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ORIGINAL RESEARCH



# Mutation profiling of both cfDNAs and CTCs in gynecological cancer patients

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**Abstract:**

**Background:** Cell-free circulating DNA (cfDNA) and circulating tumor cells (CTCs) are emerging minimally invasive cancer biomarkers that help with early diagnosis, prognosis and therapeutic target selection. Combined use of cfDNA and CTCs provides complementary information about tumor cell heterogeneity thus helping to identify genetic mutations relevant in clinical decision making.

**Patients and methods:** cfDNA and CTCs were isolated from whole blood specimens of 20 gynecological cancer patients by CD-PRIME™. We performed targeted sequencing across 51 actionable genes in 20 cfDNA and ctcDNA, and then analyzed genetic mutations and clinical significance.

**Results:** A total of 33 somatic variants were found in 16 actionable genes. A genetic variant analysis revealed 15 somatic variants in the cfDNA and 20 somatic variants in CTCs sample, only two variants were found in common. The most frequently altered genes in cfDNA samples were *TP53, PTCH1, FGFR, and BRCA2*. in contrast, the most frequently altered genes in CTCs sample were *TP53, BRCA1, TSC2, ERBB2* and *PTCH1*. An *in silico* analysis revealed that 60% of somatic variants (20 out of 33) were potentially pathogenic mutations as expected. Detected *BRCA1* p.S573 frameshift mutation and *BRCA2* p.Q1683 nonsense mutation lead to loss-of-function of *BRCA1* and *BRCA2*.

**Conclusion:** Our study shows that the genetic profiling of cfDNA and CTCs together provides more enriched genomic information for guiding preclinical and clinical strategies and targeted therapies.

**Keywords:** cfDNA, CTC, Gynecologic cancer, NGS, precision medicine

**Abbreviations:** cfDNA, cell-free circulating DNA; CTC, circulating tumor cells; ctcDNA, CTC-derived DNA; NGS, next-generation sequencing; VUS, variant of uncertain significance; VAF, variant allele frequency; SNV, single nucleotide variant; INDEL, insertion and deletion.

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# Introduction

Cancer is a disease of uncontrolled cell growth, largely caused by genetic mutations that dysregulate cell functioning. Mutations can be inherited however, most occur during the lifetime of an individual. DNA mutations have been linked to both the development of cancer and the specific biological characteristics of any given cancer. Traits such as cancer initiation, progression, metastasis, treatment response and drug resistance, all depend on specific mutations. Therefore, accurate genetic profiling is required for precision medicine applications. The genetic analysis of individual mutations offers both useful information to help design medical treatments and avoid ineffective therapies.[1]

Targeted next-generation sequencing (NGS) is an effective method of analyzing specific regions of DNA of interest in a genome. Targeted sequencing is more time- and cost-effective than whole genome sequencing, and provides significant advantages including higher sequencing depth, multiplexing capacity, mutation resolution and ease of data analysis.[2] Applications of NGS to profiling tumor characteristics are facilitating personalized cancer therapies and improving therapeutic outcomes.[3,4]

Liquid biopsy is a reliable surrogate for tissue biopsies for obtaining prognostic and predictive information about cancers. Evidence shows that the blood serves as a reservoir of both cfDNA and CTCs and they reflect tumor genomic diversity better than tissue biopsies.[5,6] Therefore, using cell-free DNA (cfDNA) and circulating tumor cells (CTCs) from blood only, liquid biopsies offer a non-invasive method that overcomes the limitations of traditional tissue biopsies.[7,8] The analysis of genomic mutations in cfDNA or CTC can be used to monitor tumor behavior and treatment response over time, as high rates of DNA instability in cancer cells lead to continuous mutations of clinical significance.[9,10] Although these two methods are similar, they are fundamentally different. The origin of cfDNA is thought to be mainly from cells that have undergone apoptotic or necrotic cell death. The half-life of cfDNA has been reported to range from 16 minutes to a few hours, making cfDNA derived from tumor cells one of the easiest ways to detect up-to-date information about tumor status.[11,12] On the other hand, CTCs captured from blood are directly shed from the primary tumor or metastatic sites and can even be cultured for in-depth analyses providing much more definite information about cancer origin and status. Studies have reported advantages such as concordance between genetic mutation in CTCs and primary tissue[13], and extensive information about DNA, RNA and protein.[14,15] Hence, the isolation and

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detection of CTCs offers one of the most promising methods for accurate and precise diagnosing many cancers and predicting metastasis.[16-18] As cfDNA and CTCs possess different analytical properties, when analyzed in combination, may provide complementary and augmented information on tumor cell heterogeneity that is critical in identifying key molecular targets for improved cancer therapies.[19]

Gynecologic cancer is a cancer developed in female reproductive organs. Each gynecologic cancer has different signs, symptoms, and causes. Early detection of gynecologic cancer and an understanding of specific mutations involved can lead to more effective treatments. In this study, we performed targeted sequencing of a customized cancer panel of 51 actionable genes in cfDNA and CTCs samples of gynecologic cancers, including vulvar, endometrial, ovarian, cervical and uterine cancers. This study shows that the actionable mutations of cfDNA and CTCs from patients can be readily profiled using NGS, making them available for clinical application in guiding treatment decisions during diagnosis, disease monitoring and recommending the appropriate drug for each individual patient over time through non-invasive assays.

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# Material and methods

## Clinical samples

cfDNA and CTCs samples obtained from 20 patients with confirmed diagnoses of gynecologic cancer were subjected to targeted sequencing using our customized cancer panel. All subjects provided informed consent to participate and all clinical specimens were collected in accordance with IRBs at Chonbuk National University Hospital. Detailed clinicopathologic information for the 20 cases are provided in Table 1 and Supplementary Table S1. A total of 7 ml of whole peripheral blood was collected in EDTA tubes from each patient. 2 ml of blood was processed with CTC enrichment and 5 ml used for the plasma preparation.

**Table 1** Clinical characteristics of the 20 gynecological cancer patients in this study.

| Characteristic | | Number | Range (%) |
|---|---|---|---|
| **Age** | median (IQR) | 62 | 42 - 91 |
| **Stage** | I | 8 | (40%) |
| | II | 4 | (20%) |
| | III | 5 | (25%) |
| | IV | 3 | (15%) |
| **Cancer type** | Ovarian cancer | 9 | (45%) |
| | Uterine cancer | 3 | (15%) |
| | Cervical cancer | 4 | (20%) |
| | Endometrial cancer | 3 | (15%) |
| | Vulvar cancer | 1 | (5%) |

## Sample Preparation

Plasma was isolated from 5 ml of whole blood using density gradient centrifugation in Ficoll-Paque™ PLUS (GE Healthcare). cfDNA was extracted from isolated plasma samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. cfDNA was extracted from 2 ml to 4 ml of plasma and quantified using Qubit 3.0 fluorometer (Invitrogen). The quality of cfDNA was measured using the 2100 Bioanalyzer (Agilent) to confirm absence of contamination with genomic DNA.

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2 ml of whole blood was processed to enrich CTCs using the CD-PRIME™ system (Clinomics Inc.). Whole blood was centrifugated using an equal volume of Ficoll-Paque™ PLUS solution. After centrifugation, the peripheral blood mononuclear cell (PBMC) fraction was recovered from the separated blood cell fraction and applied to the CD-PRIME™ platform[20,21] for the enrichment of CTCs. CTCs were enriched on the membranes of CD-CTC-discs and membranes placed into collection tubes followed by DNA extraction using QIAamp DNA Micro Kit (Qiagen). Whole genome amplification was performed on extracted DNA from CTCs using REPLI-g Mini Kit (Qiagen).

## Panel development

We designed a customized NGS panel to characterize somatic SNVs, INDELs, and CNVs in 51 actionable genes. Candidate genes were included on the basis of associated FDA-approved therapies or reported clinical trials. The cancer panel was designed using Ampliseq Designer (5.4.1, Thermo Fisher Scientific). A total of 1,355 amplicons are designed in two primer pools. Amplicon size was designed to lie within the 125-175 bp range and the total number of bases covered by the amplicons is 136.75 kb.

## Library preparation and sequencing

A total of 10 ng of cfDNA or CTC-derived DNA (ctcDNA) was used for the library construction. Library preparation was performed using Ion Ampliseq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer's instruction. We used the Ion Express Barcode Adaptors Kit (Thermo Fisher Scientific) for sample multiplexing and libraries were purified using the Agencourt AMPure XP (Beckman Coulter) reagent. Libraries were quantified using the Qubit 3.0 fluorometer and the 2100 Bioanalyzer. Template preparation for the libraries was performed using the Ion Chef Instrument (Thermo Fisher Scientific) with Ion 540 Chef Kit (Thermo Fisher Scientific). Multiplexed templates were subjected to sequencing on the Ion S5 XL system (Thermo Fisher Scientific).

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## Sequencing data analysis

The human genome sequence hg19 was used as the reference. Sequence and data analysis were performed using Torrent Suite software (5.8.0). Sequencing coverage analysis was performed using coverage Analysis (5.8.0.1) plugins and VCF files were generated using the variantCaller (5.8.0.19) plugins. Annotation of the variants was obtained using the Ion Reporter (5.10.2.0) software. To filter out potential sequencing background noise, we excluded control variants detected in cfDNA or CTCs samples from 30 healthy individuals. Common Korean SNVs which are included in KoVariome whole genome sequence (WGS) database from 50 healthy unrelated Korean individuals[22,23] were also excluded. We identified variants of uncertain significance (VUS) using SIFT, PolyPhen-2 and used OncoKDM to predict the effect of genetic variants on protein function [24-27]. In addition, variants were annotated using ClinVar, COSMIC, and TCGA to match them to previously reported variants. To provide further clinical implications of the annotated tumor variants, we used a precision oncology knowledge base, OncoKB[28], which provides the guide information for FDA-approved therapies in clinical trials.

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# RESULTS

## Patient characteristics

We performed sequencing of cfDNA and ctcDNA obtained from 20 gynecologic cancer patients to characterize the diversity of genomic variants found. The cancers grouped into five distinct types. The most frequent type was ovarian (n=9, 45%), followed by cervical (n=4, 20%), uterine (n=3, 15%), endometrial (n=3, 15%) and vulvar cancer (n=1, 5%). Eight patients had Stage I (40%), four had Stage II (20%), four had Stage III (20%), two had Stage IV (10%) and two were not determined (10%). The median age of the patients was 62 years (range 42-91) (Table 1, Supplementary table S1).

## Mutation profiling of cfDNA and ctcDNA using caner panel

We isolated cfDNA and CTCs from each gynecological patient blood and performed NGS sequencing using our customized-designed cancer panel of 51 actionable genes with known drug or therapeutic relevance (Supplementary table S2). The average sequencing coverage obtained with our cancer panel was higher than 1000X, with sequencing data covering approximately 136.75 kb. We identified genetic variants in 19 out of 51 actionable genes using the cancer panel. The variants of germline, 30 normal individuals and common Korean SNPs were eliminated from detected total variants. Table 2 shows the total variants detected from cfDNA and ctcDNA of patients in these two groups. A total of 33 somatic variants were found in 16 actionable genes. We performed an *in silico* analysis of VUS to predict impact of genetic variant on protein function (see Methods) revealing that 60% of the somatic variants (20 out of 33) and 30% of the germline variants (five out of 17) were potentially pathogenic mutations as expected. The most frequently mutated genes were *BRCA1*, *BRCA2*, and *TP53*. In *BRCA1*, where three missense, two frameshift and one deletion mutations were observed. Four of these variants (K110R, Q148H, S1736I, S573fs*) were determined by *in silico* analyses as potential pathogenic mutations (Figure 1A). In *BRCA2*, seven missense and one nonsense mutations were identified. The Q1683* mutation leads to a truncation at BRC repeat sequences that bind to DNA meiotic recombinase 1[29] and N1100T, A3122T and K1445T were also determined to be potential pathogenic mutations. Interestingly, the V2466A germline variant was detected in all patients except patient number 10 but was determined to be a tolerated mutation (Figure 1B). We also observed the *TP53* mutation in nine patients.

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**Table 2. List of variants identified by cancer panel**

Somatic mutations

| Gene | Patient | | cDNA | Protein | Predictive algorithms | | |
|---|---|---|---|---|---|---|---|
| | cfDNA | CTCs | | | SIFT[a] | PolyPhen-2[b] | OncoKDM[c] |
| *BAP1* | | 1 | c.548T>C | p.L183P | D | PD | |
| | | 17 | c.1168C>A | p.P390T | D | B | |
| *BRCA1* | 4 | | c.444G>T | p.Q148H | | PD | |
| | | 4 | c.5207G>T | p.S1736I | | PD | |
| | | 10 | c.329A>G | p.K110R | | PS | |
| | | 18 | c.3114delA | p.A1039fs | | | |
| *BRCA2* | 5 | | c.5047C>T | p.Q1683* | D | | D |
| | 11 | | c.3299A>C | p.N1100T | T | PS | |
| | 11 | | c.9364G>A | p.A3122T | D | PD | |
| *BTK* | 8 | | c.1475G>A | p.R492H | T | B | |
| *CDK4* | | 9 | c.753_754insC | p.R252fs | | | |
| *ERBB2* | | 5 | c.1966T>C | p.S656P | | B | |
| | | 9 | c.1147G>A | p.G383R | D | PD | |
| | | 16 | c.1642C>T | p.Q548* | D | | D |
| *ERBB3* | | 18 | c.1959_1960insT | p.V654fs | | | |
| *FGFR1* | | 2 | c.1741delG | p.A581fs | | | |
| | 15 | | c.947C>T | p.P316L | | PD | |
| *FGFR3* | 5, 7, 14 | | c.1295G>A | p.S432N | T | B | |
| *MYCN* | 16 | | c.134_135delCG | p.P45fs | | | |
| *NOTCH1* | 5 | | c.2982C>G | p.N994K | D | PD | |
| *PIK3CA* | 10 | | c.3136G>A | p.A1046T | D | PD | |
| *PTCH1* | 2, 7, 16 | 20 | c.3785C>T | p.P1262L | | PD | |
| | 4 | 4, 20 | c.3943C>T | p.P1315S | | B | |
| *TP53* | | 2, 4, 5, 12 | c.214C>G | p.P72A | T | B | |
| | 8 | | c.785G>T | p.G262V | D | PD | |
| | 9 | | c.743G>A | p.R248Q | | PD | PD |
| | 10 | | c.602delT | p.L201fs | | | |
| | | 11 | c.31G>C | p.E11Q | T | PD | |
| | 13 | | c.827C>A | p.A276D | D | PD | |
| *TSC1* | | 12 | c.1585G>A | p.A529T | | B | |
| *TSC2* | | 15 | c.2509G>A | p.A837T | | PD | |
| | | 15 | c.3178T>C | p.W1060R | | PD | |
| | | 18 | c.3299T>C | p.V1100A | T | B | |

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Germline mutations

| Gene | Patient | | cDNA | Protein | Predictive algorithms | | |
|---|---|---|---|---|---|---|---|
| | cfDNA | CTCs | | | SIFT[a] | PolyPhen-2[b] | OncoKDM[c] |
| *ALK* | 1~20 | 6, 8, 10~12, 14, 17, 19 | c.4381A>G | p.I1461V | | B | |
| *BAP1* | 16 | | c.1168C>A | p.P390T | | B | |
| *BRCA1* | 8 | | c.5080_5082delCAC | p.H1694del | | | |
| | 1 | | c.1716_1717insA | p.S573fs* | | | |
| *BRCA2* | 4 | | c.4334A>C | p.K1445T | D | PD | |
| | 8 | | c.7088A>G | p.Y2363C | T | B | |
| | 1~9, 11~20 | 4, 5, 8, 11, 12, 15, 16, 19 | c.7397T>C | p.V2466A | | B | |
| | 7 | 8 | c.7469T>C | p.I2490T | T | B | |
| *ERBB2* | 11 | 11 | c.3149C>T | p.S1050L | | B | |
| *FGFR2* | 1 | | c.238G>A | p.G80R | T | B | |
| *FGFR4* | 1~7, 13~15, 17, 18, 20 | 2, 5, 6, 8, 9, 13~16, 19 | c.28G>A | p.V10I | T | B | |
| | 1~20 | 2~6, 8~20 | c.407C>T | p.P136L | T | B | |
| *NOTCH1* | 16 | | c.6788G>A | p.R2263Q | T | B | |
| | 2~9, 11~20 | 5~9, 11, 13, 15~19 | c.3569A>G | p.H1190R | T | B | |
| *PTCH1* | 4 | | c.3232C>T | p.L1078F | | PD | |
| *TP53* | 17 | | c.722C>A | p.S241Y | | PD | D |
| *TSC2* | 19 | | c.2153G>A | p.R718H | | PD | |

[a] SIFT: D, damaging; T, tolerated

[b]PolyPhen-2: PD, probably damaging; PS, possibly damaging; B, benign

[c]OncoKDM: D, damaging; PD, potential damaging

The most frequently mutated genes were *BRCA1*, *BRCA2*, and *TP53*. In *BRCA1*, where three missense, two frameshift and one deletion mutations were observed. Four of these variants (K110R, Q148H, S1736I, S573fs*) were determined by *in silico* analyses as potential pathogenic mutations (Figure 1A). In *BRCA2*, seven missense and one nonsense mutations were identified. The Q1683* mutation leads to a truncation at BRC repeat sequences that bind to DNA meiotic recombinase 1[29] and N1100T, A3122T and K1445T were also determined to be potential pathogenic mutations. Interestingly, the V2466A germline variant was detected in all patients except patient number 10 but was determined to be a tolerated mutation (Figure 1B). We also observed the *TP53* mutation in nine patients. The seven *TP53* mutations consisted of six missense mutations and one frameshift. Moreover, alterations, such as p.L201fs, p.S241Y, p.248Q, p.G262V, and p.A276D, were all detected at its DNA binding domain. Many studies have reported that mutations in the DNA binding domain of p53 can result in loss-of-function activities and promote tumor growth[30,31] (Figure 1C).

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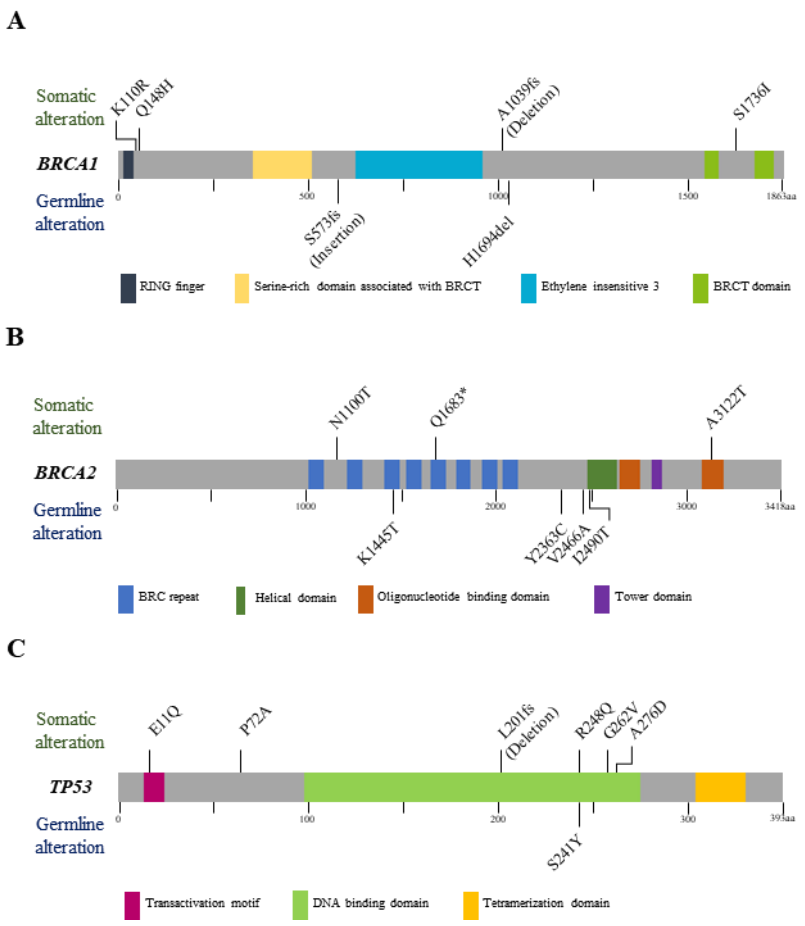


**Figure 1** Schematic diagram of alteration distribution per gene. **Notes:** The *BRCA1* (A), *BRCA2* (B) and *TP53* (C) are displayed in the context of protein domain models derived from cBioPortal. Number indicates amino acid residues and the colored boxes are specific functional domains.

## The mutation frequencies in cfDNA and ctcDNA of gynecologic cancers

To explore the clonal heterogeneity of cfDNA and ctcDNA, sequence data from cfDNA and ctcDNA were compared. An independent mutation analysis revealed 13 somatic variants in the cfDNA sample (Supplementary Figure S1A) and 18 somatic variants in CTCs sample (Supplementary Figure S1B). This analysis confirmed that variants detected in cfDNA or ctcDNA, have only a 6% overlap (two out of 33 variants; Supplementary Figure S1C) indicating that the combined approach of using both methods improves accuracy of diagnoses and monitoring of tumor progression.

We detected a total of 31 genetic variants in 15 genes and 85% patients have least one variant

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in either cfDNA or ctcDNA. The median number of mutated genes per patients was two (range of 0-5). The most frequently altered genes in cfDNA samples were *TP53* (20%, four out of 20 cfDNA samples), *PTCH1* (20%), *FGFR* (15%) and *BRCA2* (15%). In contrast, the most frequently altered genes in ctcDNA samples were *TP53* (25%, five out of 20 ctcDNA samples), *BRCA1* (15%), *TSC2* (15%), *ERBB2* (15%) and *PTCH1* (15%). The distribution of these genetic mutations in the whole population is shown in Figure 2A and 2B.

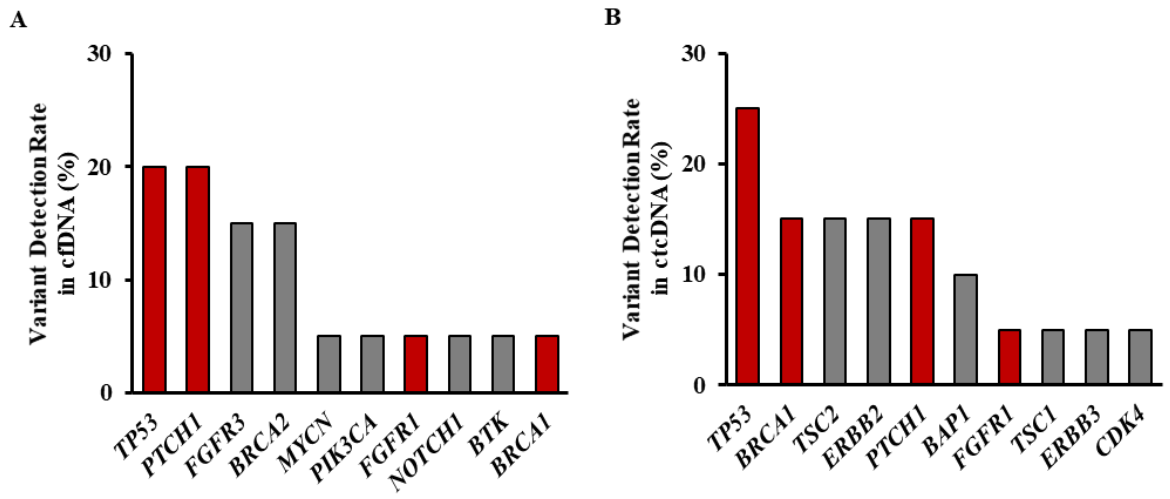


**Figure 2.** Distribution of the genetic alterations in the entire population. **Notes:** The frequency of alteration in (A) 20 cfDNA samples and (B) 20 ctcDNA samples. Multiple variants within the same gene in each patient were counted as one. Red boxes indicate that the genes in which mutations were found in common with cfDNA and ctcDNA.

## Complementary genomic profiling of cfDNA and CTCs

Although profiling of cfDNA and CTCs liquid biopsies offer convenient analysis of genetic mutations, low levels of cfDNA and CTCs in blood limit thresholds of detection.[32] Our data found mutations in 65% and 70% of patients by analyzing cfDNA and ctcDNA, respectively. However, simultaneous analysis of both cfDNA and ctcDNA raises this number to 85% (Figure 3A).

Some genes in the panel were specific to either cfDNA (*BRCA2*, *MYCN*, *PIK3CA*, *FGFR3*, *NOTCH1*, and *BTK*) or ctcDNA (*ERBB2*, *ERBB3*, *BAP1*, *TSC1*, *TSC2,* and *CDK4* (Figure 3B). Others were mutated in both (*TP53*, *PTCH1*, *BRCA1,* and *FGFR1*). *TP53* gene variants were the most

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frequently found in both (45% of patients, nine out of 20 patients).

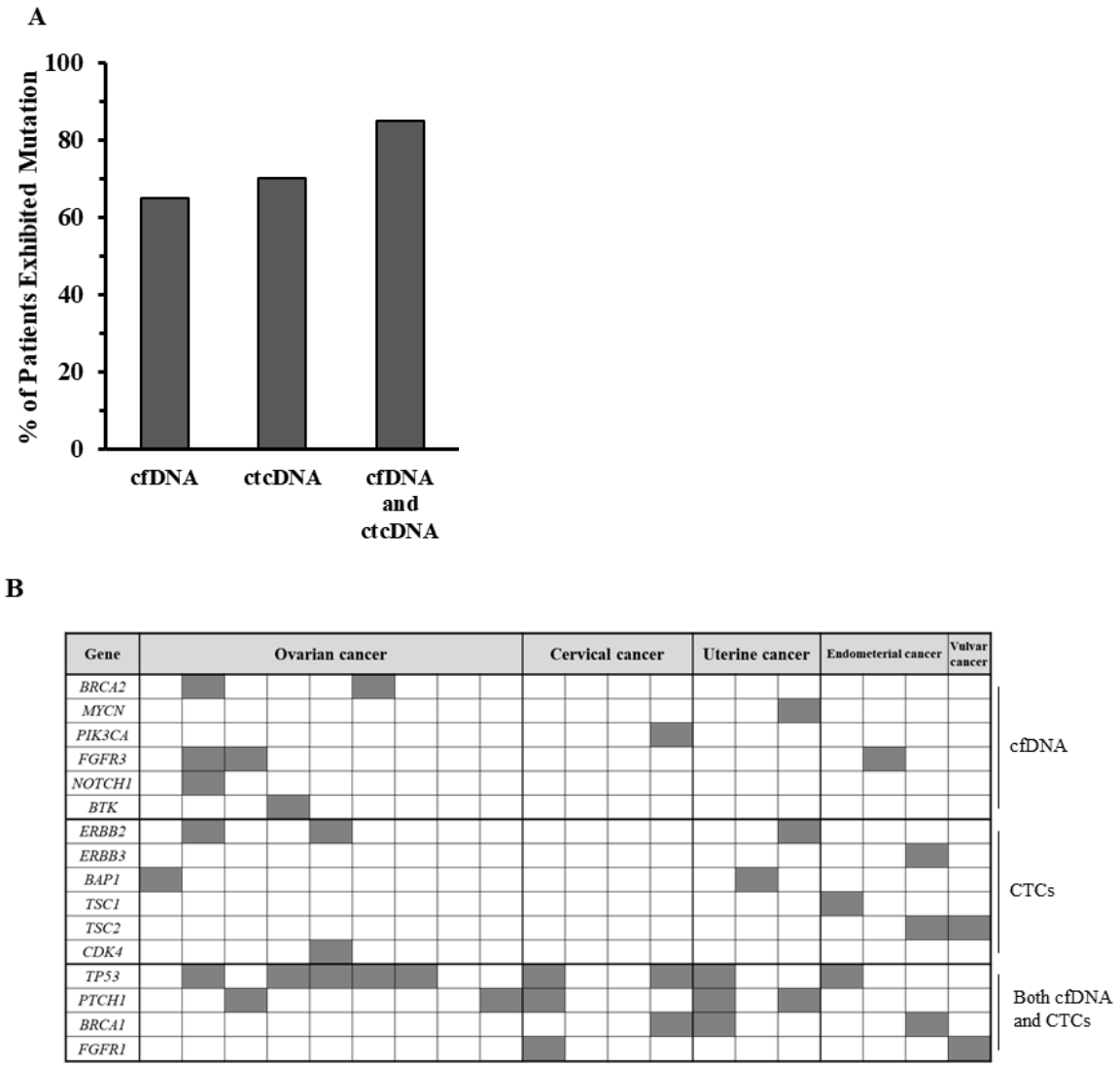


**Figure 3** The complementary genomic profile of cfDNA and ctcDNA. **Notes:** (A) The percentage of patients exhibiting mutations using cfDNA and ctcDNA respectively, or by analysis of cfDNA and ctcDNA together. (B) Heat map depicting somatic mutations detected by targeted sequencing of cancers. Detected mutations were classified by cancer type.

## Precision medicine based on genetic mutations

Genetic variants may lead to significant changes in the appearance and behavior of cancers in different individuals owing to tumor heterogeneity. Therefore, genetic variant analysis can offer

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potentially useful data for treatment. We detected a number of variants in cfDNA and CTCs samples of gynecologic cancers by targeted sequencing and identification of actionable variants related to drug responses using OncoKB.

For example, the germline *BRCA1* p.S573fs* mutation, which produces a truncated protein leading to a loss-of-function of the *BRCA1* gene, was detected in the cfDNA of ovarian cancer patient 1. Patient 5, also with ovarian cancer, has a somatic *BRCA2* mutation, the detected nonsense mutation (p.Q1683*) also of which leads to loss-of-function of the *BRCA2* gene. *BRCA1* or *BRCA2* deficient tumors are known to be more sensitive to cytotoxic agents such as platinum compounds and PARP inhibitor.[33] Therefore, rucaparib, niraparib, and olaparib as FDA-approved PARP inhibitors are recommended treatments these patients (Table 3).

**Table 3** Targeted therapies for deleterious mutations in actionable genes

| Patient ID | Gene | Mutation | Drugs | Classes |
|---|---|---|---|---|
| 1 | *BRCA1* | p.S573fs* (germline mutation) | Rucaparib Niraparib, Olaparib | PARP inhibitors |
| 5 | *BRCA2* | p.Q1683* (somatic mutation) | Rucaparib Niraparib, Olaparib | PARP inhibitors |

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# Discussion

As panels of biomarkers obtainable from liquid biopsies, cfDNA and CTCs offer a minimally invasive practical tool for monitoring the interplay between tumor heterogeneity and clinical relevance. Recent studies have reported that amounts of cfDNA in the blood increased in patients with cancer compared to healthy individuals and is related to tumor stage and burden in gynecologic cancers.[34,35] Moreover, cfDNA profiles accurately reflect genomic variants found in tissue biopsies.[36] The quantity and genomic variation characteristics of CTCs have also shown intra-tumor heterogeneity and can provide comprehensive diagnostic information of a number of cancers.[37,38] Although the advantages of cfDNA and CTCs make them promising tools, more sensitive techniques must be developed to exploit their full promise.[39,40] In this study, we investigated the feasibility of using a targeted sequencing panel of 51 actionable genes in cancers to identify cfDNA and ctcDNA variants in patients with the five main types of gynecologic cancers. We first performed a cfDNA assay to detect somatic mutations and verify the sensitivity of the customized cancer panel for cfDNA. Our findings show that the limit of detection (LOD) was ~0.1% allelic frequencies (Multiplex 1 cfDNA Reference Standard set, data not shown). This is not sensitive enough for a reliable detection of early cancers and at least one order of magnitude gain is required in sensitivity and detection for wide-spread future use. However, for advanced, and perhaps certain types of cancers, 0.1% allelic detection can be useful enough. The most common variants found were specific to either the cfDNA or CTCs allelic pools and only two of out 33 variants were found in both. This means that either the sensitivity of current NGS-based method is too low or the two types of DNA samples, i.e., one from cfDNA and one from whole CTCs, have drastically different characteristics. It is likely that cfDNA are selected in the blood as a result of physiological conditions and various enzymes. Therefore, although it is more difficult to filter out many CTCs, CTCs may have more complete set of tumor variants for high quality NGS data analysis. Our results confirm that analyzing cfDNA and ctcDNA together provides a far richer set of data per patient, than does examining either biomarker in isolation. Finally, in applying these technologies in a clinical setting, using NGS analysis of cfDNA and ctcDNA offers far easier access to genomic DNA suitable for diagnostic and clinical implications than traditional solid tumor analysis. Furthermore, combined solid and liquid biopsies using NGS can provide doctors with powerful detection capabilities upon which to make precise and personalized drug choices.

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The commercialization of novel technologies for the enrichment of cfDNA and CTCs has been rapid. Unfortunately, most of these nascent technologies cannot distinguish somatic and germline origins. Therefore, to bioinformatically enrich the tumor specific variants we used the normal variants of cfDNA or CTCs from 30 healthy individuals and KoVariome data set which is a standard reference of Korean population variome. The simultaneous analysis of somatic and germline mutations is able to discover potential pathogenic risk including hereditary cancers.[41] Consequently, we were able to identify somatic mutations in actionable genes in 85% of the patients.

Our cancer panel consists of actionable genes representing critical tumor pathways. Therefore, mutations in those actionable genes when detected in cfDNA and ctcDNA can have high clinical significance. Especially, loss-of-function variants including missense, nonsense, and frameshift mutations can provide potential therapeutic targets because of their role in mRNA transcript and translation.

Missense mutations induce amino acid changes in proteins, rendering the resulting protein potentially nonfunctional. Nonsense mutations induce a premature stop codon resulting in the truncation of proteins. Frameshift insertions and deletions (INDEL) add or remove one or more nucleotides in a DNA sequence producing different protein sequences, including frameshifts and premature terminations or elongated proteins. Loss-of-function variants are difficult to discriminate as either pathogenic or tolerated putatively alterations.[42,43] In this study, we observed that somatic and germline mutations have six and two INDELs respectively, and two nonsense mutations were detected in *BRCA2* and *ERBB2* genes (Table 2). Based on three deleterious mutation detection resources such as PolyPhen-2 and SIFT programs, and OncoKDM database, c.1716_1717insA INDEL (p.S573fs) in *BRCA1* and c.5047C>T (p.Q1683*) nonsense mutations in *BRCA2* yielded truncated proteins, leading to loss-of-function of these genes. The amino acids 452-1079 in *BRCA1* are a known DNA binding domain and play an important role in DNA repair by inhibiting exonuclease activity of Mre11/Rad50/Nbs1 complex.[44] Similarly, the truncation mutation within the BRC repeat in *BRCA2* has been reported to lead to loss-of-function of *BRCA2* such as DNA repair.[45] Thus, the p.S573fs and p.Q1683* mutations in *BRCA1* and *BRCA2* probably have clinical significance, however, this hypothesis remains to be confirmed. The loss-of-function of *BRCA1/2* by stop-gain variants induces malfunctions by dysregulating diverse cellular processes such as DNA repair, thus mutations in *BRCA1* and *BRCA2*

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increase the risk of breast, ovarian and prostate cancer.[46] The hereditary breast and ovarian cancer (HBOC) syndrome, known to be one of the most common hereditary cancers, has a heterozygous mutation in *BRCA1/2* that makes tumor cells susceptible to cytotoxic agents such as PARP inhibitors and platinum compounds.[33] The PARP inhibitors, such as rucaparib, niraparib, and olaparib, target tumors that have deficits in *BRCA1/2* and other DNA repair genes. Consequently, they inhibit PARP enzyme and promote PARP-DNA complex resulting in DNA damage, apoptosis, and cell death.[47] These drugs have been approved by the FDA for treatment of ovarian cancer or breast cancer with *BRCA1/2* mutations.[48,49] Rucaparib and olaparib were approved specifically to treat patients with BRCA mutation-positive ovarian cancer by the FDA-approved NGS-based companion diagnostic test. Our data showed that patient 1 and 5 with ovarian cancers have *BRCA1* or *BRCA2* mutations, respectively, in NGS data. Although the *BRCA1* somatic mutation of patient 1 was not detected, the *BRCA1* germline mutation was considered as a high risk for developing malignancy. Thus, drugs such as rucaparib, niraparib, and olaparib could be used for these patients.

# Conclusion

This study demonstrated that genetic profiling of cfDNA and CTCs together using our bespoke cancer panel covering 51 actionable genes provides enriched genomic profiling of gynecologic cancers. In addition, candidate drugs associated with pathogenic alterations were identified using *in silico* methods. Our study suggests that genetic variant profiling analysis of cfDNA and CTCs combined offers an enriched data set for guiding preclinical and clinical strategies and targeted therapies.

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# Disclosure

The authors declare no potential conflicts of interest in this work.

# Supplementary data

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