Serial circulating-tumor DNA analysis with a tumor naïve next-generation sequencing panel detects minimal residual disease and predicts outcome in ovarian cancer

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Abstract

Circulating tumor DNA (ctDNA) may aid in personalizing ovarian cancer therapeutic options. Here, we aimed to assess the clinical utility of serial ctDNA testing using tumor-naïve, small-sized next-generation sequencing (NGS) panels. A total of 296 patients, including 201 with ovarian cancer and 95 with benign or borderline disease, were enrolled. Samples were collected at baseline (initial diagnosis or surgery) and every 3 months after that, resulting in a total of 811 blood samples. Patients received adjuvant therapy based on the current standard of care. Cell-free DNA was extracted and sequenced using an NGS panel of nine genes: *TP53, BRCA1, BRCA2, ARID1A, CCNE1, KRAS, MYC, PIK3CA,* and *PTEN*. Pathogenic somatic mutations were identified in 69.2% (139/201) of patients with ovarian cancer at baseline but not in those with benign or borderline disease. Detection of ctDNA at baseline and/or at 6 months follow-up was predictive of progression-free survival (PFS). PFS was significantly poorer in patients with detectable pathogenic mutations at baseline that persisted at follow-up than in patients that converted from having detectable ctDNA at baseline to being undetectable at follow-up; survival did not differ between patients without pathogenic ctDNA mutations in baseline or follow-up samples and those that converted from ctDNA positive to negative. Disease recurrence was also detected earlier with ctDNA than with conventional radiological assessment or CA125 monitoring. These findings demonstrate that serial ctDNA testing could effectively monitor patients and detect minimal residual disease, facilitating early detection of disease progression and tailoring of adjuvant therapies for ovarian cancer treatment.

Significance

In ovarian cancer, serial ctDNA testing is a highly predictive marker of patient survival with a significantly improved recurrence detection lead time compared to conventional monitoring tools.

Introduction

Ovarian cancer is a difficult-to-treat malignancy with a high recurrence rate of 70–80% in the late stage and 20–25% in the early stage (1-3). Primary debulking surgery (PDS) or interval debulking surgery (IDS), followed by platinum-based adjuvant chemotherapy, is the standard of care for ovarian cancer. The approved maintenance options include bevacizumab, a vascular endothelial growth factor inhibitor, and a poly-ADP-ribose-polymerase (PARP) inhibitor (4). According to current guidelines, ovarian cancer treatment is based on the clinical stage, breast cancer gene (*BRCA)* status, or homologous recombination deficiency (HRD) status. However, further refinement of clinical risk groups and early detection of disease progression are necessary to improve treatment outcomes in ovarian cancer. Current tools for the detection, prognostic stratification, and disease monitoring of ovarian cancer have several limitations. For instance, the sensitivity and specificity of CA125, the most widely used tumor marker at initial diagnosis are poor, especially in patients with early-stage cancer or histologic subtypes other than high-grade serous carcinoma (HGSC) or endometrioid cancer (5). CA-125 is not optimally sensitive for disease monitoring because up to 50% of patients with normal CA-125 levels have small volumes of persistent disease during second-look surgery (6). Furthermore, radiologic methods are costly, expose patients

to radiation, and have limited resolution, and therefore show a limited detection rate in the setting of small-volume tumors or low metabolic disease (7). A recent study demonstrated that programmed death-ligand 1 (PD-L1) expression and tumor-infiltrating lymphocyte dynamics in tumor biopsy samples before and after treatment are related to prognosis. However, they cannot be sampled periodically due to the risk of sample collection (8).

A newly developed method is solid tumor liquid biopsy, which is based on next-generation sequencing (NGS) of circulating tumor DNA (ctDNA) (9). The advantages of ctDNA are its high sensitivity, even in early-stage cancers, and high tumor specificity (10). Furthermore, various ctDNA applications, such as the assessment of minimal residual disease (MRD) (11) and early detection of disease progression, have been suggested in colorectal and lung cancers (12, 13). Previous pan-cancer studies investigated the diagnostic value of ctDNAs in ovarian cancer (14, 15). However, studies on prognostic stratification and disease monitoring using serially collected ctDNA in ovarian cancer are limited. Moreover, these studies are conducted either in specific contexts, such as *BRCA* reversion, or are limited in terms of the number of patients included (16-18).

Overall, the potential use of ctDNA testing in ovarian cancer has not been sufficiently demonstrated; the European Society for Medical Oncology (ESMO) recommendation on ctDNA refers to the use of ctDNA in patients without germline pathogenic *BRCA* 1/2 variant or only when tissue is unavailable (19). The finer details, such as the optimal timing of follow-up ctDNA sampling and monitoring capacity compared to those of conventional methods, remain unknown. Moreover, in the era of maintenance therapy for ovarian cancer, only a few studies (20, 21) have investigated whether serial ctDNA testing can guide disease management. Therefore, we prospectively collected ctDNA

for 3 months from a sizeable cohort of patients with ovarian cancer and subjected it to tumor-naïve, small-sized panel sequencing to investigate the dynamic changes in ctDNA before and after treatment and its value in disease monitoring.

Materials and Methods

Patient recruitment and collection of clinical data

Patients with ovarian cancer who underwent PDS or IDS for a newly diagnosed disease, diagnostic laparoscopy, or secondary debulking for a recurrent disease at the Yonsei Cancer Center, Seoul, Republic of Korea, were prospectively recruited between October 2019 and March 2022 (NCT 05504174). For the non-cancer control group, patients who underwent surgery for a suspicious ovarian mass with a CA-125 level of > 35 U/mL were enrolled. Patients with ovarian cancer received adjuvant therapy based on the current standard of care as clinicians were blinded to the ctDNA results. Patients underwent whole blood sampling for ctDNA analysis at initial diagnosis or surgery and then every 3 months (Fig. 1A). Concurrently, routine monitoring was performed based on CA-125 every 3 weeks and radiological assessment with abdominopelvic computed tomography (APCT) every 3 months. Additional evaluations with magnetic resonance imaging or positron emission tomography/computed tomography or both were performed at the discretion of the clinician. This study was approved by the Institutional Review Board of Yonsei University (approval number 4-2019-0698). This study was conducted in accordance with the principles of the Declaration of Helsinki, and all patients provided written informed consent prior to enrollment.

Sample size calculation

In a pilot study of 50 patients (22), the baseline ctDNA detection rate was 70%. Assuming a hazard ratio (HR) of 0.2 in the not-detected group and a loss to follow-up of 10%, a minimum of 152 patients would need to be enrolled for progression-free survival (PFS) as the endpoint.

Collection of clinical variables and outcome

Clinical variables, such as age, FIGO stage, histological subtype, type of primary surgery, and adjuvant therapy with or without maintenance, were recorded. Moreover, CA-125 levels and radiological findings at each time point during disease monitoring were collected. The outcomes (PFS and overall survival [OS]) were defined as the time from primary surgery (PDS or IDS) to the time of first progression or death, respectively.

Cell-free DNA (cfDNA) extraction and sequencing

For each sampling time point, whole blood (9 mL) was collected in cfDNA collection tubes (Dxome, Seongnam, Republic of Korea), and ctDNA was extracted from plasma samples (3–4 mL) using the Magnetic Serum/Plasma Circulating DNA Kit (Dxome, Seongnam, Republic of Korea). The size of the cfDNA was measured using TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA). cfDNA concentration was measured using a 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 ovarian cancer-related genes (*TP53*, *BRCA1*, *BRCA2*,

157 *ARID1A, CCNE1, KRAS, MYC, PIK3CA,* and *PTEN*), which are frequently mutated in
158 ovarian cancer, as shown in previous studies (16, 23-25). The enriched DNA was amplified,
159 and clusters were generated and sequenced using a NovaSeq 6000 System (Illumina) with 2 ×
160 151 bp reads. A mean sequencing depth of 30,000× was targeted. All procedures were
161 performed according to the manufacturer's instructions. Sequence reads were aligned to
162 human genomic reference sequences (GRCh37) using the Burrows–Wheeler alignment tool
163 version 0.7.12 (Wellcome Trust Sanger Institute, Cambridge, UK). To identify single
164 nucleotide variants (SNVs) and indels, the HaplotypeCaller and Mutect2 in the genome
165 analysis tool kit (GATK) package version 3.8-0 (Broad Institute of MIT and Harvard,
166 Cambridge, MA, USA) and VarScan2 version 2.4.0 (Washington University, St. Louis, MO,
167 USA) were used. To increase the accuracy of the sequencing reads, the position indexing
168 sequencing (Pi-Seq) algorithm (Dxome, Seongnam, Republic of Korea), which uses the
169 genomic positions of sequencing reads as unique identifiers for DNA fragments, was used.
170 This algorithm refines the accuracy of molecular barcoding and efficiently filters read
171 amplification and sequencing errors, thereby enabling accurate determination of variants with
172 low variant allele frequency (VAF) (26). Matched peripheral blood mononuclear cell (PBMC)
173 sample sequencing was done to eliminate clonal hematopoiesis of indeterminate potential
174 (CHIP),. Additionally, we reviewed the previous germline-NGS tests performed on the
175 samples in which the ctDNA variants were detected. The cutoff for variant allele frequency
176 was set to 0.2% for the baseline samples, according to our previous study (26), and 0.1% for
177 serial samples due to systematic error in the Illumina NGS platform (27).
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Variant classification and copy number variant (CNV) analysis

All somatic variant interpretations were made uniformly in tumor-naïve settings using a small panel of ovarian cancer-relevant genes. The Pi-Seq pipeline (Dxome, Seongnam, Republic of Korea) was used to call and annotate somatic variants. The pathogenicity of these variants was predicted using multiple computational algorithms. We also used various somatic mutation databases, including Catalog of Somatic Mutations in Cancer (COSMIC), OncoKB, and The Cancer Genome Atlas (TCGA). The Exome Aggregation Consortium, Single Nucleotide Polymorphism Database (dbSNP), 1000 Genomes, and ClinVar databases were 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 (28). CNVs were analyzed using the CABANA (number analysis by base-level normalization) (https://github.com/lstlab/CABANA) algorithm (29).

Tumor tissue sequencing

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

procedures were performed according to the manufacturer's instructions.

Comparison of ctDNA and tissue NGS results

The concordance between ctDNA analysis and tissue NGS was assessed in a subset of patients with tissue NGS data. Because ctDNA analysis involved 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*) and SNVs and CNVs were identified. The analysis results were considered concordant when one or more Tier 1 or 2 mutations were detected at the same chromosomal position in each test.

Classification based on the dynamic changes in ctDNA

Based on the dynamic changes in ctDNA at baseline and 3 or 6 months, patients were classified into three groups: non-detected, zero-converted, and persistent. The non-detected group included patients without pathogenic mutations in baseline ctDNA samples. The zero-conversion group included those with pathogenic mutations in baseline ctDNA but showed no detectable pathogenic mutations in the 3 or 6-month follow-up samples. The persistent group included those with pathogenic mutations at baseline and persistently exhibited pathogenic mutations at 3 or 6 months of follow-up.

Statistical analysis

The primary endpoint was PFS according to the ctDNA dynamics after the surgery. PFS and OS were calculated using the Kaplan–Meier method with the log-rank test. All statistical analyses were conducted using R Statistical software (v.4.2.1), and differences with

a p-value of < 0.05 were considered significant. Statistical comparisons between groups were performed using the Mann–Whitney U or the Kruskal–Wallis tests. HR and significance were obtained using the R package 'survival' based on Cox Proportional Hazard models. P-values reported in forest plots for multivariate Cox regression were obtained using two-sided Wald test. Oncoplots to explore ctDNA characteristics and Oncoprint plots to identify positive concordant somatic variants were generated using the maftools package (Bioconductor) and the ComplexHeatmap package (Bioconductor), respectively.

Data and materials availability

Data for this study were generated at Severance Hospital, Seoul, Republic of Korea. Raw sequencing data are available in ArrayExpress (accession number E-MTAB-13555). Other data analyzed in this study were obtained from TCGA (https://portal.gdc.cancer.gov), COSMIC (https://cancer.sanger.ac.uk/cosmic), OncoKB (https://www.oncokb.org), dbSNP (https://www.ncbi.nlm.nih.gov/snp), 1000 Genomes (https://www.internationalgenome.org) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar). All other raw data supporting the findings of this study are available from the corresponding authors upon request.

Results

Patient enrollment and demographics

Between October 2019 and March 2022, 328 patients with suspected ovarian malignancy or benign-to-borderline ovarian masses and elevated CA-125 levels (>35 U/mL) were prospectively identified. After excluding patients diagnosed with synchronous cancer (n = 6) and those with sample quality failure (n = 26), analysis was performed on 296 patients,

including 201 with ovarian cancer and 95 with benign or borderline ovarian masses. The ctDNA sampling scheme and patient selection flowchart are shown in Fig. 1B. The demographics of patients with ovarian cancer are shown in Supplementary Table S1. Most patients were in the late stage (76.6%, 154/201) with a high-grade serous histology subtype (74.1%, 149/201) and underwent PDS or neoadjuvant chemotherapy followed by IDS (80.6%, 162/201). Germline *BRCA* mutations were found in 24.9% (50/201) of the patients with ovarian cancer. The median follow-up duration was 12.7 months (range: 1.6–33.1 months). During follow-up, 811 samples were collected, and the median number of samples collected per patient was 4 (range: 1–8 samples per patient; Supplementary Table S2).

Baseline ctDNA analysis

Among the 201 patients with ovarian cancer, 149 were identified with HGSC. Analysis of baseline samples identified pathogenic somatic mutations in 74.5% (111/149) of patients with HGSC and 53.8% (28/52) of those with non-HGSC. Overall, 69.2% (139/201) of patients with ovarian cancer had pathogenic somatic mutations, whereas no mutations were identified in the 95 patients with benign or borderline disease. The most frequently identified mutations were *TP53* (55.7%, 112/201), *BRCA2* (8.0%, 16/201), *ARID1A* (7.5%, 15/201), *BRCA1* (4.0%, 8/201), *PIK3CA* (5.0%, 10/201), *KRAS* (2.5%, 5/201), and *PTEN* (2.5%, 5/201) mutations. The overall landscape of pathogenic somatic mutations stratified based on histological subtypes is shown in Fig. 2A. Moreover, the findings revealed specific mutations associated with histological subtypes: *TP53* mutation with HGSC, *ARID1A* and *PIK3CA* mutations with clear cell carcinoma, and *PTEN* and *KRAS* mutations with endometrioid carcinoma. The detection rate of pathogenic *TP53* mutations in patients with

HGSC was 67.1% (100/149). The most frequent mutations in *TP53* were R273H, R248Q, R273C, R175H, R248W, and Y220C, which were consistent with mutations listed in the COSMIC serous carcinoma database (Fig. 2B).

The comparison between the tumor NGS samples obtained from a subset of patients with NGS data (n = 79) and ctDNA analysis identified pathogenic SNVs in 88.6% (70/79) of the patients with an overall concordance rate of 91.1% between the two sequencing modalities (Fig. 2C and Supplementary Table S3A). The coverage of the nine genes panel was 78.6% (110/140 mutations) compared to TSO500 panel (comprising 532 genes) based on FFPE samples with Tier 1 and 2 mutations from 79 patients. However, the nine genes panel did not cover the following Tier 1 or 2 mutations: *ARID1B, BRAF, CDH1, CDK12, CDKN2A, CTNNB1, FBXW7, KMT2A, LZTR1, MSH3, MSH6, MUTYH, NF1, NF2, NOTCH1, NRAS, PTCH1, RAD51D, SETBP1, SMAD4, SMARCA4,* and *WT1* (Fig. 2C(e)). The most frequent CNVs were in *CCNE1* (2.5%, 2/79), *MYC* (7.6%, 6/79), *KRAS* (1.3%, 1/79), and *PIK3CA* (3.8%, 3/79). Overall, we detected CNVs in 13.9% (11/79) of the patients using the CABANA algorithm, and the concordance rate between ctDNA-based CNV and tissue NGS-based CNV was 88.6% (Supplementary Table S3B). *TP53* mutations in tissue samples were detected in 92.8% of the ctDNA samples (64/69 with *TP53* mutations in tissue NGS samples). The concordance of *TP53* mutation was 90.4%, with a sensitivity of 92.8% and specificity of 84.0% (Supplementary Table S3C).

Classification based on ctDNA trend in sampling time-points

Of the 201 patients with ovarian cancer, 143 had ctDNA samples at baseline (T0), 3 months (T1), or 6 months (T2). To determine the optimal time-point for follow-up ctDNA

sampling, both time-point pairs were investigated separately. The proportions of patients classified into the non-detected, zero-converted, and persistent groups were 28.7%, 32.2%, and 39.2% for the T0–T1 pair and 28.7%, 49.7%, and 21.7% for the T0–T2 pair, respectively. Both time-point options led to a significant PFS difference among the three clinical groups ($p < 0.0001$ for both); however, the T0–T2 pair demonstrated greater separation (Supplementary Figs. S1A–S1B). Moreover, patients with persistent detection of pathogenic somatic mutations in ctDNA at 6 months, which marks the end of the conventional six cycles of adjuvant treatment, showed significantly worse PFS among the three clinical groups.

Therefore, further subgroup analyses were performed using the baseline (T0) and 6-month (T2) pair. The median PFS was 17.9 months (range: 5.8–29.8 months) in the not-detected, 19.0 months (range: 4.3–32.8 months) in the zero-converted, and 6.7 months (range: 1.6–33.1 months) in the persistent groups. Pairwise comparisons demonstrated that the PFS of the patients in the zero-conversion group did not differ from that in the non-detected group ($p = 0.41$). However, PFS significantly differed between the zero-converted and persistent groups ($p = 0.001$) and the non-detected and persistent groups ($p = 0.001$). Subgroup analysis further confirmed that ctDNA trends, based on baseline (T0) and 6-month (T2) time-points, were highly predictive of PFS, irrespective of the clinical stage (early vs. late stage) (Fig. 3A), treatment setting (newly diagnosed vs. recurrent; Supplementary Fig. S1C), the use of maintenance therapy (Supplementary Fig. S1D), and the type of surgery undergone (PDS vs. IDS; Supplementary Fig. S2A–S2B). Cox proportional-hazards model analysis showed that persistent detection of ctDNA mutations at 6 months after initial treatment was significantly associated with a worse PFS (HR = 10.7 [4.4–25.9), $p < 0.001$, after adjusting for the above-mentioned clinical variables; Supplementary Fig. S3). Fig. 3B shows the ctDNA dynamic

status at baseline and 3 and 6 months. Among the 104 patients who were ctDNA-positive at baseline, 45% (46/104) were zero-converted at T1. Twenty-six of the fifty-seven patients with ctDNA positivity at T1 were zero-converted at T2. Additionally, among 32 patients with persistent ctDNA positivity at T2, 29 (91% of 32) experienced disease progression. At T3 (9 months after surgery), 83.5% (91/109) of patients held the same ctDNA status (positive or negative) of T2, 4.6% (5/109) of patients were zero-converted from persistent, and 11.9% (13/109) of patients were converted to positive.

Longitudinal monitoring with serially collected ctDNA

After a median follow-up of 12.7 months (range: 1.6–33.1 months), 73 of the 201 patients exhibited disease progression. In longitudinal sample analysis, 27 patients were excluded due to fewer than three serial samples (n = 19), lack of follow-up ctDNA sample collection within 3 months of clinical recurrence (n = 2), and no mutation detected at baseline (n = 6). The findings from serial ctDNA, 3-weekly CA-125, and 3-monthly radiological assessments are shown in Fig. 4A. The mutation at baseline was consistently identified in serially collected samples from 44 of 46 patients (95.7%). Furthermore, compared to CT, serial ctDNA testing detected recurrence at an earlier time-point with a median lead time of 2.0 months (range: 0–8.8 months), with a lead time >1 month in 39.1% (18/46) of patients. Of the 26 patients whose CA-125 levels dropped below the upper limit of normal after debulking and adjuvant chemotherapy, ctDNA testing resulted in an earlier identification of disease progression than CA-125 assessment based on the GCIG criteria by a median of 2.3 months (range: 0–8.3 months), with 57.7% of patients (15/26) having a lead time of > 1 month with ctDNA testing compared with CA-125 assessment; an example is shown in Fig.

4B. The lead time for detecting disease recurrence with ctDNA testing did not differ between the patients who received maintenance therapy and those who did not (p = 0.717; Supplementary Table S4).

Discussion

We prospectively obtained ctDNA samples from 296 patients, including 201 with ovarian cancer and 95 with benign or borderline adnexal masses. Based on our findings, ctDNAs reflect patient-specific tumor mutations and can effectively determine disease recurrence in longitudinal samples. Moreover, ctDNA dynamics at baseline and 6 months after diagnosis allowed patients to be classified into non-detected, zero-converted, and persistent groups, which were highly predictive of PFS. The prognostic value of ctDNA was maintained regardless of the clinical stage and the use of adjuvants or maintenance therapy. Furthermore, when assessed at 3-monthly intervals, disease progression could be detected earlier with ctDNA than with conventional monitoring tools such as APCT or CA-125 assessment.

Earlier studies on cfDNA or ctDNA in ovarian cancer focused on analyzing baseline samples. For instance, cfDNA at baseline is associated with tumor burden and disease progression (30-32). The prognostic value of baseline ctDNA has also been reported (20, 33). Two previous studies demonstrated the feasibility of surveillance based on ctDNA using a multi-gene panel (16, 34); however, the number of patients analyzed was small, with 2 and 12 patients, respectively. In this context, our ovarian cancer cohort was one of the largest cohorts in terms of the number of patients and samples. Moreover, our study is the only one to date that includes information on the use of maintenance therapy, such as PARP inhibitors and

bevacizumab. As we prospectively included all patients with ovarian cancer from a single institution during the given time window, the patient demographics were comprehensive and reflective of the clinical setting at a tertiary hospital.

In terms of the interpretation of ctDNA mutation profiles, we only included Tier 1 and 2 pathogenic variants because the focus on specificity was important for MRD detection. If we included Tier 3 variants of unknown significance, the sensitivity might have improved at the expense of specificity. Using this approach, we discovered that the pathogenic mutation in ctDNA was largely concordant with that obtained from tissue NGS analysis, not only in terms of mutated genes but also for the specific pathogenic variant involved. The concordance rate of 88.6% was higher than the previously reported range of 79–81% (16, 34). These data support that ctDNA based on blood samples reflects patient-specific tumor molecular profiles.

We also identified several mutations in ctDNA but not in tumor NGS. Moreover, our analysis did not identify pathogenic mutations in benign/borderline patients, suggesting high specificity of ctDNA-based analysis. Thus, we cannot assume that ctDNA-only mutations are false positive, as tumor-tissue NGS could have failed to detect true mutations due to intra-tumor heterogeneity or other unknown underlying causes (35). Parallel molecular testing of tumors appears to be the best strategy for providing precise information. However, since tumor tissue biopsy is invasive and frequently impossible, ctDNA samples can provide valuable clinical information.

The pathogenic variants in our ctDNA samples were largely consistent with previously reported variants in the COSMIC database, and the type of mutation was

associated with the histological type. The rate of pathogenic *TP53* mutations detected in patients with HGSC in our study was 67.1%, which is within the range of 66.7–86% reported previously in ovarian cancer ctDNA studies (16, 32, 36). Nevertheless, some tumor tissue-based studies have also reported a higher detection rate (72–96.7%) of pathogenic mutations in *TP53* (37, 38). The rate of *ARID1A/PIKC3CA* mutations among patients with clear cell carcinoma was 50%—within the previously reported range of 50.0–66.7% (39-41). In addition to SNV, ctDNA samples provided CNV information, albeit with a lower detection rate (13.9%) than that of tissue-based NGS (20.3%). Our ctDNA-based CNV detection rate was lower than that reported by Noguchi et al. (16), who assessed MET and EGFR mutations in ctDNA (19.6%) and that by Nakabayashi et al. (42), who utilized cfDNA analysis for non-invasive prenatal testing (16.7%). Therefore, we speculate that using a larger sequencing panel may improve the CNV detection rate.

The size of our panel is notable. Previous studies on ctDNA in ovarian cancer used NGS panels with 55–500 genes (16, 43, 44). During the pilot phase of our study, we designed a panel using genes that are frequently altered in ovarian cancer (16, 23, 24). Utilizing a more compact panel (three genes) containing *TP53, BRCA1,* and *BRCA2* can easily detect pathogenic mutations. However, adding *ARID1A, PIK3CA, KRAS,* and *PTEN* genes to the panel (seven genes) may increase the sensitivity because they are specific to non-serous histological subtypes. We also evaluated the coverage of the nine genes panel compared with that of the tissue-based 532 genes panel (78.6%). We expect the sensitivity and specificity of our custom panel to improve if we add *NF1* and *CDK12* for additional HGSC coverage and *NRAS* for low-grade serous carcinoma, all of which are often detected in FFPE analysis. Furthermore, to capture CNV in addition to SNV, the *MYC* and *CCNE1* genes need to be

included in the panel. Our current nine genes panel is both cost-effective (Supplementary Table S5) and efficient, with the potential to be incorporated into a large-scale screening program, as well as an adjunct test with CA-125 assessment in managing ovarian cancer.

Our findings based on the analysis of ctDNA dynamics have important clinical implications. We identified the optimum time point for follow-up sampling in the adjuvant setting based on the Kaplan–Meier curve. For OR, ctDNA testing at 3 months, corresponding to three cycles of adjuvant therapy and the timing of radiologic assessment, was not as good as that at 6 months, which marks the end of the conventional six cycles of adjuvant platinum-based chemotherapy. This finding is similar to that of a previous study by Elena et al., who showed that undetectable levels of ctDNA, based on droplet PCR testing, at 6 months were associated with improved PFS (45). Persistent ctDNA at six months was independently associated with poor PFS with an HR of 10.7, even when other relevant clinical parameters were considered. Based on our results, ctDNA analysis based on two sampling time points may provide clinically useful information, as most patients with ovarian cancer are diagnosed at a late stage and receive adjuvant chemotherapy. The MRD status at 6 months may help identify patients who may benefit from more intensive monitoring or treatment, such as additional cycles of chemotherapy, use of a combination regimen, or addition of maintenance therapy. In contrast, the absence of pathogenic mutations at 6 months suggests the possibility of less intensive monitoring or withholding maintenance with bevacizumab or PARP inhibitors.

With respect to monitoring, we found the same pathogenic variant in the follow-up samples as in the baseline samples in 94% of patients with disease progression, which suggested that the mutation profile of the patients at baseline is highly specific and useful in

terms of a ctDNA-based monitoring scheme. Similar to a previous study on the usefulness of *TP53* mutations in monitoring ovarian cancer (17), we found that *TP53* serves as an important monitoring biomarker. Furthermore, for detection timing, disease progression was detected earlier with serial ctDNA testing than with the currently used modalities (3-monthly APCT or 3-weekly CA-125 assessments). For approximately 15% of patients with progressive ovarian cancer who did not show elevated CA-125 levels, ctDNA-based monitoring effectively identified recurrence. The ability to identify disease progression early may offer clinicians considerable flexibility. For example, patients may undergo screening and additional immunohistochemical testing to participate in clinical trials. In addition, the ability to act early on signs of recurrence may increase the efficacy of specific treatments that are more effective in low-disease-burden settings.

Study limitations

Our study has several limitations. First, tissue NGS was performed in only a small proportion of patients. Second, due to the short observation period and the loss of some patients to follow-up, longitudinal monitoring analysis was only possible for patients with at least four serial samples, including the one at baseline. Third, the subgroup of patients enrolled in the later phase of the study had a relatively short observation period, increasing the right-censored samples. However, the log-rank test used in Kaplan–Meier plots is highly effective in comparing the equality of survival distributions within various observation periods. Fourth, our panel was limited to the nine genes in our sequencing panel. Although our panel is advantageous in terms of cost-effectiveness and efficiency, genetic information was limited to the genes included in our panel.

Conclusion

Analysis of serial ctDNA with a tumor-naïve, small-sized sequencing panel was effective in terms of MRD detection and early detection of disease progression compared to that of conventional modalities such as 3-monthly APCT or CA-125 assessments. In a sizeable cohort of patients with ovarian cancer, we further determined the optimal follow-up sampling time point for MRD detection to be 6 months. Furthermore, the capacity for early detection of disease progression was not compromised, even in patients receiving maintenance therapy with bevacizumab or PARP inhibitors. Our findings highlight the potential of incorporating serial ctDNA sequencing into the clinical management of ovarian cancer. These findings are hypothesis-generating and have the potential to be used as a reference for future clinical trials.

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Figure legends

Figure 1. (A) Sampling schema for patients with newly diagnosed ovarian cancer. (B) Consort diagram of patient enrollment.

Figure 2. Mutation based on baseline ctDNA. (A) Pathogenic, somatic mutation profile stratified by histology. (B) Specific mutation location for *TP53* gene; Plot of variants done using St. Jude Cloud protein paint (https://pecan.stjude.cloud/proteinpaint). (C) Concordance/Coverage of Tier 1 or 2 mutations between ctDNA and tissue NGS analysis among patients who underwent both tests.

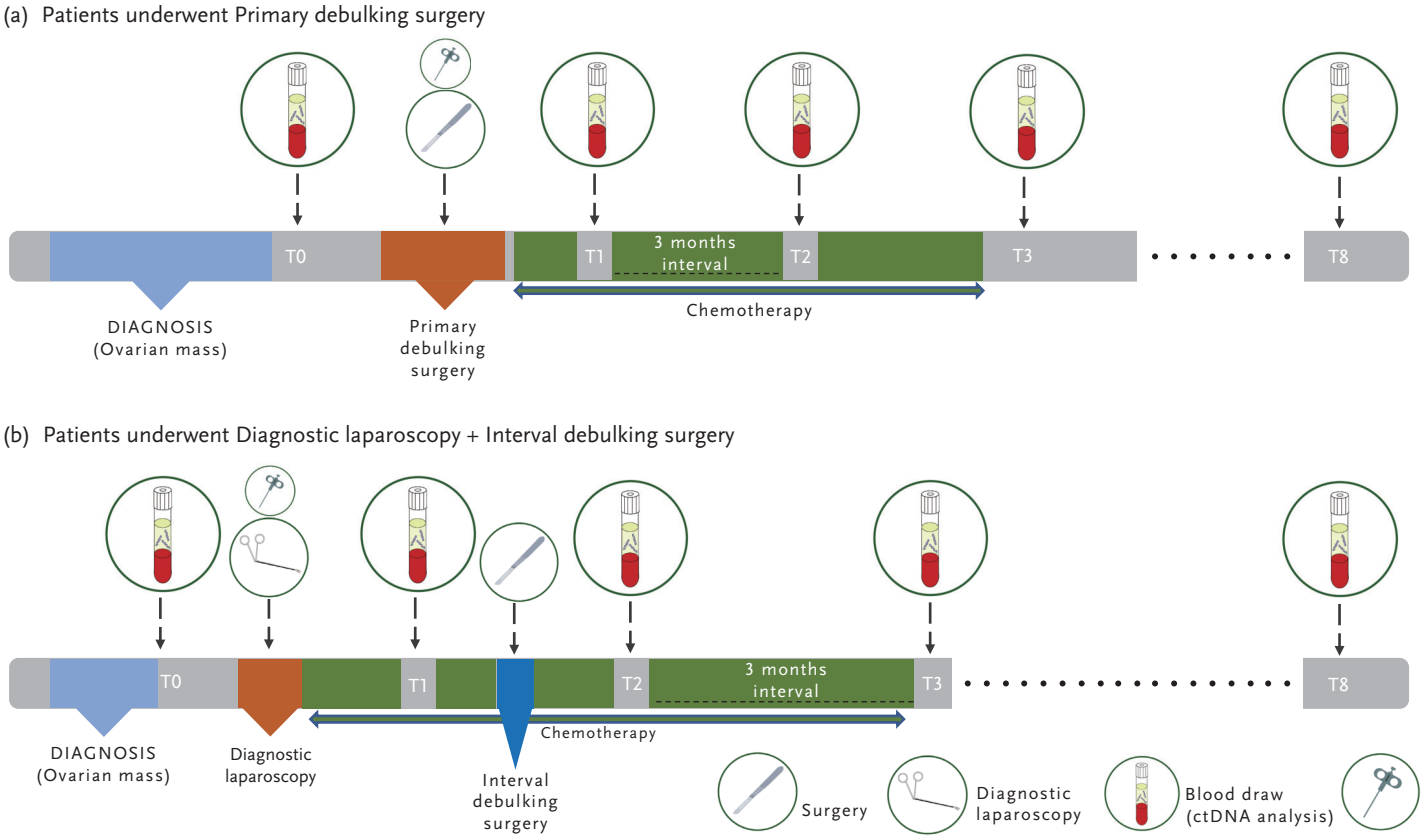
Figure 3. (A) Prognostic stratification based on samples collected at different time-points (T0: baseline, T1: 3 months, T2: 6 months). Progression-free survival with respect to the three prognostic groups based on T0 and T2 ctDNA among patients with early- and late-stage disease. Patients right-censored due to incomplete observations are shown as vertical tick marks. (B) Flow diagram showing ctDNA positivity at T0 (baseline), T1 (3 months), and T2 (6 months).

Figure 4. Disease monitoring with serial ctDNA collected at 3 monthly intervals. (A) swimmer plot showing ctDNA positivity for each sampling time point, alongside the status of conventional monitoring tools such as APCT and CA-125. (B) An exemplary case of a patient with findings from serial ctDNA and variant allele frequency, serial CA-125 levels, and clinical disease progression timing based on PET-CT.

PET-CT, Positron emission tomography-CT.

Figure 1

A



B

328 patients suspicious of ovarian malignancy (*N*=328) (CA-125 > 35 U/ml)

Exclude (*N*=33)
Patient ineligible :
- Synchronous cancer (*N*=6)
- Insufficient sample volume for analysis (*N*=26)

Primary ovarian cancer (*N*=201) (high-grade serous (*N*=149), clear cell (*N*=22), mucinous (*N*=13), low-grade serous (*N*=7), endometrioid (*N*=7) , *others (*N*=3))

Benign, borderline (benign (*N*=83), borderline (*N*=12))

ctDNA analysis (*N*=296) (baseline samples)

Clinical follow-up (CA-125, Imaging)
3 month interval ctDNA analysis
Pathological information
Tumor tissue NGS (*N*=79)

*other histologic types of ovarian cancer, granulosa cell (*N*=1), dysgerminoma (*N*=1), undifferntiated (*N*=1)

Figure 2

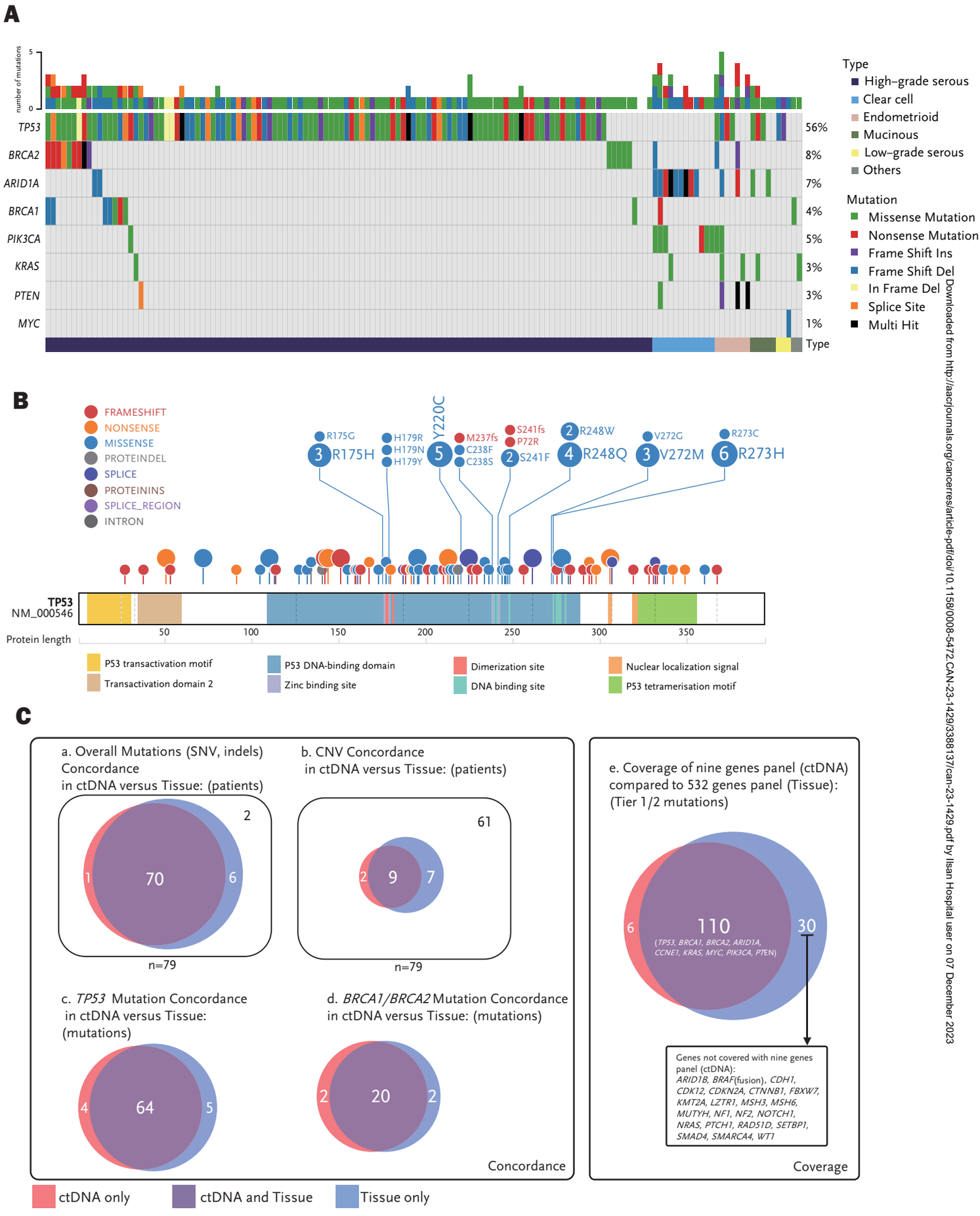


Figure 3

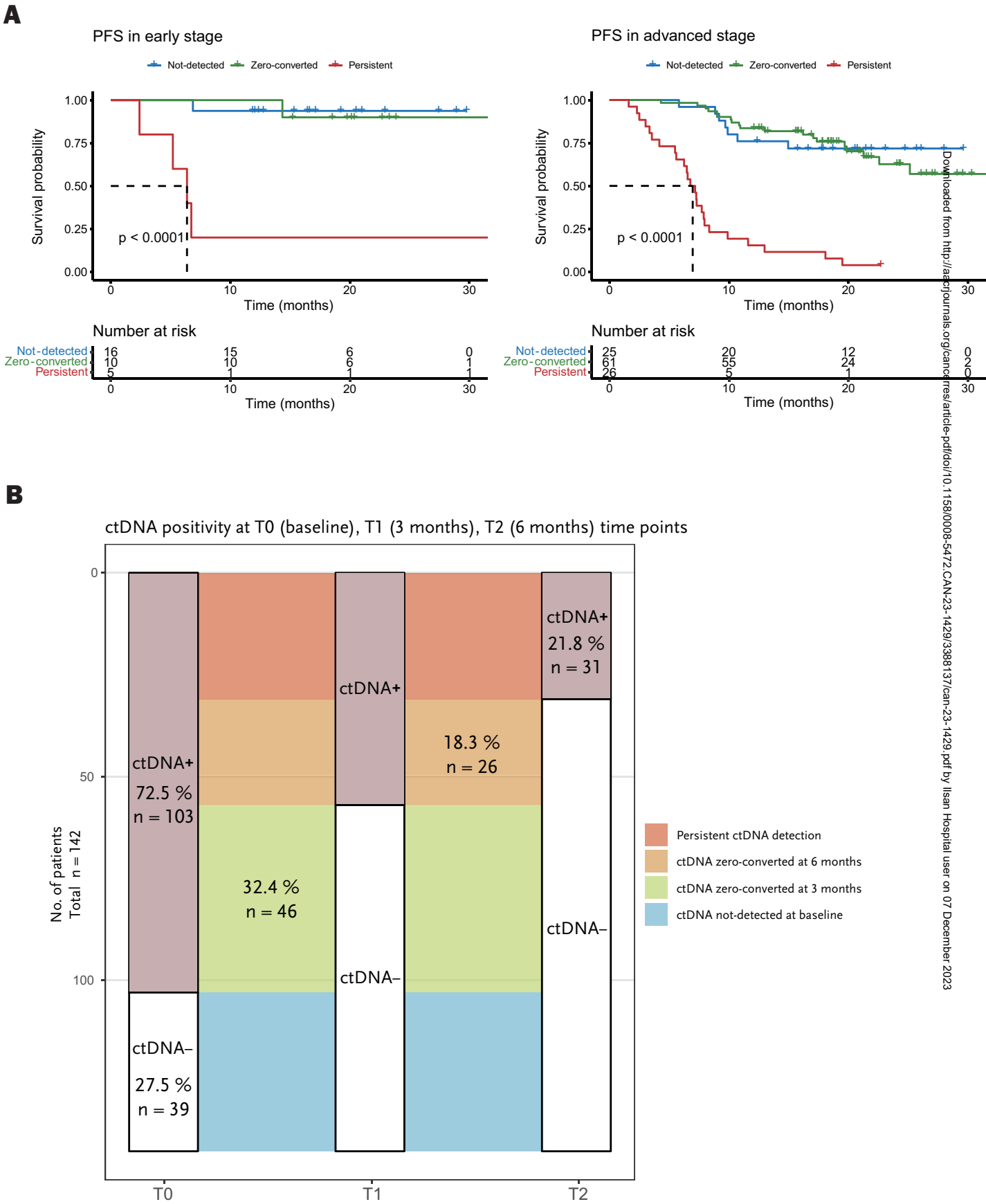


Figure 4