

Preoperative ctDNA Levels Are Associated With Poor Overall Survival in Patients With Ovarian Cancer

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Abstract. *Background/Aim:* Circulating tumor DNA (ctDNA), which is shed from cancer cells into the bloodstream, offers a potential minimally invasive approach for cancer diagnosis and monitoring. This research aimed to assess the preoperative ctDNA levels in ovarian tumors patients' plasma and establish correlations with clinicopathological parameters and patient prognosis. *Patients and Methods:* Tumor DNA was extracted from ovarian tumor tissue from 41 patients. Targeted sequencing using a panel of 127 genes recurrently mutated in cancer was performed to identify candidate somatic mutations in the tumor DNA. SAGAsafe digital PCR (dPCR) assays targeting the candidate mutations were used to measure ctDNA levels in patient plasma samples, obtained prior to surgery, to evaluate ctDNA levels in terms of mutant copy number/ml and variant allele frequency. *Results:* Somatic mutations were found in 24 tumor samples, 17 of which were from ovarian cancer patients. The most frequently mutated gene was TP53. Preoperative plasma ctDNA levels were detected in 14 of the 24 patients. With higher stage, plasma ctDNA mutant concentration increased (p for trend <0.001). The overall survival of cancer patients with more than 10 ctDNA mutant copies/ml in plasma was significantly worse ($p=0.008$). *Conclusion:* Pre-operative

ctDNA measurement in ovarian cancer patients' plasma holds promise as a predictive biomarker for tumor staging and prognosis.

Ovarian cancer (OC) is one of the most prevalent and lethal gynecological cancers with more than 300,000 new cases diagnosed and more than 200,000 deaths recorded in 2020 worldwide (1). The 5-year survival rate of ovarian cancer has remained in the range of 40-50% in the past 20 years despite diagnostic and therapeutic advancements (2). When diagnosed early, the prognosis of ovarian cancer is much better, with a 5-year survival rate of 73-92% for tumors diagnosed at stage I, compared to $<6\%$ for tumors diagnosed at stage IV (3).

Early detection of minimal disease can be crucial for early surgical intervention and improve the outcome for the patient. However, due to the lack of suitable biomarkers, effective screening of ovarian cancer is yet to be established. Population screening trials like the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO) (4) and Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) (5) have been performed to improve screening to enable earlier diagnosis of ovarian cancer; however still no excellent screening method has been found. For women with average to high risk of developing ovarian cancer, transvaginal ultrasound (TVUS) and CA-125 and HE4 blood tests are advised for screening of the disease, but no significant improvement in survival has been observed with these tests (6). Protein biomarkers and combinations of biomarkers have good sensitivity to indicate malignancy in women with pelvic mass, but the specificity needs to be improved when women with pelvic tumors are evaluated before surgery (7). At follow up of ovarian cancer after primary treatment, analysis of CA-125 and HE4 in combination with computed tomography (CT) scan is usually performed when suspicion of relapse.

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Table I. Genetic mutations found in the tumors related to histopathology.

Gene mutations in tumor	Morphology								Total	
	Benign**	Endometriosis**	Teratoma**	Serous borderline	Serous cancer	Endometrioid borderline	Endometrioid cancer	Mucinous borderline		Mucinous cancer
<i>TP53</i>	0	0	0	0	12	1	0	0	1	14
<i>KRAS</i>	0	0	0	2	0	0	1	4	0	7
<i>PIK3CA</i>	0	0	0	0	0	0	2	0	0	2
<i>ASXL1*</i>	1	0	0	0	0	0	0	0	0	1
<i>BRAF</i>	0	0	0	1	0	0	0	0	0	1
<i>FLT3*</i>	0	0	0	0	1	0	0	0	0	1
<i>KIT*</i>	1	0	0	0	0	0	0	0	0	1
<i>PIK3R1</i>	0	0	0	0	0	0	1	0	0	1
No detected	2	1	1	1	6	0	0	0	2	13
Total	4	1	1	4	19	1	4	4	3	41

*Germline mutation. **Benign histopathology.

In recent years, cell-free circulating tumor DNA (ctDNA) has emerged as a promising tumor biomarker (8). Through cell apoptosis, necrosis and other mechanisms of tumor cell death, small pieces of DNA are released into the bloodstream. These pieces are called cell-free circulating tumor DNA. ctDNA can be used for diagnosis, monitoring of treatment response and/or resistance, detection of latent disease, and prediction of outcome (8). It has been reported that elevated preoperative total plasma cell free DNA levels are correlated to events of advanced epithelial ovarian carcinoma (9). Advances in deep-sequencing technology enable comprehensive cataloguing of tumor-specific (somatic) chromosomal rearrangements and mutations, which can then be detected in follow-up plasma samples using mutation detection technologies (10).

The aim of the study was to measure tumor-specific mutations in plasma in ovarian cancer and borderline ovarian tumors after targeted sequencing of primary tumor tissues and to relate the ctDNA measurements to clinicopathological features and patient outcome. Tumor-specific mutations in ovarian tumors were identified by targeted sequencing, which informed ctDNA analyses in matched plasma samples using the ultrasensitive SAGAsafe digital PCR method.

Patients and Methods

Sample collection. Peripheral plasma samples were obtained preoperatively (the day before surgery or the same day) from 41 patients admitted for primary surgery of adnexal masses to the Department of Obstetrics and Gynecology in Lund, Sweden, between October 2004 and December 2012. Fresh frozen tumor biopsies from the same patients were also obtained and all samples stored at -80°C until analyzed. The 41 patients were selected as a

representative sample of ovarian tumor patients from the biobank. All data were grouped according to tumor type and stage of cancer: benign (B), borderline (BOT) and ovarian cancer (OC Stage I-IV).

Sequencing. DNA was extracted from tumor samples using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) between 2018 and 2020. Tumor DNA samples were sheared to an average of 250 bp using a Covaris ultrasonicator before generating Illumina-compatible sequencing libraries using the KAPA HyperPrep Kit (Roche, Pleasanton, CA, USA). Total library yields were measured using Qubit (ThermoFisher, Cambridge, UK) and size distributions checked with BioAnalyzer (Agilent, Santa Clara, CA, USA) before adding equimolar amounts of each library into pools subjected to target enrichment. Targeted regions of the 41 libraries were hybridized according to the manufacturer’s protocol with the xGen Pan-Cancer Panel (Integrated DNA Technologies; IDT Inc, Coralville, IA, USA) containing 127 cancer-associated genes or a custom xGen Lockdown Probes panel covering exons and hotspots in 14 genes recurrently mutated in cancer (IDT). The hybridized libraries were sequenced using Illumina NextSeq 550 or MiSeq (Illumina Inc, San Diego, CA, USA).

Sequencing data was processed using a Snakemake workflow (11) and Bioconda (12) software packages. Basecalls were demultiplexed by sample and converted to unmapped BAM format using Picard v2.21.1, and unique molecular barcodes (UMIs) were extracted using fgbio v0.7.0. (<https://github.com/fulcrumgenomics/fgbio>) Reads with matching UMIs (allowing for a maximum 1 base mismatch) were collapsed to generate consensus reads. These were aligned to the hg38 version of the human reference genome using BWA v0.7.17 (13). Variants were called using VarDict-Java v1.7.0 (14). Annotation was performed using vcfanno v0.3.1 (15) and the data sources COSMIC v87 (16), dbSNP build 151 (17), as well as the Swedish and Danish human variation databases. To remove germline variants and sequencing artifacts, all variant calls that were also found in normal samples from five healthy donors were filtered, and only retained candidate variants as somatic that a) passed basic VarDict-Java criteria (“PASS” variants), b) were not in sequence repeat regions,

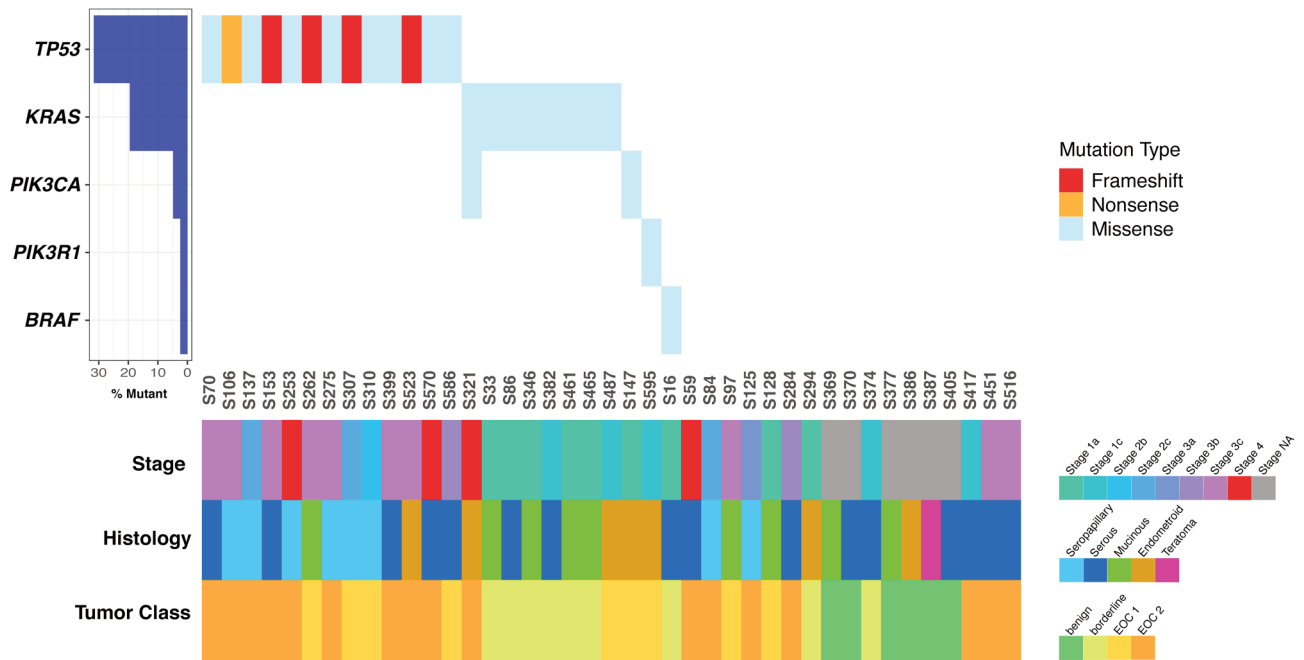


Figure 1. Waterfall plot of validated somatic mutations in patient tumors. Genes are indicated in rows, and samples in columns. Mutated samples are shown according to mutation type. Patient and tumor clinicopathological variables are shown below the patient IDs.

and c) were not known common germline variants in the population according to the dbSNP and COSMIC databases, and the Swedish and Danish variation data (defined as >1% population frequency).

Cell-free DNA was isolated from patient plasma samples using the QIAamp MinElute cfDNA kits (Qiagen). CA-125 was analyzed at the routine laboratory at Lund University Hospital with ELISA. For each patient, candidate somatic mutations were selected and SAGAsafe digital PCR (dPCR) assays or developed (SAGA Diagnostics AB, Lund, Sweden). All assays were validated using positive control tumor DNA and wild-type negative control DNA samples. One individual assay was selected for each patient. The SAGAsafe technology detects point mutations and small indels down to 0.001% variant allele frequency. These assays provide quantitative result in terms of mutant- and wild-type copy number and the mutant allele frequency (MAF) for an analyzed sample. The assays were developed and validated on the Bio-Rad QX200/QXDx Droplet Digital PCR System (Hercules, CA, USA).

Statistical analysis. One-way ANOVA with Bonferroni correction as *post hoc* test and trends across ordered groups were analyzed using linear regression with log-transformed values. Overall survival probabilities were calculated using the Kaplan–Meier method and the log-rank test. All comparisons were two-sided, and a 5% level of significance was used. The statistical analyses were performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA).

Ethics approval and consent to participate. Written informed consent was obtained from all study participants. Ethical approval was granted by the Ethical Review Board at the Faculty of

Medicine, Lund University, Sweden. Dnr 495 2016 (amendment to Dnr 558-2004 and 94-2006).

Results

Patient data were grouped according to tumor type as follows: Benign ($n=6$), BOT ($n=9$) and ovarian cancers ($n=26$). The benign group generally consisted of younger patients (mean age=48 years \pm 13.3) compared to the cancer group (64 years \pm 11.9). Preoperative CA-125 levels in the BOT group were 293.7 ± 627.3 units/ml and in the cancer group $1,264.3 \pm 1,147.1$ units/ml. Somatic mutations were found in 24 tumor samples (seven were in BOT and 17 in cancer patients). *TP53*, *KRAS*, *PIK3CA*, *PIK3R1*, and *BRAF* were the most recurrently mutated genes in this cohort (Table I and Figure 1). In stage III ovarian cancer, there were eight tumors with a *TP53* mutation and one with *PIK3R1* mutations (Figure 1). *KRAS* mutations were found in four mucinous borderline tumors and two serous borderline tumors.

The mutations detected in plasma were in *TP53*, *KRAS*, *PIK3CA*, and *PIK3R1*. Seven patients with stage III, three with stage IV and four with stages I and II of ovarian cancer had detectable ctDNA in the plasma. All serous cancer patients with detectable ctDNA showed *TP53* mutations, and one serous borderline patient had a *KRAS* mutation in plasma. The concentration of ctDNA increased with higher stage (p for

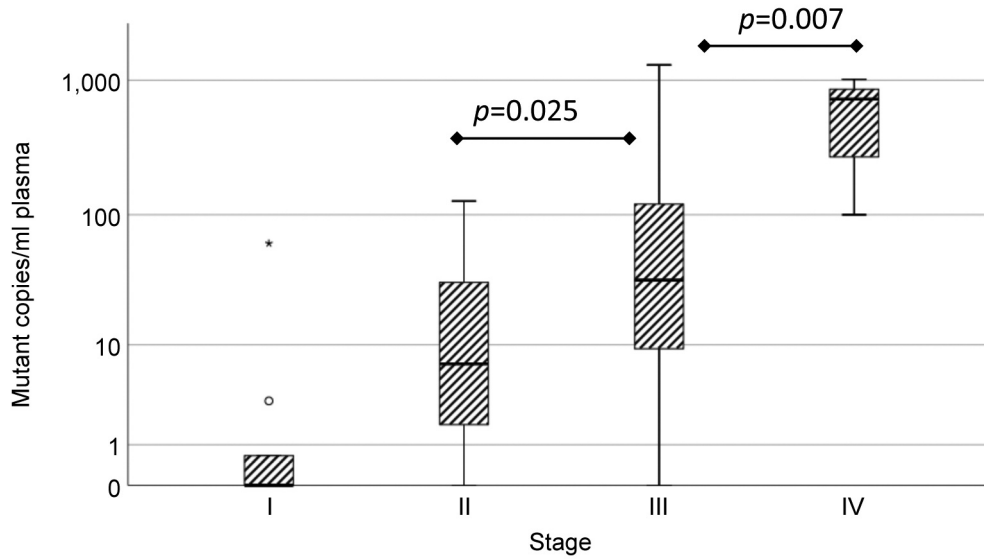


Figure 2. Plasma circulating tumor DNA (ctDNA) mutant concentration increased with higher stage (p for trend <0.001). Bars show the highest and lowest values, except outliers (\circ), which are 1.5 to 3 box lengths from the end of the box, and extremes ($*$) which are more than 3 box lengths from the end of the box. Concentrations of ctDNA in stage III and IV were significantly higher compared with stage I ($p=0.025$ and 0.007 , respectively). One-way ANOVA with Bonferroni correction and trends across ordered groups were analyzed using linear regression with log-transformed values.

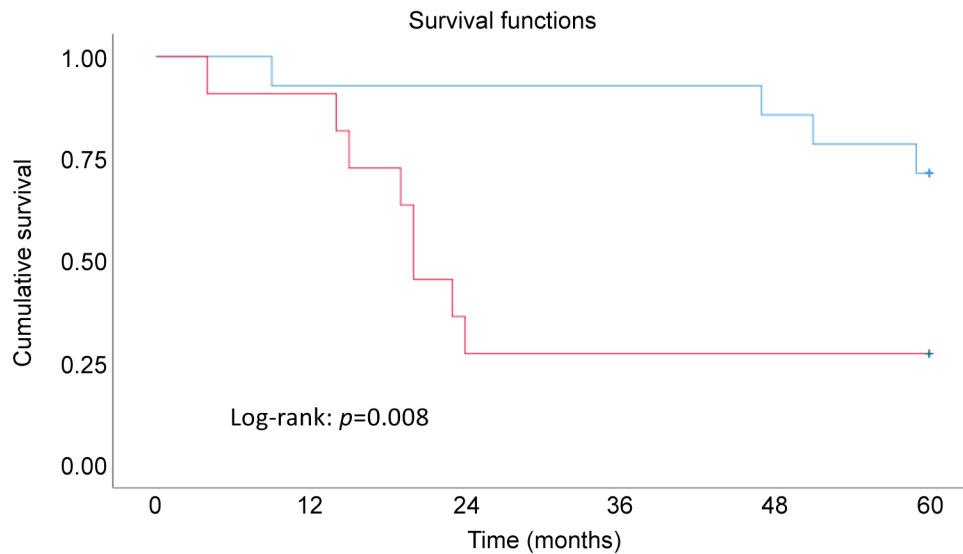


Figure 3. Kaplan Meier analysis of overall survival in patients related to genetic mutation found in plasma (OS, $p=0.008$ by log-rank test). The blue line corresponds to <10 copies/ml and the red line >10 copies/ml. Among patients with ctDNA levels below 10 copies/ml, 2 out of 3 experienced mortality, while among those with ctDNA levels exceeding 10 copies/ml, 10 out of 11 patients faced mortality.

$trend <0.001$). Concentrations of ctDNA in stage III and IV were significantly higher compared with stage I ($p=0.025$ and 0.007 , respectively) (Figure 2). The number of 10 mutant copies per ml for the survival analyses was chosen after

studying the results (Figure 2), in which the number of mutant copies is shown related to stage. Cancer patients with more than 10 mutant copies/ml in plasma showed significantly worse overall survival (OS, $p=0.008$ by log-rank test) (Figure 3).

Discussion

In this study, ctDNA was measured in plasma samples from ovarian cancer patients and borderline ovarian tumor patients using the SAGAsafe dPCR technology. The plasma levels of ctDNA mutations were increased in patients with higher stage of disease. In patients with ovarian cancer, higher levels of ctDNA in plasma were associated with worse OS. These results indicate that ctDNA analyzed in plasma can be useful pre-operatively both as a diagnostic and as a prognostic biomarker in ovarian cancer.

Specific genomic alterations in ctDNA can guide cancer treatment. The most frequently mutated gene in these patients with ovarian tumors was *TP53*, especially in advanced stage OC. Several studies have shown that *TP53* mutations are significantly associated with advanced OC since they are found in more than 90% of the high-grade serous ovarian carcinomas (18). Loss of p53 function makes cells unable to induce apoptosis and therefore primes these cells for transformation into malignancy (19, 20). Most mutations are single-base substitutions distributed throughout the coding sequence. *TP53* mutations have been shown to be prognostic and targets for pharmacological intervention (21). In the current Swedish national guidelines (RCC), PARP inhibitors are recommended for use in treatment of BRCA-mutated epithelial ovarian cancer (22). PARP inhibitors have demonstrated efficacy in BRCA-mutated ovarian cancer (22) as well as in other malignancies characterized by homologous recombination deficiency. Only a few studies have been performed to evaluate the efficacy of PARP inhibitors in terms of *TP53* status (23), and it has been presumed that targeting mutant p53, which destabilizes PARP repair function, may impede the distant spread of cancer cells (24).

KRAS gene mutations were found in seven tissue specimens, and one was found in plasma ctDNA. *KRAS* is a member of the Ras family of oncogenes, which also includes two other genes: *HRAS* and *NRAS*. The proteins these genes encode play important roles in cell division, cell differentiation, and apoptosis. Six mutations in *KRAS* were found in borderline tumors. Low-grade serous carcinomas (LGSOC) may progress from borderline tumors with frequent mutations of the *KRAS*, *BRAF*, or *ERBB2* genes and lack of *TP53* mutations (25). Two multicenter trials studying MEK inhibitors in LGSOC demonstrated activity in LGSOC, especially in *KRAS*-mutated disease. Accordingly, MEK inhibitors could be an alternative treatment in LGSOC (26). In patients with advanced malignant melanoma with *BRAF* mutations, *KRAS* inhibition (KRASi) has shown good results (27). In lung adenocarcinoma patients with co-occurring *TP53* mutations, clinical benefit has been demonstrated from PD-1 inhibitors and mutation status may guide anti-PD-1/PD-L1 immunotherapy (28).

Concordance between tumor DNA and ctDNA. Various plasma ctDNA technologies are being tested in academic and commercial laboratories (29). Plasma ctDNA testing has a promising role in follow up as a tumor marker, especially when treatment is based or monitored on a gene mutation profile (30). In this study, it was found that 15 out of 24 patients with tumor DNA mutations had mutations detected in plasma ctDNA, which corresponds to a sensitivity of 62.5%, in line with previous reports (31). However, the possibility to detect ctDNA mutations in plasma depends on the morphology of the tumor, the stage of the disease, tumor burden, as well as DNA degradation (31).

ctDNA in relation to stage. The plasma ctDNA mutant concentration increased with higher stage, which is in accordance with previous studies (32). In late-stage cancer, especially in abdominal organs, such as colon, pancreas, or ovaries, ctDNA has been commonly detected in more than 60% of patients (33). In this study ctDNA was detected in two patients in stage I OC. Interestingly, our study also detected ctDNA mutation in plasma in one patient with a borderline tumor, which has not previously been reported. Other studies observed that tumor volume assessed by CT imaging correlated with ctDNA levels in patients with relapsed high-grade serous ovarian cancer (34). Patients with advanced ovarian cancer have had median concentrations of 100–1,000 mutated gene copies per 5 ml of plasma (34). The analyses in this study were able to detect less than 10 copies/ml in plasma in the early stage of the disease, indicating very high sensitivity. The quantity of ctDNA in plasma correlates with tumor size and disease stage, as indicated by our observations and previous research (35).

ctDNA mutations/ml plasma as a prognostic factor. Confirming the aim of our study, that it is possible to detect ctDNA in ovarian cancer patients, we can further conclude that our results showed that patients in advanced stage of OC had more mutated gene copies than in early stage of disease. The prognosis for ovarian cancer patients is highly related to stage (36). Similar studies with gynecologic cancers have found that higher percentage of ctDNA correlated with worse survival (37). In colorectal cancers ctDNA has found to be a prognostic factor for disease recurrence (38), and another study identified ctDNA as a potential independent factor (39). In addition, higher ctDNA concentration significantly correlated with worse progression-free survival (PFS) (40). Our results showed that patients with more than 10 ctDNA mutation copies/ml plasma had significantly worse overall survival.

Strengths and limitations. The findings of this study have demonstrated the potential clinical utility of ctDNA analysis in detecting ovarian cancer and predicting survival. However, it is important to note that the study was

conducted on a relatively small patient cohort and only genetic variants in the coding regions of genes were investigated. Previous studies have found that variants located in the intronic, and non-coding regions of genomes may play a role in identifying individuals at genetic risk of developing ovarian cancer (41). This and other sequencing studies have limitations in identifying genetic variants associated with OC in non-coding regions. The presence of ctDNA in the bloodstream is directly proportional to the tumor burden in the body, and patients with a larger tumor burden are more likely to have detectable ctDNA. The sensitivity of ctDNA detection can be influenced by the technology employed for genetic analysis. In this study, high-sensitivity sequencing data and coverage were used to detect ctDNA. The quality and quantity of ctDNA can also be influenced by other factors, such as age and overall health status. For example, ctDNA levels may be higher in older patients due to the natural aging process, while patients with other medical conditions or treatments may have lower levels.

Implications for practice and future research. The study demonstrated the potential clinical utility of ctDNA analysis in detecting ovarian cancer and predicting survival, particularly in advanced stages. However, the results need to be validated in larger patient cohorts before clinical implementation. Further research is needed to explore the detection of genetic variants in non-coding regions and improve the accuracy of ovarian cancer detection using ctDNA analysis. The concentration of ctDNA mutated copies in plasma was found to be related to stage and overall survival, suggesting its potential as a prognostic tool; in larger studies, the optimal cut-off level of mutant copies per ml should be evaluated. In this study, candidate somatic mutations were sequenced from the available tumor material for ctDNA analysis in plasma. In cases where tumor material is not available, broad genomic panels must be designed for ctDNA analysis in plasma. The future use of broader genomic panels may simplify the ctDNA analyses excluding the need for high-sensitivity DNA sequencing analyses of tumor tissue, retaining the high sensitivity of ctDNA analysis and potentially improving the accuracy of OC detection.

Conclusion

This study found that ctDNA mutations could be detected in 15 out of 24 patients with ovarian cancer and pre-malignant ovarian borderline tumors. The most mutated gene was *TP53*. The concentration of ctDNA mutated copies in plasma was related to stage and was higher in advanced OC stages. Patients with an increased amount of ctDNA in plasma experienced worse overall survival. Plasma is easily accessible and ctDNA may be used for prediction of prognosis.

Conflicts of Interest

The Authors are solely responsible for the content and writing of the paper. Lao H Saal has received honoraria from Novartis and Boehringer-Ingelheim. Yilun Chen, Christian Brueffer and Lao H Saal have ownership interests in SAGA Diagnostics. The Authors declare that they have no other conflicts of interest.

Authors' Contributions

AD: Study conception and design; data acquisition, analysis, and interpretation; drafting and revising the manuscript. PL: Data acquisition and revising the manuscript. YC: Data acquisition, analysis, and interpretation; drafting and revising the manuscript. ChB: Data acquisition, analysis, and interpretation; drafting and revising the manuscript. LHS: Study conception and design; data acquisition, analysis, and interpretation; revising the manuscript. ChBo: Study conception and design; data acquisition, analysis, and interpretation; revising the manuscript.

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