Detection of circulating-tumor DNA through liquid biopsies in ovarian cancer and utilization as prognostic factors

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Detection of circulating-tumor DNA through liquid biopsies in ovarian cancer and utilization as prognostic factors

Directed by Professor **Seung-Tae Lee**

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ABSTRACT

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Background: Effective detection of ovarian cancer progression and recurrence is crucial in improving patient prognosis. Existing tests based on biomarkers (CA-125) and radiological imaging are insufficient for the minimal residual disease detection (MRD) of ovarian cancer. This study aimed to assess the feasibility of using circulating tumor DNA (ctDNA) as an additional biomarker for disease progression in ovarian cancer patients undergoing debulking surgery followed by adjuvant therapy.

Methods: We recruited 330 patients suspicious of ovarian malignancy (CA-125 > 35 U/ml). Blood samples were collected between Oct 2019–Mar 2022. Samples were collected at baseline just prior to surgery and every three months thereafter. Conventional post-operative monitoring was performed using CA125, HE4, MRI, and PET-CT. Custom target gene panel targeting nine genes (*TP53, BRCA1, BRCA2, ARID1A, CCNE1, KRAS, MYC, PIK3CA* and *PTEN*). Next-generation sequencing was done with the NextSeq System (Illumina, USA). Data analysis was performed using the custom pipeline PiSeq (Dxome,

Korea). Retrospective chart review was done to obtain relevant clinical information.

Results: We analyzed a total of 813 blood samples from 296 patients, including 201 patients with carcinoma (high-grade serous, low-grade serous, mucinous, clear cell, endometrioid, and others) and 96 patients with benign/borderline ovarian disease. 69.8% (139/199) of epithelial ovarian cancer patients were identified with tier I/II (pathogenic) somatic mutations from preoperative samples at baseline. No pathogenic mutations were identified in benign/borderline tumor patients (0/96). Of the 38 progressive patients with baseline ctDNA mutation, 89.8% (44/49) patients were identified with the same list of mutations at the baseline. In these patients, ctDNA enabled early detection of future progression by an average of 50.9 days (maximum of 267 days) than the conventional diagnostic methods. Based on 6 months follow up ctDNA analysis, persistent elevated group showed a worse median progression free survival (PFS) compared with zero conversion group (7.7 vs 25.3 months; $P < 0.001$).

Conclusions: Our analysis suggests that ctDNA-based surveillance may serve an essential role in the detection of disease progression in ovarian cancer, providing genetic characteristics of ovarian cancer, and applicability of ctDNA in clinical decision making and might help establish personalized therapeutic strategies.

Key words: circulating-tumor DNA; ctDNA; epithelial ovarian cancer; high-grade serous carcinoma

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I. INTRODUCTION

Epithelial ovarian cancer (EOC), the most lethal gynecologic malignancy is the fifth most common cause of cancer-related deaths in women worldwide.[1,2] Most ovarian cancer patients are diagnosed in the advanced stages, approximately 70% of patients with OC are diagnosed at advanced stages (stage III and IV).[3] The most common subtype of epithelial ovarian cancer (EOC) is the high-grade serous carcinoma (HGSC) with a prevalence of 52%, followed by endometrioid (10%), mucinous (6%), and clear cell carcinoma (6%).[4]

The current standard of care (SOC) is primary debulking surgery followed by platinum-based chemotherapy. Recently, targeted therapy agents have been applied, including the vascular endothelial growth factor (VEGF) inhibitor bevacizumab and the poly-ADP-ribose-polymerase (PARP) inhibitor olaparib.[5,6]

Neoplasms of the ovary are diagnosed and monitored by conventional biopsy methods, computerized tomography (CT) scan, positron emitting tomography (PET), and detection of the membrane glycoprotein, known as cancer antigen-125 (CA-125). Imaging studies can help identify mass in the ovary or other organs but do not provide a precise diagnosis or distinguish between malignant or benign lesions.[7] Moreover, detecting possible

metastasis at other organs may be difficult until reaching a sufficient size. On the other hand, tissue biopsy can be complicated and invasive and does not reveal tumor heterogeneity.[8,9]

CA-125 and human epidural protein 4 (HE4) is a characteristic biomarker of OC and is currently a clinical standard for monitoring. However, CA-125 lacks specificity as a screening tool because it can be elevated in other benign diseases (pelvic inflammatory disease (PID) or endometriosis) and malignant tumors (lung cancer or gastrointestinal cancer). Therefore, CA-125 is helpful for disease and treatment monitoring, but it is less reliable for screening or initial diagnosis of OC. The use of alternative biomarkers to support and supplement CA-125 is an unmet need.

Over the past decade, tissue-based NGS studies reported that high-grade serous ovarian cancer is characterized by *TP53* mutations in almost all tumors, some harboring the amplifications of cell cycle regulatory genes, including *MYC* and *CCNE1*.[10,11] Several studies have demonstrated the potential use of liquid biopsies for solid tumor diagnosis and monitoring of response to treatment.[12-14] Circulatory tumor DNA (ctDNA) increases during somatic cell deaths, and DNA fragments are released into blood circulation. ctDNA has very high tumor specificity and can accurately detect the presence of metastatic and minimal residual diseases in many solid tumors.[15,16] Previous studies have discovered the detection of ctDNA in ovarian cancer.[17,18] Few reports analyzed progression-free survival (PFS) with groups divided with *BRCA* reversion mutation or ctDNA concentration.[19,20]

Despite reports of ctDNA detection in ovarian cancer patients, it is unclear if ctDNA analysis is helpful, along with testing tumor markers. Additionally, it is unknown if ctDNA analysis should be tested postoperatively, preoperatively, or both. Recently, ESMO (European society for medical oncology) recommended the use of circulating tumor DNA assays for patients with no germline pathogenic *BRCA1/2* variant [21], mentioning ctDNA analysis might be more informative than CA-125 levels [22]. In this study, we conducted a comprehensive gene mutation profiling of ctDNA in a larger number with serial sampling from ovarian cancer patients. At this point, there seems to be no reports analyzing the

relationship between ctDNA dynamics and patient prognosis. We evaluated the association with ctDNA dynamics and prognostic outcomes to clarify its clinical feasibility in genetic profile-based strategies.

II. MATERIALS AND METHODS

1. Study design

We conducted a prospective cohort study enrolling 330 female patients (median age = 51.0 years) suspicious of ovarian malignancy, and serum CA-125 level is above reference value (CA-125 >35 U/mL). Patients enrolled at Yonsei University Health System from October 2019 to March 2022. This study was conducted according to the Ethics Committee of Yonsei University College of Medicine in Seoul, Korea (approval no: IRB No: 4-2019-0698, ClinicalTrials.gov Identifier : NCT05504174). The patients' primary cancers were staged according to the International Federation of Gynecology and Obstetrics (FIGO) surgical staging criteria. Conventional post-operative monitoring was performed using CA-125, HE4, abdominopelvic computed tomography (APCT), MRI, and PET-CT. All treatments were delivered as per standard of care and blinded to ctDNA results. We performed a retrospective chart review for 3 years to obtain relevant clinical information, including pathological diagnosis, the extension of disease and operation, and adjuvant therapy.

2. Samples

9 mL of blood samples were collected in cell-free DNA collection tubes (Dxome, Korea) for ctDNA analysis. Blood samples were collected at baseline prior to debulking surgery and serial sampled every three months thereafter. In NAC cases, blood samples were obtained pre-, post-NAC and prior to Interval debulking surgery (IDS) (Figure 1).

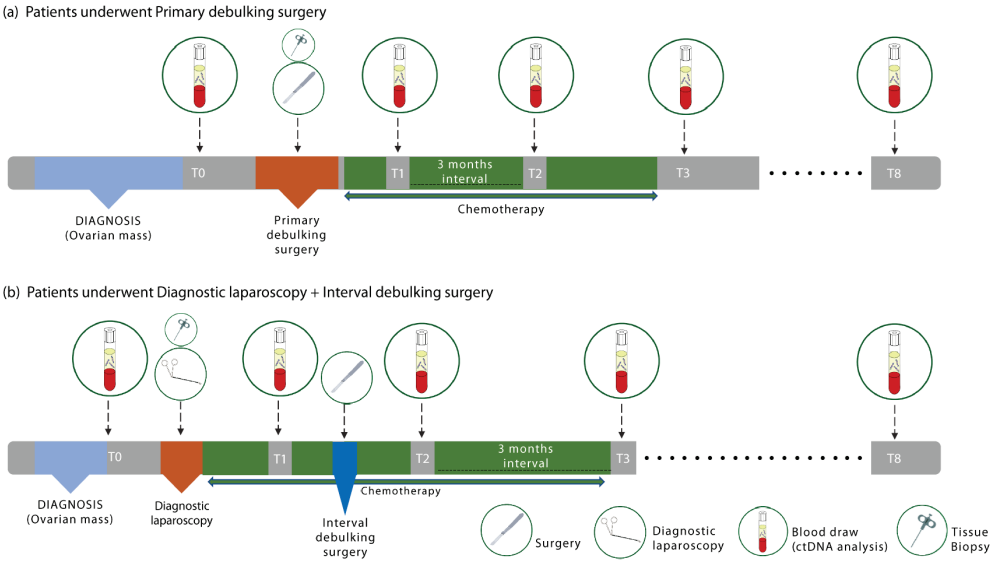


Figure 1. A. Study schema

(a) Diagram showing the study schema of the NCT05504174 trial. Blood samples were collected at baseline prior to debulking surgery and serial sampled every three months thereafter.

(b) Diagram showing NAC cases. Blood samples were obtained pre-, post-NAC and prior to Interval debulking surgery (IDS)

3. ctDNA sequencing (NGS : next-generation sequencing), analysis

Blood samples were aliquoted into ethylenediaminetetraacetic acid-containing tubes, centrifuged at 1600× g for 10 min at 4 °C, and then transferred to fresh tubes. The samples were further centrifuged at 4000× g for 10 min at 4 °C. Plasma samples were stored at –80 °C until ctDNA analysis.

Circulating cell-free DNA (cfDNA) was extracted from 3-4 ml of plasma samples using the Magnetic Serum/Plasma Circulating DNA Kit (Dxome, Korea). The size of cfDNA was measured using the TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA). The cfDNA concentration was measured using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The resulting DNA was ligated using Illumina adapters and then indexed using unique dual indices for duplex sequencing (Illumina, San Diego, CA, USA). Sequencing libraries were hybridized with customized probes targeting nine OC-related genes (*TP53, BRCA1, BRCA2, ARID1A, CCNE1, KRAS, MYC, PIK3CA* and *PTEN*), which are frequently mutated in OC, as shown in previous studies (Supplementary Table. S1).[11,20,23] Enriched DNA was amplified, and the clusters were generated and sequenced on a NovaSeq 6000 System (Illumina, USA) with 2 × 151 bp reads. Mean sequencing depth of 30,000× was targeted. All procedures were performed in accordance with the manufacturer's instructions.

Pi-seq ctDNA analysis pipeline (Dxome, Korea) was used call variants and annotate somatic variants. In Pi-seq, reads were aligned using Burrows-Wheeler alignment tool version 0.7.12 (Wellcome Trust Sanger Institute, Cambridge, UK) to human genomic reference sequences (GRCh37). To identify SNVs and indels, the HaplotypeCaller and Mutect2 in the genome analysis tool kit (GATK) package version 3.8-0 (Broad Institute of MIT and Harvard, Cambridge, MA, USA) and VarScan2 version 2.4.0 (Washington University, St. Louis, MO, USA) was used.

A matched peripheral blood mononuclear cell (PBMC) sample sequencing and review of the previous germline-NGS test were performed on the sample in which the ctDNA variants were detected.

The cut-off of variant allele frequency (VAF) was set to 0.2% when analysing the baseline samples of patients, and cut-off was set to 0.1% when analysing the following serial samples.

4. Genetic variant classification

Pathogenicity of variants was predicted using multiple computational tools (BayesDel addAF, BayesDel noAF, DANN, DEOGEN2, EIGEN, EIGEN PC, FATHMM, FATHMM-MKL, FATHMM-XF, LIST-S2, LRT, M-CAP, MVP, MutPred, Mutation assessor, MutationTaster, PROVEAN, PrimateAI, SIFT, SIFT4G, dbscSNV). We also used various somatic mutation databases including Catalog of Somatic Mutations in Cancer (COSMIC), OncoKB, and The Cancer Genome Atlas (TCGA). Exome Aggregation Consortium, Single Nucleotide Polymorphism Database (dbSNP), 1000 Genomes and Clinvar was also used to check previous reports of variants.

Variants were classified into four tiers based on their clinical significance in cancer diagnosis, prognosis, and therapeutics following the standards and guidelines established by the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists.

5. Tumor-tissue DNA analysis

DNA was extracted from the frozen tissue samples using QIAamp DNA Blood Mini Kit (Qiagen) and from the FFPE tissue using QIAGEN AllPrep FFPE Kit (Qiagen). DNA from five frozen tissues was sequenced using the Twist Human Core Exome Kit (Twist Bioscience, San Francisco, CA, USA), and DNA from the three FFPE tissues was sequenced using the TruSight Oncology 500 (Illumina). After hybridization, paired-end sequencing with 2 × 151 bp reads was performed using a NovaSeq 6000 System (Illumina) for DNA from two types of tissues. All procedures were performed according to the manufacturer's instructions.

6. Comparison of ctDNA and Tissue Alterations

Intra-patient concordance analysis was performed on patients who had done both ctDNA and Tissue-NGS analysis. Since ctDNA has nine target genes, and Tissue-based-NGS analyzed 523 genes, a comparative analysis was performed only on nine genes (*TP53, BRCA1, BRCA2, ARID1A, CCNE1, KRAS, MYC, PIK3CA,* and *PTEN*). Single-nucleotide variants (SNVs) and Copy number variants (CNVs) were included in the analysis. We judged that the analysis results had concordance when one or more tier 1/2 mutations were both detected in the same chromosomal position on each test.

7. Grouping patients

We divided the patients into three groups to identify differences in prognosis according to the ctDNA dynamics.

Not detected group: Patients without tier 1/2 (pathogenic) mutation in baseline-ctDNA samples.

Zero-conversion group: Patients with tier 1/2 mutation were detected in baseline-ctDNA samples, but no mutation was detected in ctDNA analysis in the 6th-month (T2) follow-up sample.

Persistently elevated group: Patients with tier 1/2 mutation detected in baseline-ctDNA samples, also mutation was persistently detected in the 6th-month follow-up sample.

8. Statistical analysis

PFS and OS were calculated using the Kaplan-Meier method with log-rank test. Progression-free survival (PFS) was defined as the time from the date of diagnosis of ovarian cancer to the date of disease progression. Statistical analysis was carried out using the R 4.2.1 software, where P values<0.05 were considered significant. Oncoplot for exploring characteristics of ctDNA and Oncoprint plot for identifying positive concordant somatic variants were generated using the maftool package (Bioconductor) and the Complex Heatmaps package (Bioconductor), respectively, using the R 4.2.1 software.

III. RESULTS

1. Patients Enrollment and Clinical Characteristics

Between October 2019 to March 2022, 330 patients suspicious of ovarian malignancy with elevated serum CA-125 (CA-125 >35 U/mL) were enrolled. Figure 1.B. summarizes the flow of patients through the study, including reasons for exclusion from the analysis. Baseline patient characteristics are summarized in Table 1.

Six patients were excluded due to the final pathological diagnosis of synchronous cancers (n=6) consisting of peritoneal cancer, diffuse large B cell lymphoma, signet ring cell adenocarcinoma, colorectal cancer, gastric adenocarcinoma and pseudomyxoma peritonei. 27 patients were excluded due to insufficient blood sample volume for analysis (n=27). The remaining 298 patients were eligible for the study.

298 patients' samples were analyzed. 202 patients were diagnosed with primary ovarian cancer and 96 patients were diagnosed with benign or borderline cancers. Precisely 150 of the patients (74%) were diagnosed with high-grade serous carcinoma (HGSC) followed by 22 patients with clear cell carcinoma, 13 with mucinous carcinoma, 7 with low-grade serous carcinoma, 7 with endometrioid and 3 with other types of cancer consisting of granulosa cell tumor, dysgerminoma and intraepithelial carcinoma.

We were able to acquire total 813 serial samples in addition to 298 baseline samples during the study period, and ctDNA analysis was carried out pre-operatively in 298 cases and post-operatively in 150 cases. The presence of tier 1/2 mutation was identified in 70% (139/199) of the epithelial ovarian cancer (EOC). In our analysis, the tier 1/2 mutation was not identified in patients with benign/borderline tumors (0/96).

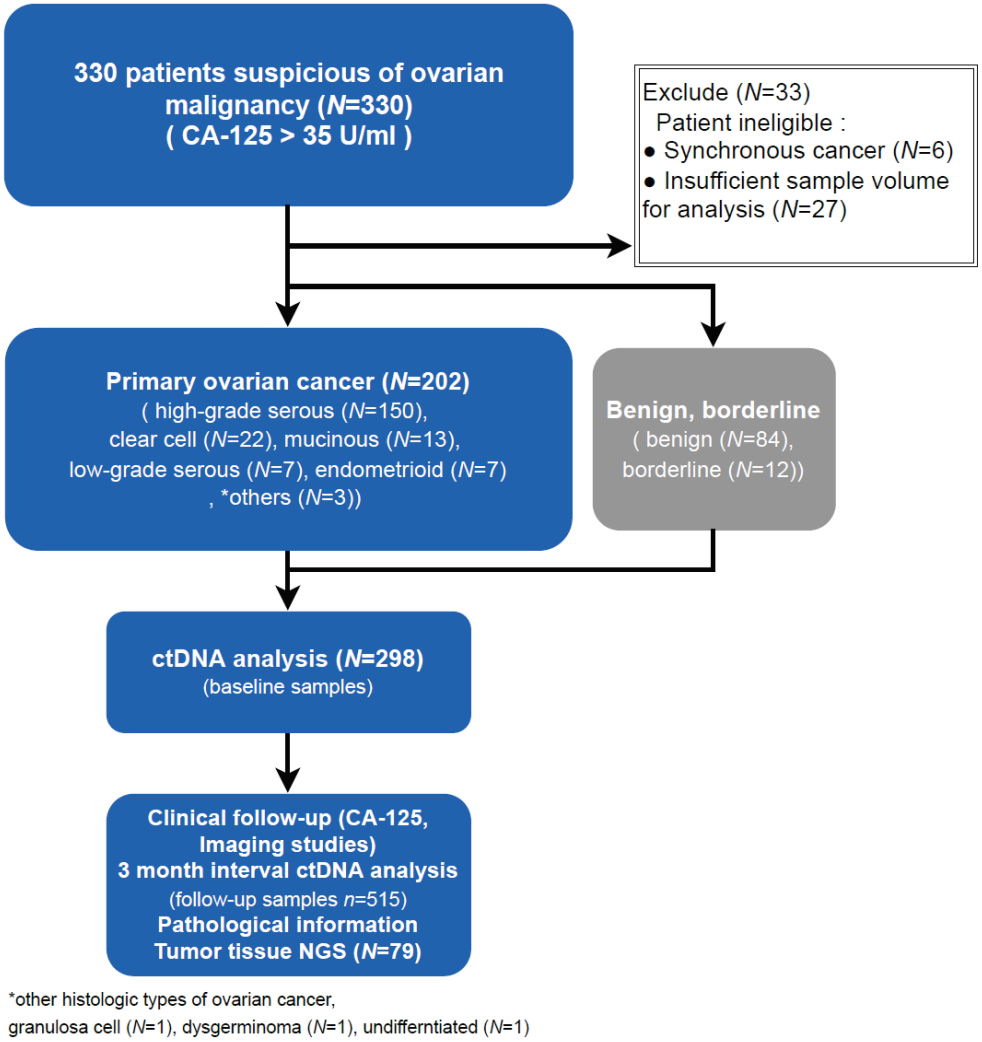


Figure 1. B. Consort diagram of patient enrollment

Table 1. Patient characteristics and baseline-ctDNA positivity

Variables	Total (N=202)	ctDNA-Positive (N=140)	ctDNA-Negative (N=62)	P value
Age	58.2 ± 12.3	58.2 ± 12.1	58.1 ± 12.7	0.946
Stage				0.001
- I	32 (15.8%)	11 (7.9%)	21 (33.9%)	
- II	15 (7.4%)	12 (8.6%)	3 (4.8%)	
- III	71 (35.1%)	51 (36.4%)	20 (32.3%)	
- IV	84 (41.6%)	66 (47.1%)	18 (29.0%)	
Histology				0.102
- High-grade serous carcinoma	150 (74.3%)	112 (80.0%)	38 (61.3%)	
- Clear cell carcinoma	22 (10.9%)	13 (9.3%)	9 (14.5%)	
- Mucinous carcinoma	13 (6.4%)	7 (5.0%)	6 (9.7%)	
- Endometrioid carcinoma	7 (3.5%)	4 (2.9%)	3 (4.8%)	
- Low-grade serous carcinoma	7 (3.5%)	3 (2.1%)	4 (6.5%)	
- Others	3 (1.5%)	1 (0.7%)	2 (3.2%)	
Treatment				0.117
- PDS	89 (44.1%)	55 (39.3%)	34 (54.8%)	
- NACT-IDS	74 (36.6%)	55 (39.3%)	19 (30.6%)	
- recurrent	39 (19.3%)	30 (21.4%)	9 (14.5%)	
Germline BRCA mutation				0.012
- Yes	51 (25.2%)	43 (30.7%)	8 (12.9%)	
- No	151 (74.8%)	97 (69.3%)	54 (87.1%)	

PDS, primary debulking surgery;

NACT-IDS, neoadjuvant chemotherapy with interval debulking surgery;

2. Concordance of ctDNA- and Tissue- Based NGS analysis

Concordance of ctDNA-Tissue Cohort comprised a subset of 79 patients with tissue-based NGS test results, the proportion of samples harboring pathogenic genomic alterations in both NGS platforms was 88.6% (N = 70), whereas in 2.5% (N = 2) of patients' samples (Figure 2.A.), no alterations were detected by either test (Supplementary Table. S2). The rate of patients who have genomic alterations detected only in ctDNA was 1.3% (n = 1), and the rate of alterations detected only by the tissue-based NGS test was 7.6% (n = 6). Compared with tissue-based NGS testing, the ctDNA-based NGS test showed a 91.1% concordance rate between the tests. We also compared detection of copy number variation (CNV) between two tests. Copy number alterations were evaluated in *CCNE1*, *MYC*, *KRAS* and *PIK3CA* genes (Supplementary Table. S3). This resulted in a 88.6% concordance rate between the tests. Of the patients with *TP53* mutations detected in tissue, these mutations were also detected in ctDNA in 92.8% (64/69) of patients. Of the patients with *BRCA1/2* mutations detected in tissue, these mutations were also detected in ctDNA in 90.9% (20/22) of patients.

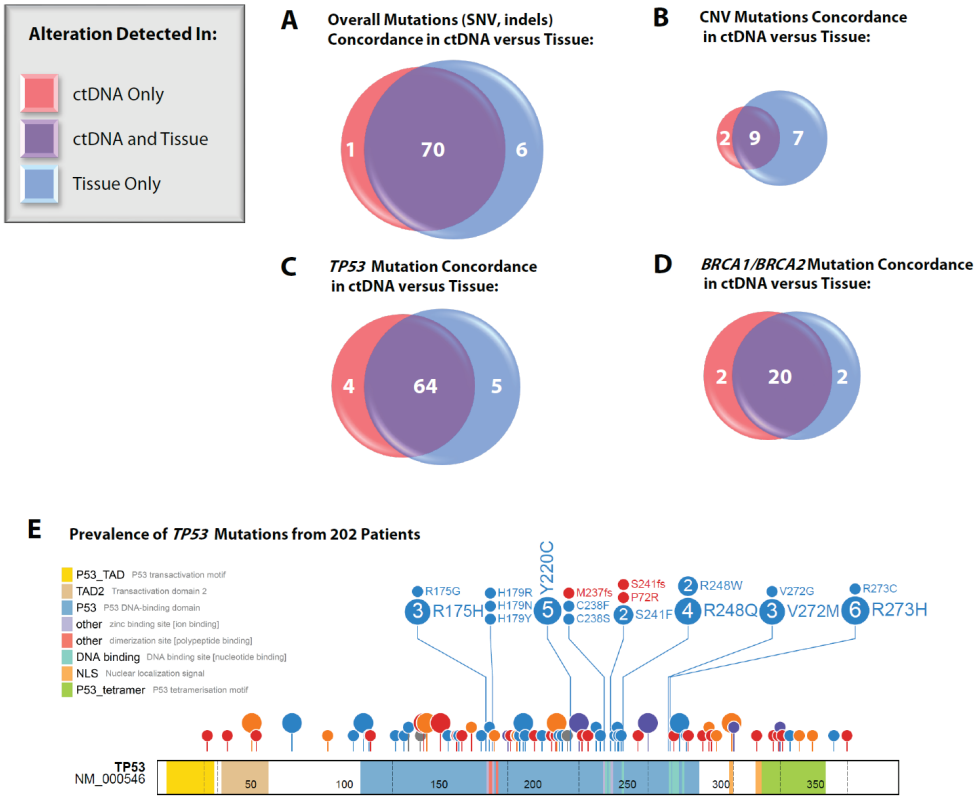


Figure 2. Intra-patient concordance of genitic alterations between ctDNA and tissue in a cohort of 79 patients with OC. Concordance in detection of *TP53, BRCA1/2, ARID1A, PIK3CA* and *PTEN* mutations (N = 77 patients) (A) overall (SNV, small indels) and (B) Copy Number Variation mutations in *CCNE1, MYC, KRAS*, and *PIK3CA*. (C) Concordance in detection of *TP53* mutations among 72 patients. (D) Concordance in detection of *BRCA1/2* mutations among 24 patients. (E) Prevalence of *TP53* mutations from 118 patients on ctDNA profiling. This diagram was created using the ProteinPaint (Copyright: St. Jude Children's Research Hospital.; https://proteinpaint.stjude.org/).

3. Profiling of Genetic Somatic Mutation in Ovarian Cancer

To identify individual genetic profiles for each patient, we analyzed baseline sample ctDNA results. Our custom NGS panel targets OC-related nine genes.

Among the 202 primary ovarian cancer patients, 139 (69%) showed one or more tier 1/2 somatic mutations. Frequencies of tier 1/2 somatic mutations detected from all patients were *TP53* (57%), *BRCA2* (7%), *ARID1A* (7%), *BRCA1* (4%), *PIK3CA* (4%), *KRAS* (3%), and *PTEN* (2%). Pathogenic or likely pathogenic germline *BRCA1/2* (g*BRCA*) mutation was detected in 52 cases (25%) (Figure 3).

We identified the type and frequency of somatic mutations in other histologic subtypes. The most frequently mutated gene in HGSC was *TP53* (70%), which was significantly higher than non-HGSC ($p = 0.002$), followed by *BRCA2* (9%) and *BRCA1* (6%) mutations. Of the 105 patients with the somatic *TP53* mutations, the germline *BRCA* 1/2 mutation prevalence was relatively high (64.8%). In clear cell carcinoma, *ARID1A* and *PIK3CA* were mutated preferentially (50%), followed by *PTEN* and *KRAS* mutations. In endometrioid carcinoma, *PTEN* and *KRAS* mutations were detected (25%).

The *TP53* mutation profile was compared with the COSMIC serous carcinoma database (Figure 2.E). We confirmed that the frequently reported R273H, R248Q, R273C, R175H, R248W, and Y220C mutations in the existing online database were equally reported in our analysis.

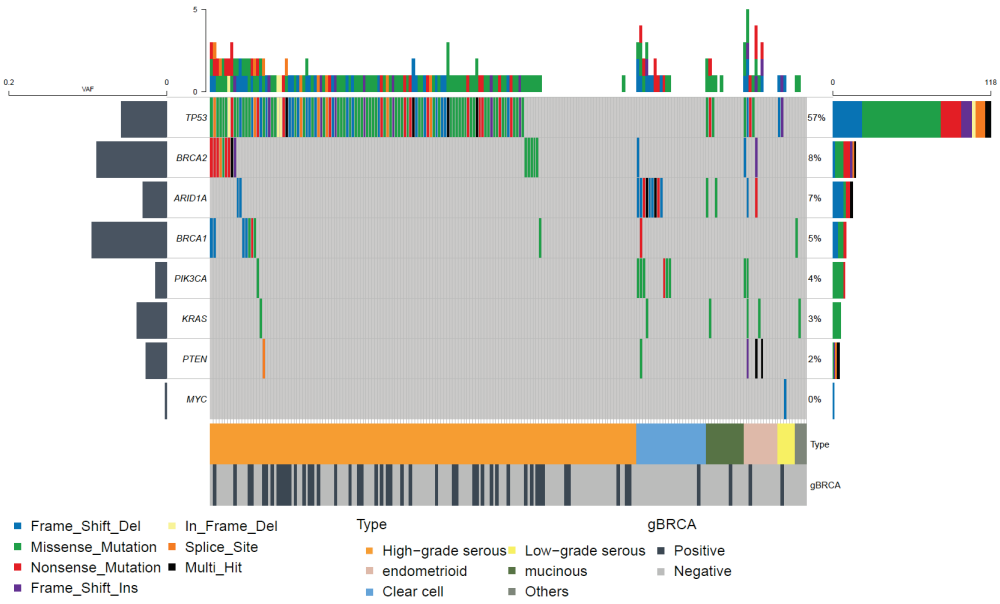
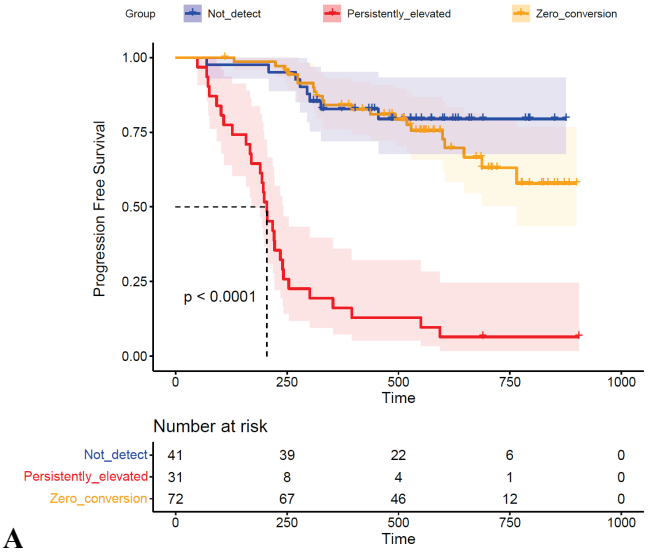


Figure 3. Profiling of genetic mutation in 201 ovarian cancer patients

4. Prognostic significance of the dynamic change in ctDNA

The median follow-up of patients was 372 days, ranging from 19 to 907. In total, 813 samples were collected, and the average number of samples collected per patient was 2.6 (maximum = 9). We divided 144 patients into three groups (not detected group, zero-conversion group, and persistently elevated group) to identify prognostic differences according to the ctDNA dynamics. To set a landmark timepoint that reflects prognostic value most, we compared two time points (T1 (3 months after debulking surgery) and time T2 (6 months after debulking surgery)) at which ctDNA decreases to zero-level. Patients who had detectable levels of ctDNA at T1 (persistently elevated group ; 3 months after baseline sample) had a shorter progression-free survival (PFS) and overall survival (OS) compared to patients with undetectable mutation (not detected group and zero-conversion group) ($P < 0.001$; Supplementary Figure S1, S2). Also, patients who had detectable levels of ctDNA at T2 (persistently elevated group ; 6 months after baseline sample) had a shorter PFS and OS compared to patients with undetectable mutation (not detected group and zero-conversion group) ($P < 0.001$; Figure 4. A; Supplementary Figure S3). Comparing two time points, persistently elevated group in T2 had shorter median disease-free interval compared to persistently elevated group in T1 (0.7 years vs 0.9 years). Median disease-free interval (DFI) was 0.7 years in patients with detectable ctDNA at T2 (persistent group) compared with 2.5 years in zero conversion group. Patients with detectable ctDNA at follow up test had significantly increased risk of progression of disease compared with those with undetectable ctDNA (Figure 4. B). When comparing the two groups without ctDNA mutation at the time of T2 (zero conversion and not detected group), no significant difference was found in PFS ($P= 0.41$; Supplementary Figure S8). A difference in PFS was observed when comparing patients in whom ctDNA was detected in baseline sample versus not detected at any timepoint during observation period ($P= 0.003$; Supplementary Figure S6).



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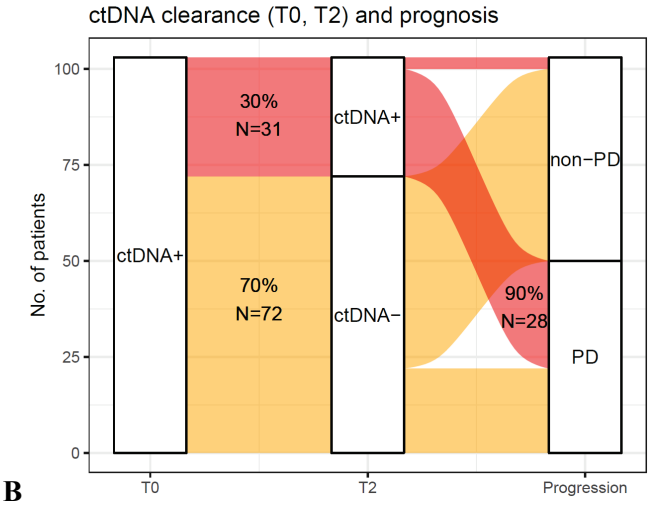

B

Figure 4. **A. Kaplan-Meier curve of progression-free survival** Association of ctDNA dynamic based groups with progression free survival (PFS)

B. Sankey plot showing ctDNA dynamics (clearance or non-clearance) Analysis was focused on patients who were ctDNA positive at baseline (T0) and had corresponding ctDNA testing results at T2, 6 months after initiation of therapy.

5. Longitudinal monitoring of ctDNA to detect residual disease and recurrence

We serially analyzed ctDNA samples from 202 patients diagnosed with carcinoma every three months during the observation period. The number of serial samples collected from the patients and the average observation period are summarized in Supplementary Table S4. Clinical recurrence information, including CA-125, and CT image result, was compared with ctDNA-detected recurrence (Figure 5.A.). During the 30 months of the observation period, 73 out of 202 patients clinically recurred (36.1%). 26 patients were excluded from the longitudinal recurrence analysis for the following criterias (17 patients: insufficient number of samples for longitudinal monitoring analysis (less than three serial samples), three patients were failed to collect ctDNA samples for three months before and after the clinical diagnosis of recurrence, and six patients were those with no mutations detected in the baseline sample. Finally, a longitudinal monitoring analysis was conducted on 47 patients. The pathogenic mutation found in the baseline sample was also detected in the serially collected samples from 44 out of 47 patients (93.6%). In 23 out of 47 patients (48.9%), ctDNA-detected recurrence had a lead time of more than one month than CT-detected recurrence, and in 19 patients (40.4%), both methods could detect recurrence in similar time points (lead time : average 56.3 days, maximum 267 days). Of 28 patients with CA-125 level dropped below the upper limit of normal (ULN) after debulking surgery and chemotherapy, 14 patients (50.0%) had a lead time of more than one month compared to CA-125 based surveillance (using GCIG criteria) (lead time : average 49.3 days, maximum 231 days). As in the example of patient 006 (Figure 5.B.), there was also lead time in a group of seven patients with CT-detected recurrence but no change in CA-125 level at the recurrence (lead time : average 58.3 days, maximum 228 days).

Figure 5. A.

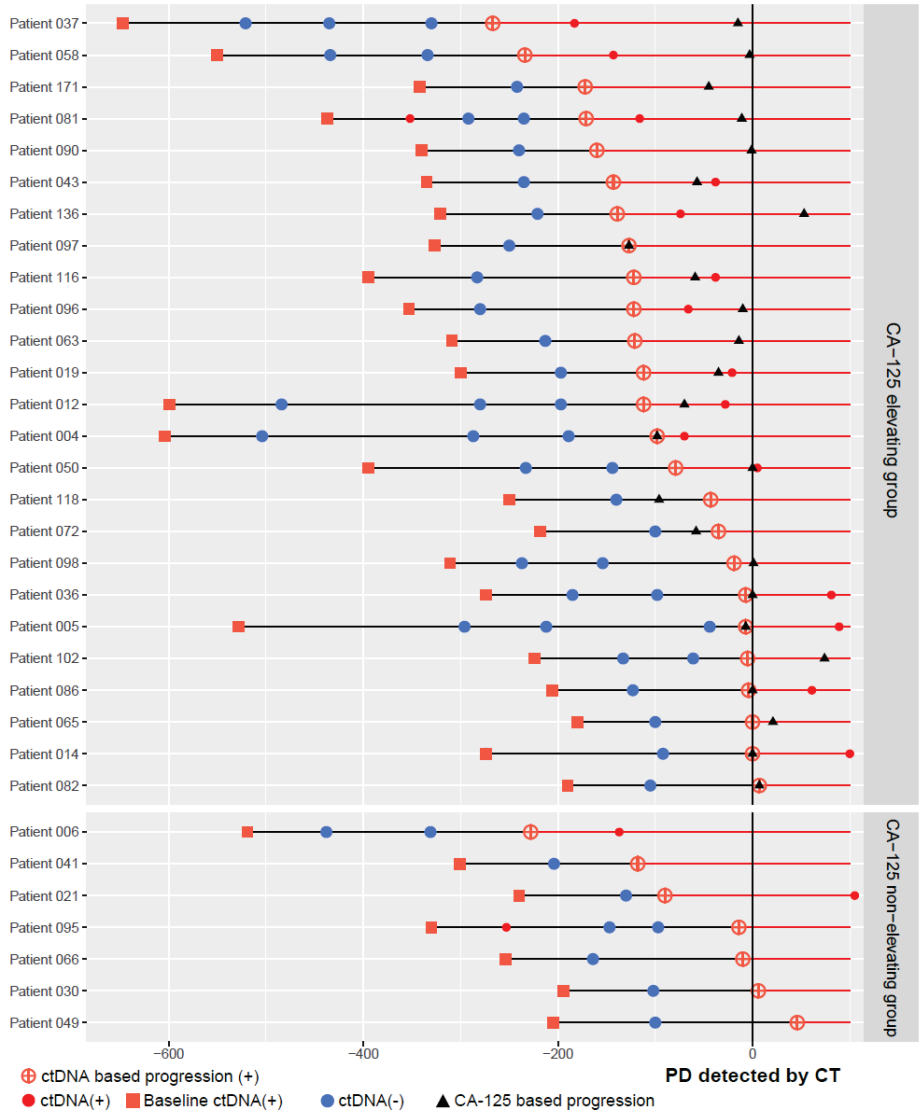

Figure 5. B

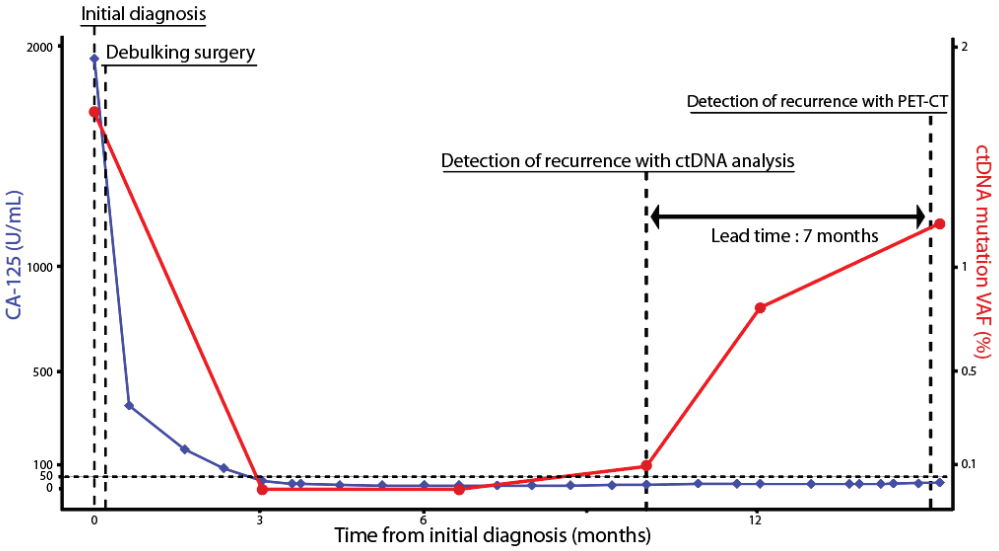

Figure 5. Longitudinal monitoring of ctDNA

A. Summary of 149 serial plasma samples from 33 patients indicating when ctDNA was detected (red dots) or not detected (blue dots). Clinical recurrence based on CA-125 GCIG criteria is indicated with a triangle. In this figure, two patients were excluded, who are ctDNA progression was detected later than conventional CT surveillance (203 and 149 days).

B. Patient 006 with high-grade serous carcinoma stage IV disease, treated by primary debulking surgery and Olaparib. Red dots indicate samples with ctDNA, and blue circles indicate CA-125 level. Showing 7 months of lead time compared to conventional surveillance methods.

IV. DISCUSSION

In this study, we prospectively validated ctDNA dynamics as a marker of treatment. We utilized NGS-based liquid biopsy approach, blood-derived ctDNA samples obtained from 202 patients with major histopathological subtypes of EOC, and 96 benign/borderline patients' samples. Our study is the largest to date on the use of ctDNA in ovarian cancer. We characterized comprehensive genomic profiles in ovarian cancer. From subgroup analysis of 144 ovarian cancer patients with serial samples, we demonstrated that dynamics of ctDNA can provide prognostic information. We found that baseline ctDNA is associated with PFS, and persistently elevated ctDNA mutation after 3-6 months sample is more associated with worse PFS in OC. These findings suggest that ctDNA liquid biopsy might be helpful not only as a non-invasive biomarker but also as a prognostic biomarker of clinical situation for ovarian cancer.

Previous reports show that cell-free DNA (cfDNA) concentrations corresponded to the tumor burden and cancer stage[24,25], and our analysis also demonstrated it. However, cfDNA concentration may increase in infectious circumstances when apoptosis occurs.[26-28] To increase the specificity of the ctDNA analysis, our study focused on the qualitative detection of genomic alterations(SNV, indel) from ctDNA samples through the NGS method.

Our analysis showed that the *TP53* mutation detected in the ctDNA was the most frequent in ovarian cancer cohort (113/202; 55.9%), and in the HGSC cases (100/149; 67.1%), which is similar to previous ctDNA studies (66.7%) [20] and lesser than previous tissue-based studies (86%). [29] *ARID1A/PIK3CA* mutation was detected in 50% (11/22) of clear cell carcinoma patients which is similar to previous studies (50% - 66.7%).[30-32]

Validating the concordance between tumor-NGS and ctDNA, we analyzed 79 patients' tissue-ctDNA paired samples. Focusing on tier 1/2 pathogenic mutations detected in tissue-based-NGS was also detected in ctDNA samples (70/79; 88.6%). High concordance with tumor NGS, previous studies have looked at the concordance, reporting a range of 79 to 81%.[20,33] We also compared our mutational profiles of *TP53* with COSMIC database and

previous studies [22,34]. We confirmed that locus distribution of mutations (SNV and indel) of our analysis is consistent with existing somatic *TP53* mutation database. These results suggest that analysis of ctDNA reflects tumor molecular landscape.

We demonstrated that comprehensive gene mutation profiling and serial follow-up with ctDNA analysis could be helpful for monitor treatment response in ovarian cancer patients, and each variant detected by ctDNA analysis could be a personalized biomarker in ovarian cancer patients.

To define the zero-conversion group's time-point, we compared the time-point set to T1 (3 months after surgery) and T2 (6 months after surgery) and confirmed that setting zero-conversion based on T2 is a better predictor of patient PFS, although both time-point, T1 and T2 are significant for predicting clinical PFS. In this study, we set T2 as the landmark time point, and the previous non-small cell lung cancer ctDNA study, which set the landmark time point as 2-4 weeks after the end of treatment, showed similarities in the clinical prognosis prediction time point setting.[14]

Through longitudinal monitoring analysis, we found the same pathogenic mutations initially found in 94% of recurrent patients with baseline pathogenic mutations. We also demonstrated that the patient's baseline mutation profile could be used as a high-specific tumor marker. We also demonstrated that faster recurrence diagnosis is possible by having an average lead time of 55 days (maximum 267 days) compared to the existing methods using CT and CA-125. Also, 49% of PD patients could be detected more than one month earlier than traditional surveillance methods. Especially, in about 15% of patients with recurrent ovarian cancer with no elevation of CA-125 or gradual increases, ctDNA showed the possibility of being the only biomarker except CT-based surveillance.

Specific panel design of nine genes. Previous studies on ctDNA in OC have utilized panels including 55 to 500 genes.[19,20,35] We have narrowed down to the most frequently altered genes in ovarian cancer based on occurrence frequency.[11,20,23] The advantage of our customized panel is that it is cost-effective and efficient, considering the variety of clinical information it can provide. Our current panel has the potential to be incorporated into a

large scale screening program as well as an adjunct test with CA-125 in the management of OC.

Our analysis data and recent study [36] suggest that liquid biopsy might enable early detection of tumor relapse/progression and assessment. Ability to identify early, It may offer flexibility for clinicians, especially in patients with recurrent disease who often need to be screened for clinical trials and frequently need additional sequencing/IHC tests to qualify. We also demonstrated the possibility of using ctDNA analysis for minimal residual disease (MRD) purposes. With serially obtained 3 monthly samples, patients at high risk of disease progression can be identified early, allowing for clinicians to act early on the choice of next therapy and allow personalized treatment based on additional biomarker tests.

Obtaining samples with tumor biopsy is sometimes risky, particularly in cases of advanced/recurrent ovarian cancer. Therefore, comprehensive noninvasive ctDNA-based liquid biopsy, reflecting heterogeneity, might provide effective treatment for individual patients and monitor response to treatment. ESMO published a recommendation on using ctDNA for NSCLC, breast cancer, and patients without tissue testing results [21], but large-scale studies that can be readily implemented in OC are limited. Our study effectively addresses this point.

Study limitations

In this study, calculating concordance between Tissue-based NGS and ctDNA-based NGS, the entire cohort did not perform tissue-based NGS analysis, so only 77 subset patient cohorts out of 298 patients were included in the comparison. CtDNA analysis was done regardless of the patient's histology type (including both carcinoma and benign/borderline), but tissue-based NGS was only done on patients diagnosed with carcinoma, so there was a limit to the evaluation of inter-rater reliability. When analyzing longitudinal monitoring, it was necessary to compare at least four serial samples, including baseline samples. In this study, which was conducted for about 30 months, the enrollment time was different for

each participating patient. Therefore, the observation period was composed of patients with as short as 3 months and long as 30 months, so there were only 109 patients who could perform longitudinal monitoring (Supplementary Table S4). We only compared patients with clinical recurrence status by conventional methods such as CT or CA-125. We excluded three patients who suspected recurrence only with ctDNA analysis during the observation period but have not yet been clinically identified as recurrence. In follow-up of patients, CA-125 is usually tested at 4-week intervals. However, the ctDNA analysis was performed every 3 months, so there was a limitation in comparing performance with CA-125 analysis.

V. CONCLUSION

Our analysis suggests that ctDNA-based surveillance may serve an essential role in the detection of disease progression in ovarian cancer, providing genetic characteristics of ovarian cancer, and applicability of ctDNA in clinical decision making and might help establish personalized therapeutic strategies.

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APPENDICIES

Supplementary Tables

Supplementary Table S1.

Gene list of the target panel for ovarian cancer

TP53	*BRCA1*	*BRCA2*	*ARID1A*	*PIK3CA*
CCNE1	*KRAS*	*MYC*	*PTEN*	

Supplementary Table S2.

Comparison of Tissue-Based NGS and ctDNA-Based NGS Test Results in Patients with Epithelial Ovarian cancer

		Tissue-NGS		
(n=79)		Positive	Negative	Total
ctDNA-NGS	Positive	70 (88.6%)	1 (1.3%)	71 (89.9%)
	Negative	6 (7.6%)	2 (2.5%)	8 (10.1%)
	Total	76 (96.2%)	3 (3.8%)	79 (100%)

Supplementary Table S3.

Comparison of Tissue- and ctDNA-NGS based CNV detection

(n=79)		Tissue-NGS		
		Positive	Negative	Total
ctDNA-NGS	Positive	9 (11.4%)	2 (2.5%)	11 (13.9%)
	Negative	7 (8.9%)	61 (77.2%)	68 (86.1%)
	Total	16 (20.3%)	63 (79.7%)	79 (100%)

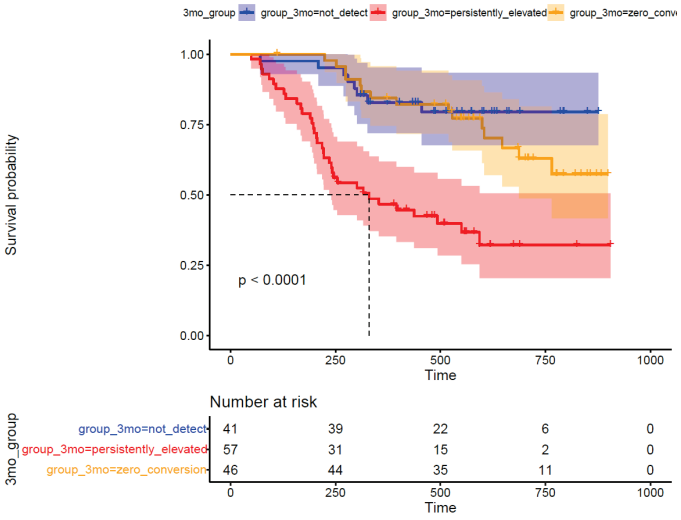
Supplementary Table S4.

Collected ctDNA Sample count

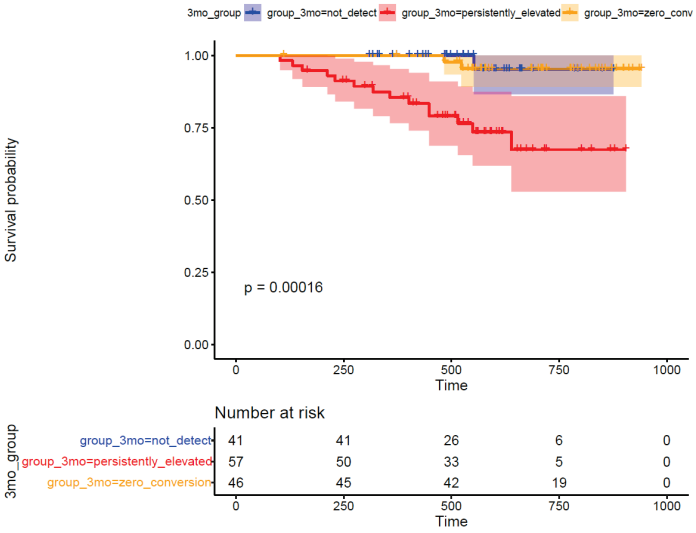
	T0	T1	T2	T3	T4	T5	T6	T7	T8
Number of samples	298	146	119	109	72	40	21	8	3
Observation days (median)	0	101	201	298	407	497	551	582	630

Supplementary Figures

Supplementary Figure S1. Kaplan-Meier curve of ctDNA dynamic based groups (T1 : 3 months) with progression free survival (PFS)

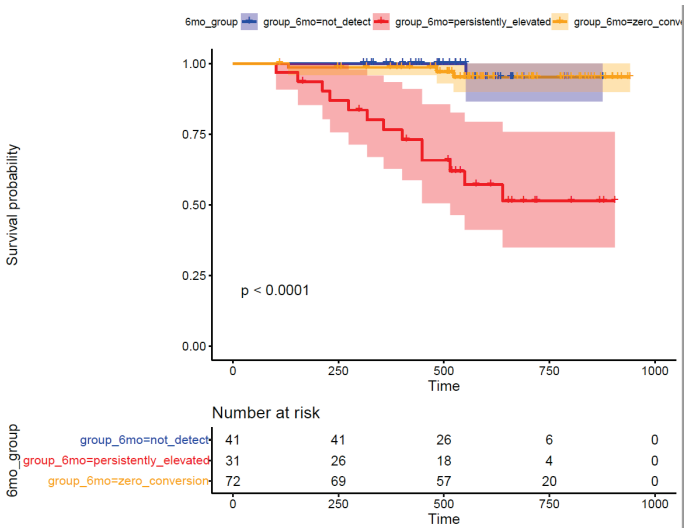


Supplementary Figure S2. Kaplan-Meier curve of ctDNA dynamic based groups (T1 : 3 months) with overall survival (OS)

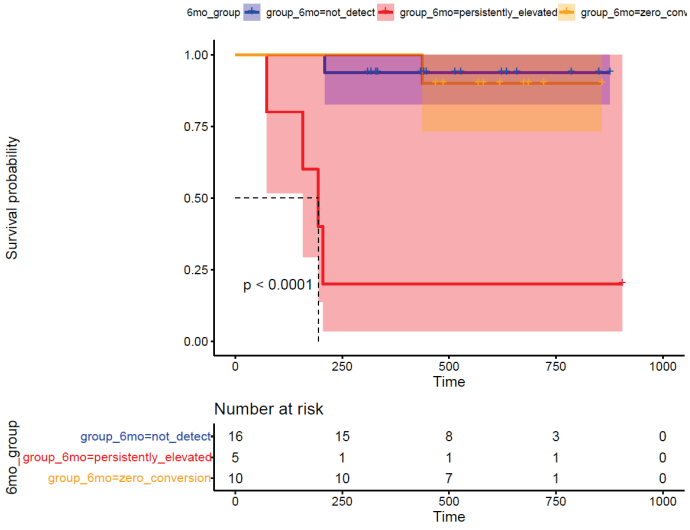


Supplementary Figure S3. Kaplan-Meier curve of ctDNA dynamic based groups (T2 : 6

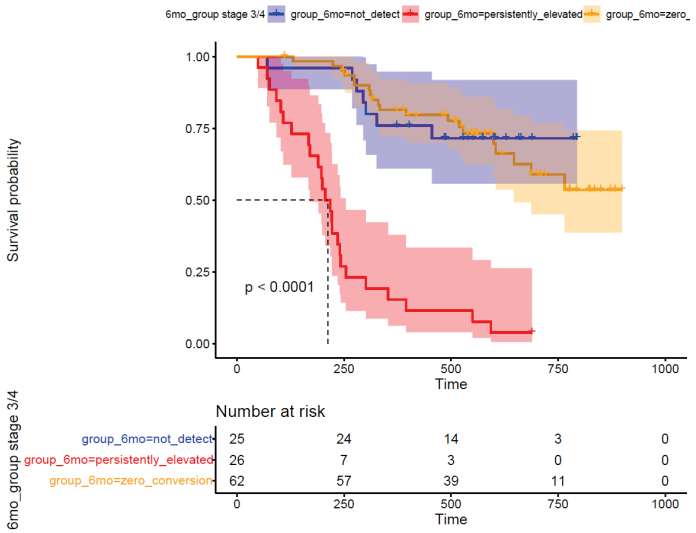
months) with overall survival (OS)



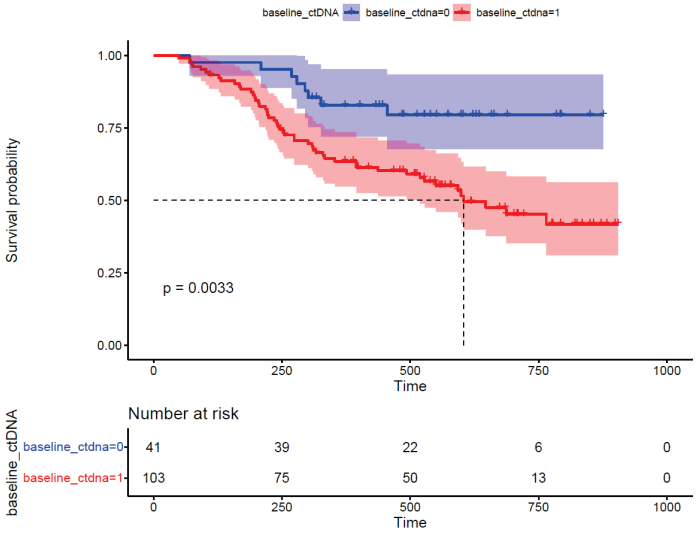
Supplementary Figure S4. Kaplan-Meier curve of ctDNA dynamic based groups (T2 : 6 months) with progression free survival (PFS) in stage 1/2 ovarian cancer



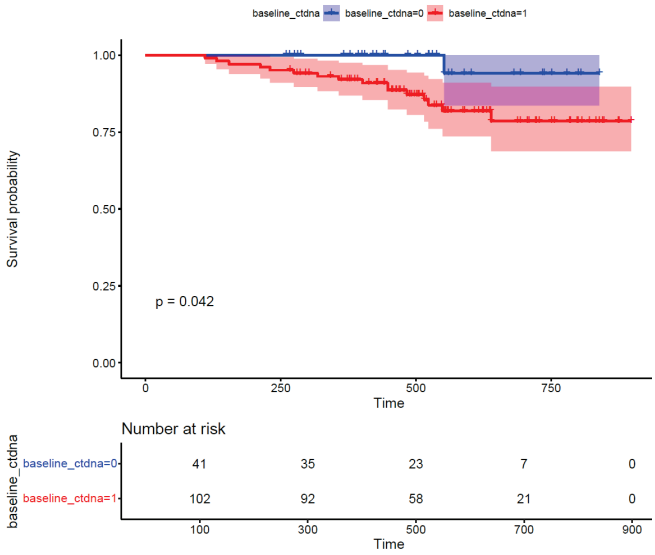
Supplementary Figure S5. Kaplan-Meier curve of ctDNA dynamic based groups (T2 : 6 months) with progression free survival (PFS) in stage 3/4 ovarian cancer



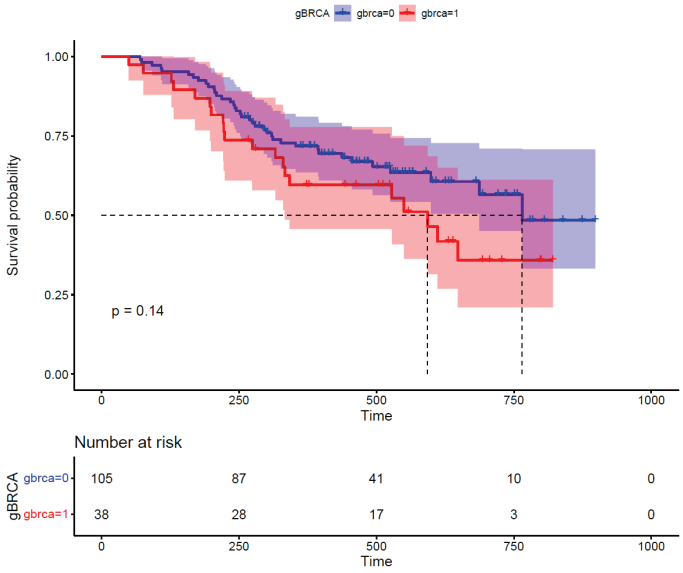
Supplementary Figure S6. Kaplan-Meier curve of association with ctDNA mutation detection at baseline with progression free survival (PFS)



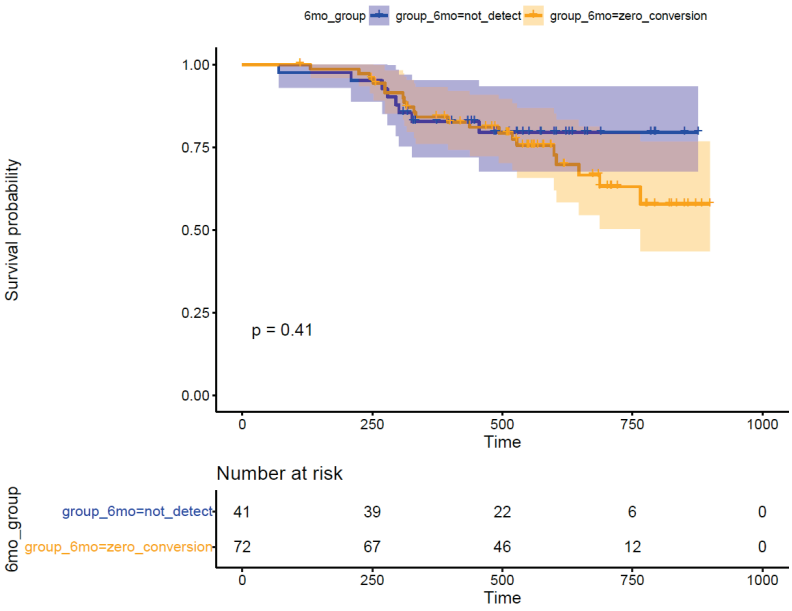
Supplementary Figure S7. Kaplan-Meier curve of association with ctDNA mutation detection at baseline with overall survival (OS)



Supplementary Figure S8. Kaplan-Meier curve of association with germline *BRCA1/2* mutation detection with progression free survival (PFS)



Supplementary Figure S9. Kaplan-Meier curve of ctDNA dynamic based groups (not detected VS zero conversion) with progression free survival (PFS)



ABSTRACT(IN KOREAN)

난소암 환자에서 액체생검을 통한 순환종양 DNA 검출 및 예후인자로서 활용도 평가

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허 진 호

난소암의 진행 및 재발을 효과적으로 감지하는 것은 환자 예후를 개선하는 데 중요하다. 기존의 종양 바이오마커(CA-125) 및 영상 검사(CT)는 난소암의 최소 잔류 질환 검출(MRD)에 충분하지 않다. 본 연구는 수술 후 보조 요법을 받는 난소암 환자의 질병 진행을 위한 추가 바이오마커로 순환종양 DNA(ctDNA)를 사용하는 것의 타당성을 평가하는 것을 목표로 했다.

본 연구에서는 난소 악성 종양이 의심되는 330명의 환자를 모집했다 (CA-125 > 35 U/ml). 수술 직전과 그 후 3개월마다 채혈을 진행했으며, 기존의 수술 후 모니터링은 CA125, HE4, MRI 및 PET-CT를 사용하여 수행되었다. 9개의 유전자(*TP53, BRCA1, BRCA2, ARID1A, CCNE1, KRAS, MYC, PIK3CA* 및 *PTEN*)을 대상으로 하는 유전자 패널을 이용하여 염기서열을 분석했다.

암환자 201명(고등급/저등급 점액성, 투명세포, 자궁내막 등)과 양성/경계성 난소 질환 환자 96명을 포함하여 296명의 환자로부터 총 813개의 혈액 검체를 분석했다. 상피성 난소암 환자의 69.8%(139/199)에서 병원성

돌연변이를 확인할 수 있었다. 양성/경계성 종양 환자(0/96)에서는 병원성 돌연변이가 확인되지 않았다. 진단시점 ctDNA 돌연변이가 있는 38명의 진행성 환자 중 89.8%의 환자가 재발시점에서 동일한 돌연변이가 확인되었다. 이러한 환자의 경우 순환종양분석을 통해 기존 진단 방법보다 평균 50.9일(최대 267일)이 재발을 조기에 발견할 수 있었다. 진단 이후 시점 6개월의 추적 분석에 따르면, 지속적으로 변이가 관찰되는 그룹이 음성전환 그룹과 비교해서 무진행 생존율(PFS)이 더 나빴다 (7.7 대 25.3개월; $P < 0.001$).

본 연구를 통해 순환종양 DNA 기반 추적검사가 난소암의 진행을 감지하고 난소암의 유전적 특성을 제공하며 임상 의사 결정에서 적용 가능성에 필수적인 역할을 할 수 있으며 개인화된 치료 전략을 수립하는 데 도움이 될 수 있음을 확인했다.

핵심되는 말 : 순환종양 DNA; 상피성 난소암; 고등급 장액성 난소암