Next-Generation Sequencing's Application in ctDNA Detection and Quantification

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Circulating tumour DNA (ctDNA) facilitates longitudinal study of the tumour genome, which, unlike tumour tissue biopsies, globally reflects intratumor and intermetastatis heterogeneity. Despite its costs, next-generation sequencing (NGS) has revolutionised the study of ctDNA, ensuring a more comprehensive and multimodal approach, increasing data collection, and introducing new variables that can be correlated with clinical outcomes. Current NGS strategies can comprise a tumour-informed set of genes or the entire genome and detect a tumour fraction as low as 10^{-5}.

ovarian cancer | circulating tumour DNA | next-generation sequencing | cell-free DNA (cfDNA) | detection | quantification

1. Introduction

Ovarian cancer (OC) is the 7th most frequent gynaecological malignancy worldwide and the main cause of gynaecological cancer death [1][2]. OC is of epithelial origin in 90% of cases, and these can be classified into five different histological subgroups based on the World Health Organization's (WHO) current classification: high-grade serous ovarian carcinoma (HGSOC), endometrioid carcinoma, clear-cell carcinoma, low-grade serous carcinoma, and mucinous carcinoma [3]. Most cases are diagnosed at advanced stages with peritoneal involvement, indicating poor overall survival (OS), despite the best therapeutic efforts [1][4]. However, different subtypes have diverse molecular and phenotypical behaviours, as well as distinct prognosis and treatment options [1].

HEA-4 and CA-125 are the two clinically useful serum protein biomarkers for OC. Only CA-125 is approved for evaluating treatment response and disease recurrence [5][6]. The absence of higher-sensitivity biomarkers capable of early detection and prognostication remains an area of need in the management of EOC [1][4][5][7][8]. In numerous cancers, cell-free DNA (cfDNA) has shown promise in predicting prognosis, assessing treatment response, and recurrence detection [4][7][9]. **Table 1** compares CA-125 and ctDNA as biomarkers of OC.

Table 1. Comparison between Ca-125 and ctDNA as biomarkers of OC.

CA-125	ctDNA
Non-invasive	

CA-125	ctDNA
Can be altered by other coexisting physiological and pathological conditions	
Inexpensive and highly available	Expensive and restricted to specialist centres
Simple methodology	Complex methodology
Results in minutes-hours	Results in days and weeks
Quantitative marker	Quantitative and qualitative markers
One continuous variable	Can generate multiple continuous and discrete variables
Only informative regarding the presence/absence of treatment response and recurrence	Yields more information regarding treatment response and tumour recurrence, like resistance mechanisms and targetable genetic alterations
Directly interpreted by the clinician	Requires specialised interpretation
Easily detected in blood and urine	Low concentrations in biological fluids
The utility is limited to producing tumours (mainly restricted to HGSOC)	Theoretically applicable to all histological subtypes
Established and recognised clinical utility in trials	Clinical utility is debatable and requires confirmation in prospective trials

HGSOC—High grade serous ovarian cancer.

Cell-free DNA (cfDNA) is released by both malignant and healthy cells through apoptosis and other cell death mechanisms. At the same time, additional biological processes, like active secretion and phagocytosis, may also be involved [4][10][11][12]. It can be found in many biological fluids, and its levels are altered by many pathological and physiological conditions, such as physical activity or sepsis [4]. Particularly, cancer-bearing patients have more plasma cfDNA in the bloodstream than healthy individuals. Subtracting the amount of cfDNA originated in healthy cells and in cells from the tumour microenvironment under hypoxia, only a small percentage of cfDNA originates from tumour cells and is considered circulating tumour DNA (ctDNA), varying between 0.01 and 93% of all plasma cfDNA [4][10][13].

The quantity of plasma ctDNA differs according to the type and location of the tumour, the stage of the disease, and the treatment received by the patient [10][14]. Despite evidence of a lower ctDNA shed from peritoneal metastasis of gastrointestinal cancers when compared with visceral metastasis [15], ctDNA can be easily obtained in samples of ovarian cancer, particularly in those with advanced-stage disease [11][14][16][17]. Also, genetic alterations may influence ctDNA release. In *TP53* and *KRAS*-mutated lung cancer, higher ctDNA rates were detected, possibly due to higher tumour aggressiveness or cellular turnover [11][18]. *TP53* mutations are the defining feature of the most common type of ovarian cancer—HGSOC—and they may help explain, together with the

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frequently advanced stage of the disease, the literature reports a high ctDNA detection in HGSOC compared with other histological subtypes of OC, ranging from 75–100% of all cases [4][14].

cfDNA has a variable half-life depending on the amount shed and clearance capability of metabolizing organs, like the liver and spleen, and plasma circulating enzymes, ranging from 16 min to 2.5 h [10][11][12]. Whilst this characteristic challenges cfDNA sample collection and analysis, it allows for real-time assessment of tumour genetic characteristics. Also, in cancer patients, impaired cfDNA clearance results in higher ctDNA concentrations in the bloodstream [10].

cfDNA includes coding and non-coding nuclear and mitochondrial DNA (mtDNA) and generally ranges from 40 to 200 bp in size [10][11]. These shorter fragments originate through caspase-dependent cleavage during apoptosis, with a peak at 160 bp corresponding to nucleosomes [10][11]. Shorter fragments (<100 bp) seem to be enriched in ctDNA, carrying tumour-driven genetic alterations. Conversely, longer cfDNA fragments (>200 bpm) suggest more DNA integrity and are more frequent in cancer patients, possibly because cancer cells die more irregularly, from necrosis and autophagy, than healthy tissues, especially in advanced tumours [11]. Enrichment by size, but also according to nucleosome positioning and the cleavage site nucleotide motifs of cfDNA, may therefore augment ctDNA study outputs [11][19][20].

Theoretically, ctDNA represents a picture of the genetic patrimony of the tumour, corresponding to all metastatic niches, that can be studied and compared longitudinally through time [11][13]. Due to its multiple origins, simple cfDNA quantification is not informative, and identification and quantification of ctDNA require a qualitative analysis, which can be informative in itself [1][10]. Tracked qualitative changes can be genomic alterations, which include insertions/deletions (indels), single nucleotide variants (SNVs), gene fusions, and copy number alterations (CNAs), but also preferred DNA end motifs, chromosomal instability, DNA methylation profiles, and nucleosome footprints [10][11]. Quantitative and digital polymerase chain reaction (PCR) and targeted and non-targeted next-generation sequencing (NGS) are common approaches for ctDNA quantification [10][13]. While PCR focuses on a few known genomic markers, non-targeted NGS allows for the identification of multiple known or new alterations, allowing for a more comprehensive analysis and improved diagnostic capacity [21][22].

2. Choosing NGS for ctDNA Detection and Quantification

NGS of cfDNA has two main goals: to detect the presence of ctDNA with the highest sensitivity and specificity possible and to characterise the tumour genome [21]. The most commonly used sequencing approaches to study cfDNA in OC patients are targeted sequencing, whole exome sequencing (WES), and whole genome sequencing (WGS) [4]. Targeted sequencing focuses on specific regions of the genome that can be predefined or personalised according to the results obtained by sequencing the primary tumour or baseline plasma samples, allowing mainly the detection of SNVs and indels [4][12]. In targeted sequencing, enrichment is applied to select regions of interest against the whole genome background, making it more accurate and reducing costs [23]. Enrichment can be amplicon-based, via targeted amplicon deep sequencing (TAm-Seq), or hybrid capture-based, using biotinylated oligonucleotide probes that hybridize with the regions of interest [4][21][23]. WGS and WES are more comprehensive

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techniques, targeting the whole genome or only the protein-coding genes, respectively [21]. They produce more data and allow the study of different genetic alterations, including the detection of CNAs, rearrangements, and chromosomal abnormalities [12].

To detect ctDNA, digital droplet PCR may be used, but it requires a pre-identified genetic target [4][24]. With a higher cost, NGS allows for the analysis of multiple somatic or germline genetic alterations, thus allowing for ctDNA detection based on the identification of multiple targets simultaneously [4][22]. A commercially available platform can be used, or it can be created based on a comprehensive genome study (WES or WGS) of each patient's tumour (tumour-informed), which allows for a more selective strategy of ctDNA detection through a single sequencing process. This is of particular importance when the quantity of cfDNA is a limiting factor [4][21].

Cancer Personalised Profiling by Deep Sequencing (CAPP-Seq) is a less expensive and highly sensitive method first implemented for non-small cell lung cancer. It uses WES data from 407 tumours to build a targeted sequencing platform to cover the most frequently altered 521 exons and 13 introns [25]. Pereira et al. (2015) used a similar approach, with digital PCR (dPCR) in OC, combining somatic NGS for identifying genetic alterations in 22 ovarian carcinomas (21 of serous histology). ctDNA quantification by NGS-guided dPCR yielded a high diagnostic sensitivity (99%) and specificity (81%) for OC, accompanied by a ctDNA detection rate superior to 93% [4][26]. dPCR is as sensitive and more cost-effective than molecular barcoded NGS in the detection of *TP53* mutations in longitudinal cfDNA if a tumour-informed approach is used [24].

Considering the use of NGS, the limit for ctDNA detection varies between 0.03% and 1% and depends on numerous factors: the quantity of cfDNA, the number of genetic alterations tracked by the test, the coverage of each test for a specific locus, and the technology used [4][21]. The percentage of mutant reads, considering the total number of reads in a sample, designated by variant allele frequencies (VAFs), is influenced by the amount of ctDNA available, which varies according to tumour burden and stage [10][21]. For this reason, in order to be generally applicable, detecting even low VAFs (<1%) is a requirement of NGS-based platforms [21].

Increasing the number of targeted regions, or conducting WES or WGS (i.e., increasing the breadth of the platform), increases the probability of encountering a mutated fragment of tumoral origin among all cfDNA fragments in an obtained plasma sample [21]. Coverage, or depth, refers to the number of reads that align with or "cover" a specific nucleotide sequence; therefore, higher depth also increases the confidence in detecting a particular alteration in a specific locus and improves the detection of rare variants caused by tumour heterogeneity (low cellular representation of a specific clone) or low ctDNA levels [21].

In all stages of cancer, including the early stages, when the number of ctDNA fragments in the plasma is lower, targeted sequencing ctDNA detection is limited to a tumour fraction (TF) of 10^{-3}, with TF being the percentage of cfDNA that is ctDNA, according to the NGS output [27]. These results were obtained in 200 patients' cfDNA samples, 42 of which had ovarian cancer. Using a targeted error correction sequencing (TEC-Seq) methodology with a [27][28]. Despite covering only 55 genes, the amplicon-based TEC-Seq method uses a predefined small number of barcodes (molecular identifiers) to identify each molecule of ctDNA and redundant sequencing to reduce

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depending on fragment length and the type of variant to be analysed, but they seem to outperform the conventional platforms [35]. AI-based variant calling helps to lower the costs and complexity of NGS protocols and yields robust results even with low sample quantities, which is of particular interest during neoadjuvant treatment and in detecting minimal residual disease. However, the clinical applicability of these tools needs to be determined within clinical trials [32].