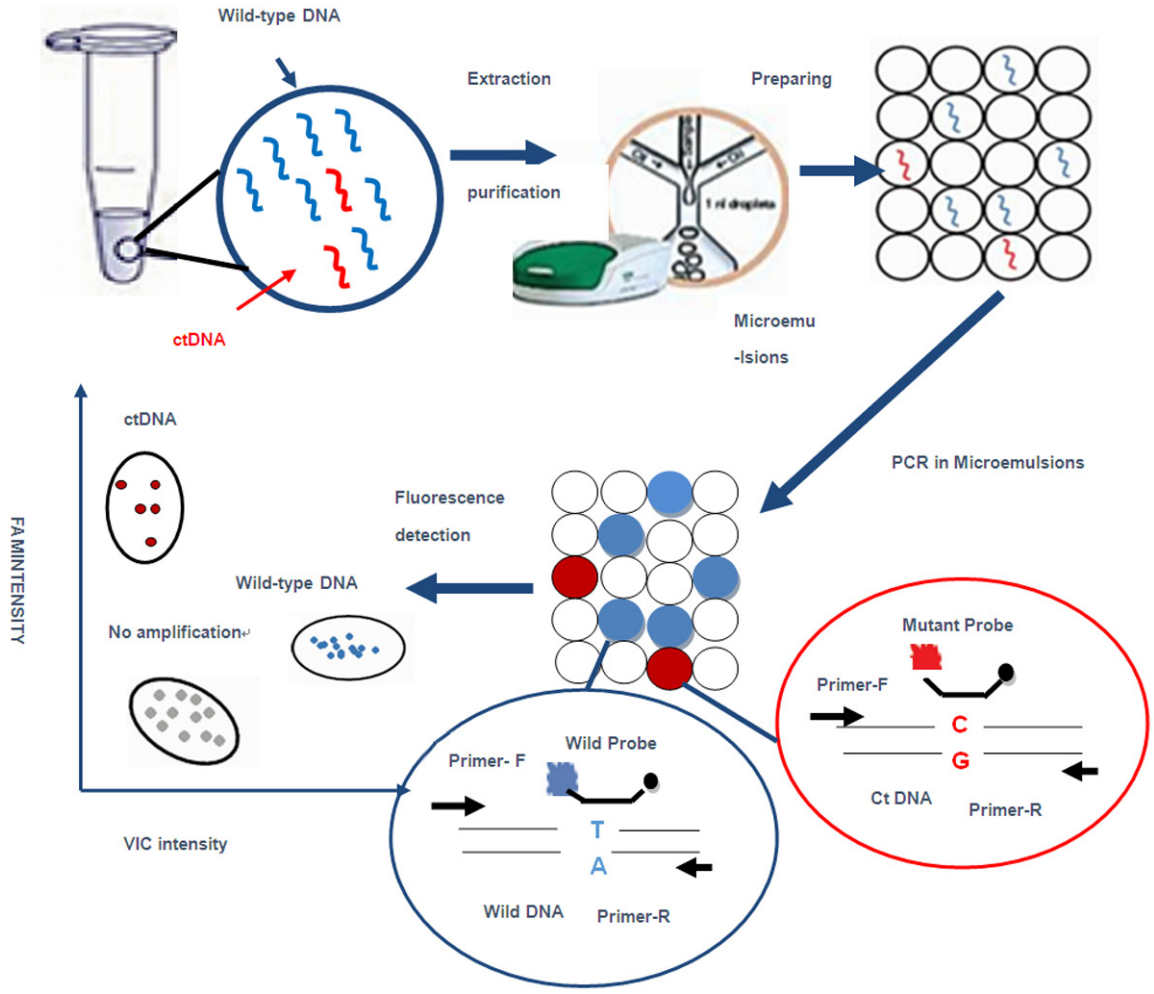

Figure 2. Schematic illustration of ctDNA analysis by droplet digital-PCR [20].

dissolved DNA released by immune cells during coagulation. Due to the low background level of wild-type DNA, plasma has been identified as an excellent source of ctDNA; (III) Extracting free DNA: Because of the low concentration and high fragmentation of ctDNA, enrichment and separation are particularly important. Therefore plasma is frozen at -80°C before DNA extraction; (IV) Downstream analysis: Due to the low proportion of ctDNA in the normal background, special cfDNA analysis methods, including sequencing and polymerase chain reaction (PCR), and based analysis (droplet digital PCR) have been developed (**Figure 2**).

ctDNA detection in gynecological tumors

Breast cancer

ctDNA testing by Olsson *et al* using blood samples of 20 postoperative breast cancer patients showed that ctDNA had a 100% specificity and a 93% sensitivity in predicting disease recurrence [13, 14]. Compared to imaging prediction, ctDNA predicted the recurrence 11 months earlier in 86% of breast cancer patients (**Table 1**). Garcia-murillas *et al* used dPCR and targeted sequencing to detect ctDNA in early stage breast cancer patients undergoing neoadjuvant chemotherapy [15], which showed that ctDNA could predict the recurrence of breast cancer after treatment. Saliou *et al* detected ctDNA of *PIK3CA* (a common mutated gene in breast cancer) in plasma, showing that ctDNA could detect cancer earlier than surgery, with a 93% consistency with preoperative plasma samples [16]. Riva *et al* also used ddPCR to track the TP53 mutation in the plasma of breast cancer patients during neoadjuvant chemotherapy. The patient's plasma (10 mL) was collected before neoadjuvant chemotherapy, after one cycle of chemotherapy, before sur-

Table 1. Application of ctDNA detection in gynecological tumors

Diseases	Target genes	Roles	Reference
Breast cancer	ctDNA	ctDNA had a 100% specificity and a 93% sensitivity in predicting disease recurrence; ctDNA predicted breast cancer recurrence 11 months earlier	[13]
Breast cancer	TP53 mutation during neoadjuvant chemotherapy	During treatment, plasma ctDNA level in patients with significant clinical efficacy was found to decline; ctDNA positivity after one cycle of neoadjuvant chemotherapy was associated with shorter DFS and overall OS (P = 0.006)	[18]
Metastatic TNBC	TP53 mutation	TP53 mutation was detected in 84% of tissue samples, and the detection rate of ctDNA TP53 mutation in patients' plasma was also up to 81%	[19]
Ovarian and uterine cancer	Existence of ctDNA in the blood after treatment	Patients with no ctDNA in the blood after treatment had significantly improved prognosis, PFS (P = 0.0011) and OS (P = 0.0194).	[31]
Cervical cancer	Plasma ctDNA	ctDNA content was significantly different in histological grade, infiltration depth, lymphatic metastasis and FIGO stage	[36]
Endometrial cancer and ovarian cancer	50 common oncogene/tumor suppressor mutations, covering 21,820 sites	ctDNA is similar to CA125 in sensitivity to the diagnosis of endometrial cancer and ovarian cancer, but with a very high specificity	[37]

ctDNA: circulating tumor DNA; DFS: disease-free survival; OS: overall survival; FIGO: international union of gynecology and obstetrics; OCCC: clear cell carcinoma of the ovary; PFS: progression free survival.

gery and during several periods of surgery (**Table 1**) [17]. During treatment, the ctDNA level in plasma of patients with significant clinical efficacy was found to decline, and no ctDNA was detected after surgery, while the patients with disease progression or poor treatment outcome had elevated ctDNA levels. The patients who were positive for ctDNA after one cycle of neoadjuvant chemotherapy had shorter disease-free survival (DFS) and overall survival (OS). Another study demonstrated the same results by measuring methylated circulating tumor DNA (met-ctDNA) levels during neoadjuvant chemotherapy [18]. ctDNA detection can predict the curative effect of neoadjuvant chemotherapy, reduce overtreatment, and timely detect poor therapeutic effect in patients. At present, it is believed that delayed clinical diagnosis and failure in early detection of micro-lesions are the major difficulties in treating metastatic breast cancer. Prediction of recurrence and metastasis and evaluation of prognosis are important for high-risk breast cancer patients. Garcia-murillas *et al* detected mutated genes in the primary lesions of 55 patients receiving neoadjuvant chemotherapy, and the tumor-specific somatic mutations in the plasma of 43 patients at different time points by ddPCR [15]. The results showed that ctDNA could be detected in about 80% patients with recurrence and metastasis during postoperative follow-up and in about 50% patients in the first postoperative test. In addition, ctDNA could predict recurrence and metastasis 7.9 months earlier than clinical imaging [15].

Triple negative breast cancer

Triple negative breast cancer (TNBC) is a highly invasive disease with a high recurrence rate and a low overall survival rate. The plasma ctDNA of TP53, a frequently mutated gene, was analyzed in a study of 50 patients with TNBC after neoadjuvant therapy (NAT) [19]. Targeted next-generation sequencing (NGS) was used to identify the mutated gene in the biopsy tissue. ddPCR was used to monitor the mutated gene in the plasma after the mutated gene was identified, so as to dynamically monitor the development of the tumor. In a study including 40 patients of early non-metastatic TNBC with TP53 mutation, ddPCR detection showed that the increased ctDNA level was associated with tumor progression during neoadjuvant chemotherapy, indicating poor prognosis. Therefore, the detection of plasma ctDNA in TNBC patients during NAT can timely identify the tumor gene mutation, dynamically monitor tumor development, and predict the recurrence of TNBC after NAT [20].

Olsson *et al* demonstrated that monitoring ctDNA could detect metastatic tumors in early breast cancer after treatment. Using ddPCR, ctDNA with genetic mutations was detected in the patient's plasma earlier than the detection of metastasis by clinical imaging. Hence, ctDNA can facilitate the identification of metastasis and recurrence, and avoid puncture biopsy when imaging cannot identify the metastasis [5]. In a study on ctDNA detection rate and prognostic significance in patients with metastatic TNBC, TP53 mutation was detected in 84% of tissue samples, and the detection rate of ctDNA in mutated TP53 in patient's plasma was up to 81%, suggesting that ctDNA with TP53 mutation may be a biomarker for TNBC patients (**Table 1**). As for the prognostic value of ctDNA, it is believed that the ctDNA content change has a great prognostic influence, and the prognostic significance of ctDNA may vary in different types of breast cancer [21].

Early diagnosis of ovarian cancer

Early detection can improve the survival rate of ovarian cancer patients. The five-year survival rate is up to 90% for early treatment, and only about 20% in the late stage. A meta-analysis by Zhou *et al* concluded that ctDNA showed a specificity of 90% and a sensitivity of 70% in the early stage of ovarian cancer. The results of RT-PCR showed the plasma ctDNA level of ovarian cancer patients was significantly higher than that of the healthy people, with a significant difference in the early stage [22]. Therefore, the quantitative detection of ctDNA can be used for the screening and early diagnosis of ovarian cancer, but whether the amount of ctDNA is related to the size and location of the ovarian tumor needs further investigation. ctDNA analysis could reveal mutations and methylation in the genome of tumor cells [23]. Liggett *et al* showed that methylation of *RASSF1A, CALCA* and EP300 could be used as biomarkers to distinguish benign and malignant ovarian cancer tissues, and the detection of multiple genes could improve the sensitivity

[24]. Bettegowda *et al* studied 640 patients with malignant tumors (including ovarian cancer), and found that 96% of the patients had mutations in one or more genes involved in the mitogen-activated protein kinase pathway [25]. These findings suggest that testing of ctDNA methylation or mutation may help the diagnosis of ovarian cancer. Genetic mutations indicate the need to modify the chemotherapy regimen or use new sensitive chemotherapy drugs in patients undergoing second-stage chemotherapy. ctDNA mutation detection can also be performed in early diagnosis of tumors. Phallen *et al* detected somatic mutation in 68% of early-stage ovarian cancer tissues, which was highly consistent with the result of ctDNA detection. Studies have used the cfDNA integrity index (the ratio of long cfDNA fragments to short cfDNA fragments) to assess and stratify tumor risk factors [26]. In a study on the ALU repeated sequence of plasma samples from cases of ovarian cancer, ovarian cyst and healthy women, the integrity index, ALU fragment and integrity index of ovarian cancer group were significantly higher than that of the control group [27]. ctDNA concentrations are higher in aggressive and metastatic tumors like high grade serous ovarian cancer (HGSC). To determine whether ctDNA detection can be applied in localized ovarian cancer, ctDNA-specific mutations in adult ovarian granulosa cell tumors (AGCTs) can be examined. Farkkila *et al* tested the plasma FOXL2 gene in 33 AGCTs patients, and found that 36% of the patients were positive for mutation, which preliminarily proved that ctDNA could be used for the diagnosis of AGCTs [28].

Monitoring and evaluating prognosis of ovarian cancer

Brca1/2 and *TP53* mutations are common in ovarian cancer. Brca1/2 mutation, which is associated with chemotherapy resistance of ovarian cancer, can be used to monitor and evaluate the prognosis of patients. Ratajska M *et al* [29] detected the brca1/2 somatic mutation of ctDNA in tumor tissues of 121 ovarian cancer patients and found that ctDNA detection could cover all brca1/2 mutations in tumor tissues, and progression free survival (PFS) was significantly longer in Brca reverse mutation-negative patients than in mutation-positive patients. Kim YM *et al* [30] detected plasma TP53 mutations in HGSC tissues. The allele count (*TP53-MAC*) of the mutant gene showed that the mutation content of *ctDNA in TP53* was positively correlated with the degree of surgical tumor reduction of ovarian cancer. According to the ctDNA count three months after the last chemotherapy, the patients were divided into the high *TP53MAC* group (≥0.2 copy/L) and the low *TP53MAC* group (<0.2 copy/L). The time to disease progression (TTP) of patients in the high *TP53MAC* group was significantly shorter, indicating the correlation between TP53MAC and TTP.

TP53 ctDNA monitoring was more sensitive than CA125 for residual lesions after treatment. After detecting ctDNA of *TP53MAC* in 318 ovarian cancer patients before treatment, Parkinson *et al* [31] found *TP53MAC* was positively correlated with tumor size, and the correlation was enhanced after drainage of cancerous as cites. When testing TP53MAC after the chemotherapy, they found patients with more than 60% decrease of *TP53MAC* were more sensitive to chemotherapy, together with longer TTP and better prognosis. Therefore, postoperative ctDNA test can evaluate the prognosis of ovarian cancer and provide evidence for postoperative residual lesions, so as to identify patients with high recurrence risk earlier. Thomsen *et al* [32] conduct a clinical trial on 23 patients with advanced platinum-resistant ovarian cancer using the combination of bevacizumab and trienol. After one cycle of chemotherapy, the patients were grouped according to the plasma level of HOXA9 tumor-specific methylated DNA (hoxa9-meth-ctdna). The median PFS was 7.8 months for those with stable or declining hoxa9-meth-ctdna levels, and the median OS was 12 months. In patients with high hoxa9-meth-ctdna level, the median PFS was 1.4 months and the median OS was 4.3 months, showing significantly shortened PFS and OS. However, this association was not found in mutations of tumor tissues, suggesting that ctDNA mutation level is extremely sensitive in treatment response and prognosis assessment.

Cervical and endometrial cancer

In recent years, the incidence of cervical cancer, a most common gynecological malignant tumor, is on the rise, especially in younger

women, posing a great threat to women's health [33]. The diagnosis approach of cervical cancer is largely based on screening, including human papillomavirus (HPV) test, cervical fluid based thin layer cytology (TCT), colonoscopy and cervical tissue biopsy, which are cumbersome and uncomfortable [34]. Liao L *et al* investigated the relationship between plasma ctDNA and cervical cancer [35] and showed that the plasma ctDNA content in cervical cancer patients was relatively higher than the healthy control. In addition, significant differences were found in the clinicopathological features such as different histological grades, depth of interstitial infiltration, lymphatic metastasis and Figo stage. ctDNA is expected to be an effective indicator for clinical diagnosis, treatment and prognosis of cervical cancer (**Table 1**) [36].

ctDNA in the serum of endometrial or ovarian cancer patients has been comprehensively studied. The results of Huang WQ's study suggested that the sensitivity of ctDNA was similar to that of CA125 in diagnos endometrial cancer and ovarian cancer, but ctDNA had a higher specificity than CA125. By comparing the levels of ctDNA before and after surgery, a significant correlation was found between the loss of ctDNA after surgery and the patient's prognosis (OS and PFS). Among the 10 patients with follow-up data, the 4 patients with an average ctDNA level >10 copies/ml all died of the disease. The 5 patients with no ctDNA detected after surgery all survived at the end of the experiment, and 2 of them survived for more than 5 years. But the researchers also found that the preoperative ctDNA level was not associated with the patient's survival. The results suggested that accurate ctDNA testing could not only predict the patient's prognosis, but also monitor and detect recurrence and metastasis (**Table 1**) [37].

Conclusion and prospects

ctDNA detection and analysis can help in the diagnosis, prognosis and treatment of gynecological tumors. ctDNA can also be used as a biomarker for noninvasive tumors. Because ctDNA testing is still in the research stage and there lacks a standardized management, the effectiveness and practicability of ctDNA testing still needs further exploration before it can be applied in clinical practice. As a new tumor marker, ctDNA promises better personalized therapy and precision medicine.

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Disclosure of conflict of interest

None.

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