ORIGINAL RESEARCH

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Comprehensive mutation profiling of both cfDNA and CTCs in gynecological cancer patients

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Abstract:

Cell-free circulating DNA (cfDNA) and circulating tumor cells (CTCs) are emerging minimally invasive biomarkers, which help in 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. Here we designed and implemented targeted sequencing of a customized cancer panel including 51 actionable genes across cfDNA and CTCs of 20 gynecologic cancer patients. As a result, we confirmed that common variants overlapped only 15.8% between cfDNA and CTCs. Most variants were found only in cfDNA or CTCs. The dual analysis of cfDNA and CTCs enabled additional mutations to be detected thus improving the accuracy of the tumor characteristics analyses. Moreover, we predicted the impact of these genetic variants on protein function using in silico methods. Overall, NGS analysis of combined cfDNA and CTCs data promise more effective methods than cfDNA or CTC alone in liquid biopsy and precision medicine for cancer therapy.

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; VAF, variant allele frequency; SNV, single nucleotide variant; INDEL, insertion and deletion.

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 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.

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.

Sample Preparation

Plasma was isolated from 5 ml of whole blood using density gradient centrifugation in Ficoll-Paque^{TM} 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.

2 ml of whole blood was processed to enrich CTCs using the CD-PRIME^{TM} system (Clinomics Inc.). Whole blood was centrifugated using an equal volume of Ficoll-Paque^{TM} 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^{TM} 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 reagent (Beckman Coulter). 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).

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 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 possible impact of variants 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.

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%), five had Stage III (25%) and three had Stage IV (15%). The median age of the patients was 62 years (range 42-91) (Table 1, Supplementary table S1).

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%)

Mutation profiling of cfDNA and ctcDNA using cancer 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 23 out of 51 actionable genes using the cancer panel. Table 2 shows the total variants detected from cfDNA and ctcDNA of patients. A total of 63 variants were found in 23 actionable genes. We performed an *in silico* analysis to predict impact of genetic variant on protein function (see Methods) revealing that that 44% of total variants (28 out of 63) were potentially pathogenic mutations as expected.

Table 2. List of variants identified by cancer panel

Genes	Coding	Amino Acid Change	Predictive algorithms		
			[a] SIFT	[b] PolyPhen-2	[c] OncoKDM
ALK	c.2158C>G	p.L720V		B	
AR	c.173A>T	p.Q58L			
BAP1	c.548T>C	p.L183P	T	PD	
	c.1168C>A	p.P390T	D	B	
BRCA1	c.3548A>G	p.K1183R		B	
	c.3113A>G	p.E1038G		PS	
	c.329A>G	p.K110R		PS	
	c.444G>T	p.Q148H		PD	
	c.5207G>T	p.S1736I		PD	
	c.2566T>C	p.Y856H		PS	
	c.3114delA	p.A1039fs			
	c.5080_5082delCAC	p.H1694del			
	c.1716_1717insA	p.S573fs*			D
BRCA2	c.865A>C	p.N289H		B	
	c.5047C>T	p.Q1683*	D		D
	c.3299A>C	p.N1100T	T	PS	
	c.9364G>A	p.A3122T	D	PD	
	c.3646T>G	p.F1216V		PD	
	c.4334A>C	p.K1445T	D	PD	
	c.7088A>G	p.Y2363C	T	B	
	c.7469T>C	p.I2490T	T	B	
BTK	c.1475G>A	p.R492H	T	B	
CDK4	c.134G>C	p.G45A		B	
	c.753_754insC	p.R252fs			
ERBB2	c.1966T>C	p.S656P		B	
	c.1147G>A	p.G383R	D	PD	
	c.1642C>T	p.Q548*	D		D
	c.3508C>G	p.P1170A		PD	
	c.3149C>T	p.S1050L		B	
ERBB3	c.1959_1960insT	p.V654fs			
ESR1	c.1451A>G	p.D484G		PD	
	c.437C>A	p.P146Q		PD	
FGFR1	c.1741delG	p.A581fs			
	c.947C>T	p.P316L		PD	
FGFR2	c.238G>A	p.G80R	T	B	
FGFR3	c.1900G>A	p.A634T		PD	
	c.1672G>A	p.A558T		PD	
	c.1295G>A	p.S432N		B	
FGFR4	c.1162G>A	p.G388R		PD	
MYCN	c.134_135delCG	p.P45fs			
NOTCH1	c.6551A>T	p.Q2184L		B	
	c.2982C>G	p.N994K	D	PD	
	c.6788G>A	p.R2263Q	T	B	
PIK3CA	c.3136G>A	p.A1046T	D	PD	
PTCH1	c.3785C>T	p.P1262L		PD	
	c.3943C>T	p.P1315S		B	
	c.3232C>T	p.L1078F		PD	
RET	c.2308C>T	p.R770*			
STK11	c.1062C>G	p.F354L		B	PD
TP53	c.31G>C	p.E11Q	T	PD	
	c.743G>A	p.R248Q		PD	PD
	c.785G>T	p.G262V	D	PD	
	c.214C>G	p.P72A	T	B	
	c.818G>C	p.R273P		PD	D
	c.602delT	p.L201fs			
	c.827C>A	p.A276D	D	PD	
	c.722C>A	p.S241Y		PD	D
TSC1	c.1585G>A	p.A529T		B	
TSC2	c.3524T>C	p.V1175A		B	
	c.2509G>A	p.A837T		PD	
	c.3178T>C	p.W1060R		PD	
	c.3299T>C	p.V1100A	T	B	
	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 six missense, two frameshift and one deletion mutations were observed. Six of these variants (K110R, Q148H, S573fs*, Y856H, E1038G, S1736I,) were determined by in silico analyses as potential pathogenic mutations (Figure 1A). The most frequently mutated genes were *BRCA1*, *BRCA2*, and *TP53*. In *BRCA1*, where six missense, two frameshift and one deletion mutations were observed. Six of these variants (K110R, Q148H, S573fs*, Y856H, E1038G, S1736I,) 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 A3122T, F1216V and K1445T were also determined to be potential pathogenic mutations (Figure 1B). We also observed the *TP53* mutation in nine patients. The eight *TP53* mutations consisted of seven missense mutations and one frameshift. Moreover, alterations, such as L201fs, S241Y, R248Q, G262V, R273P and 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).

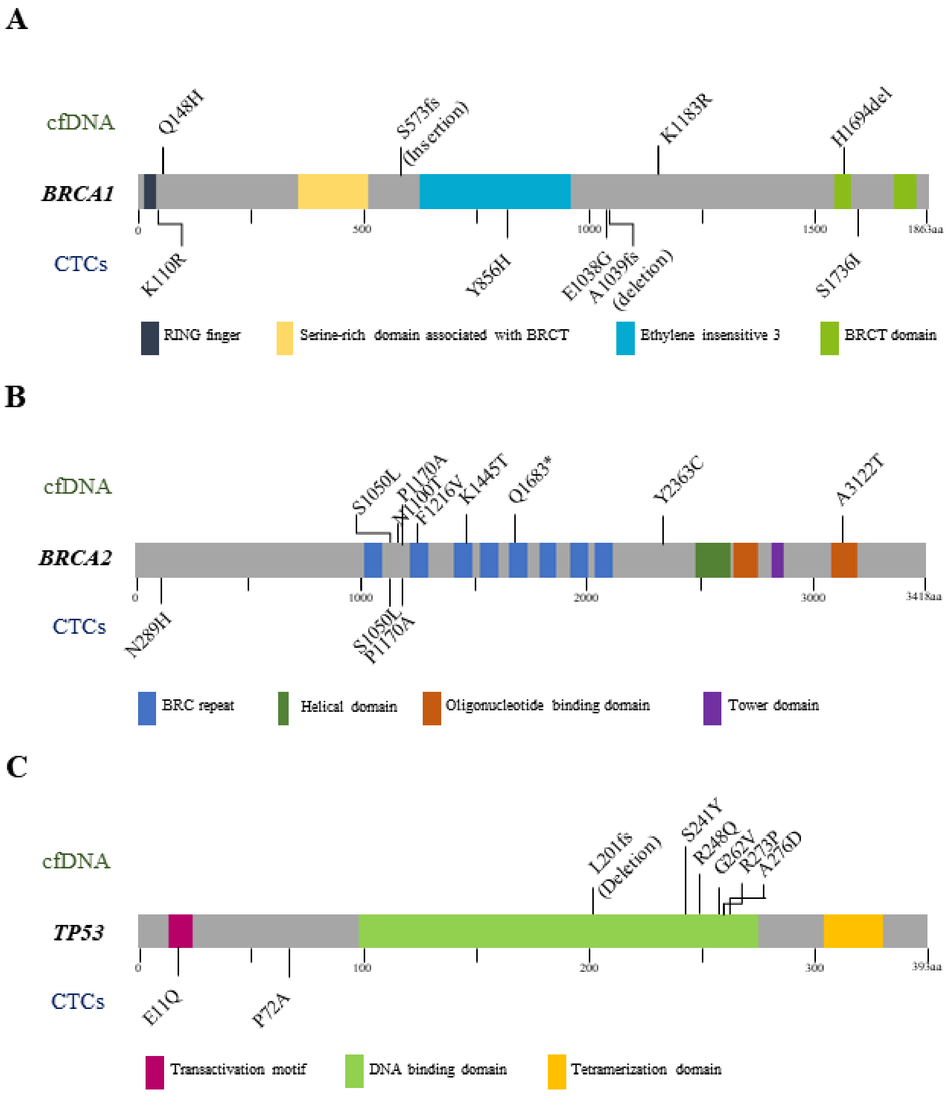
A
cfDNA
Q148H
S573fs (Insertion)
K1183R
H1694del
BRCA1
0
500
1000
1500
1863aa
CTCs
K110R
Y856H
E1038G
A1039fs (deletion)
S1736I
RING finger
Serine-rich domain associated with BRCT
Ethylene insensitive 3
BRCT domain
B
cfDNA
S1050L
P1170A
X1100T
F1216V
K1445T
Q1683*
Y2363C
A3122T
BRCA2
0
1000
2000
3000
3418aa
CTCs
N289H
S1050L
P1170A
BRC repeat
Helical domain
Oligonucleotide binding domain
Tower domain
C
cfDNA
L201fs (Deletion)
S241Y
R248Q
G262V
R273P
A276D
TP53
0
100
200
300
393aa
CTCs
E11Q
P72A
Transactivation motif
DNA binding domain
Tetramerization domain

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

We detected a total of 63 genetic variants in 23 genes and all patients have least one variant in either cfDNA or ctcDNA. The median number of mutated genes per patients was 4.8 (range of 1-11). The most frequently altered genes in cfDNA samples *ESR1* (50%, ten out of 20 cfDNA samples), *TSC2* (40%), *BRCA2* (25%), *TP53* (30%), *BRCA1* (25%), *FGFR3* (25%) and *PTCH1* (25%). In contrast, the most frequently altered genes in ctcDNA samples were *TSC2* (30%, six out of 20 ctcDNA samples), *BRCA1* (25%), *ERBB2* (25%) and *TP53* (25%). The distribution 1 genetic mutations in the whole population is shown in Figure 2A and 2B.

Our study also comprehensively analyzed the mutation spectrum in gynecological cancer patients. It was observed that *ESR1* mutations are detected in 55% of patients, followed by *TSC2* (50%), *TP53* (50%), *BRCA1* (45%) and *BRCA2* (30%). Among these genes, we found nonsense or frameshift mutations which possibly impact to an amino acid substitution on the function of *BRCA1* and *BRCA2* using predictive algorithms (Figure 2C).

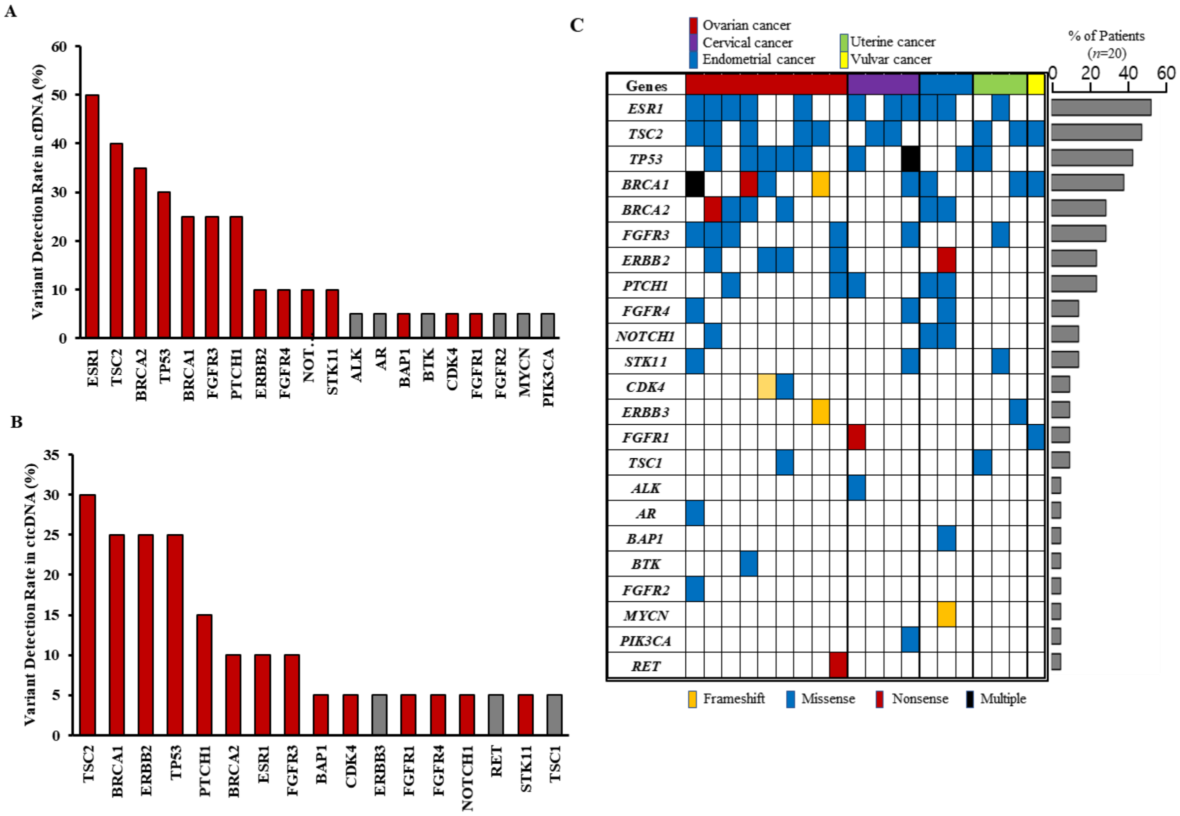

Figure 2. Distribution of the genetic alterations in the entire population. **Notes:** The frequency of alteration in (A) 20 cfDNA samples and (B) 20 ctcDNA samples. Multiple variants within the same gene in each patient were counted as one. Red boxes indicate that the genes in which mutations were found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

Complementary genomic profiling of cfDNA and CTCs

To explore the clonal heterogeneity of cfDNA and ctcDNA, sequence data from cfDNA and ctcDNA were compared. Figure 3A shows that variants out of a total of 63 variants detected, only 10 were shared by cfDNA and ctcDNA. Most variants were found only in cfDNA or ctcDNA. this result suggests that the combined approach of using both methods improves accuracy of diagnoses and monitoring of tumor progression.

An independent mutation analysis revealed 31 variants in the cfDNA sample (Supplementary Figure S1A) and 22 variants in ctcDNA sample (Supplementary Figure S1B). This analysis confirmed that variants detected in cfDNA or ctcDNA, have only a 15.8% overlap (10 out of 63 variants; Supplementary Figure S1C).

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 95% (19 out of 20 patients) and 75% (15 out of 20 patients) of patients by analyzing cfDNA and ctcDNA, respectively. However, simultaneous analysis of both cfDNA and ctcDNA raises this number to 100% (Figure 3B). Although genetic variant can be found in 95% of patients using cfDNA, simultaneous analysis of cfDNA and ctcDNA gives more variety of genomic information to treat cancer.

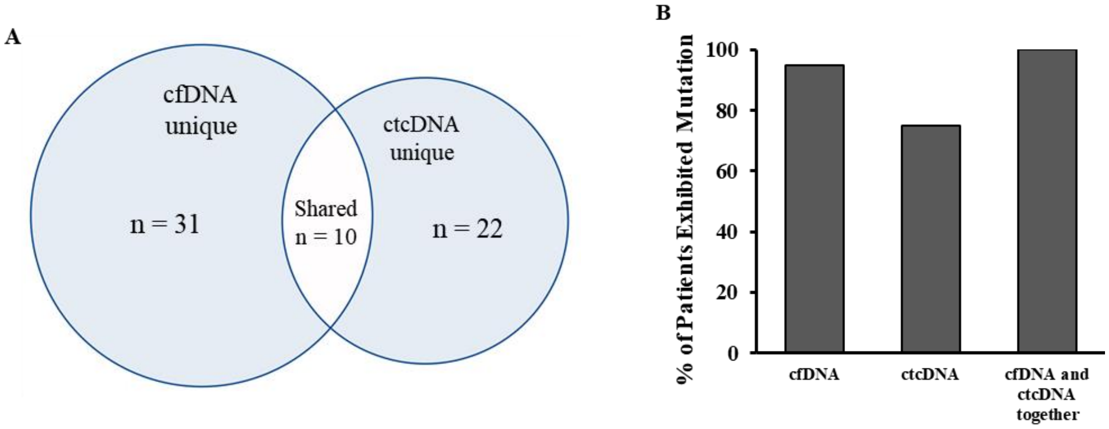

Figure 3 The complementary genomic profile of cfDNA and ctcDNA. **Notes:** (A) shared and unique genetic variants identified in cfDNA and ctcDNA samples. (B) The percentage of patients exhibiting mutations using cfDNA and ctcDNA respectively, or by analysis of cfDNA and ctcDNA together.

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 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 *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*	Rucaparib Niraparib, Olaparib	PARP inhibitors
5	*BRCA2*	p.Q1683*	Rucaparib Niraparib, Olaparib	PARP inhibitors

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 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 10 of out 63 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.

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.[41,42] In this study, we observed that mutations have seven INDELs and four nonsense mutations were detected in *BRCA1*,*BRCA2, ERBB2* and *RET* 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*, c.5047C>T (p.Q1683*) nonsense mutations in *BRCA2* and c.1642C>T (p.Q548*) nonsense mutations in *ERBB2* 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.[43] 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.[44] The alteration of *ERBB2*, a receptor tyrosine kinase, has been reported for amplification or overexpression in various cancer, but truncation of *ERBB2* might be associated with a lack of clinical benefit. 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* increase the risk of breast, ovarian and prostate cancer.[45] 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.[46] These drugs have been approved by the FDA for treatment of ovarian cancer or breast cancer with *BRCA1/2* mutations.[47,48] 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. These mutations were 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.

ACKNOWLEDGEMENTS

This study was partly supported by grant of the Basic Research Program (2017R1A2B4012353) and the Bio & Medical Technology Development Program (2017M3A9F7074175) of the Nation Research Foundation (NFR) funded by the Ministry of Science & ICT, Republic of Korea. JB was supported by the Genome Korea Project in Ul-san Research Fund (1.180024.01 and 1.180017.01) of UNIST and the Genome Korea Pro-ject in Ulsan. We thank Dr. Dawn Field for editing.

Disclosure

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

References

1. Dancey JE, Bedard PL, Onetto N, Hudson TJ. The genetic basis for cancer treatment decisions. *Cell*. 2012;148(3):409-420.
2. Rehm HL, Bale SJ, Bayrak-Toydemir P, et al. ACMG clinical laboratory standards for next-generation sequencing. *Genet Med*. 2013;15(9):733-747.
3. Pritchard CC, Salipante SJ, Koehler K, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. *J Mol Diagn*. 2014;16(1):56-67.
4. Uzilov AV, Ding W, Fink MY, et al. Development and clinical application of an integrative genomic approach to personalized cancer therapy. *Genome med*. 2016;8(1):62.
5. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin chim Acta*. 2001;313(1-2):139-142.
6. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016;164(1-2):57-68.7.
7. Rothe F, Laes JF, Lambrechts D, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. *Ann Oncol*. 2014;25(10):1959-1965.
8. Mattos-Arruda L, Weigelt B, Cortes J, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. *Ann Oncol*. 2018;29(11):2268.
9. Salvi S, Gurioli G, De Giorgi U, et al. Cell-free DNA as a diagnostic marker for cancer: current insights. *OncoTargets ther*. 2016;9:6549-6559.
10. Tseng CP, Leong KK, Liou MJ, et al. Circulating epithelial cell counts for monitoring the therapeutic outcome of patients with papillary thyroid carcinoma. *Oncotarget*. 2017;8(44):77453-77464.
11. Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med*. 1996;2(9):1033-1035.
12. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet*. 1999;64(1):218-224.

13. Buim ME, Fanelli MF, Souza VS, et al. Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer. *Cancer Biol Ther*. 2015;16(9):1289-1295.

14. Mohamadi RM, Ivanov I, Stojcic J, Nam RK, Sargent EH, Kelley SO. Sample-to-Answer Isolation and mRNA Profiling of Circulating Tumor Cells. *Anal Chem*. 2015;87(12):6258-6264.

15. Scher HI, Lu D, Schreiber NA, et al. Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer. *JAMA oncol*. 2016;2(11):1441-1449.

16. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol*. 2011;192(3):373-382.

17. Alix-Panabieres C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem*. 2013;59(1):110-118.

18. Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. *Cancer Discov*. 2016;6(5):479-491.

19. Tellez-Gabriel M, Ory B, Lamoureux F, Heymann MF, Heymann D. Tumour Heterogeneity: The Key Advantages of Single-Cell Analysis. *Int J Mol Sci*. 2016;17(12).

20. Lee A, Park J, Lim M, et al. All-in-one centrifugal microfluidic device for size-selective circulating tumor cell isolation with high purity. *Anal Chem*. 2014;86(22):11349-11356.

21. Kim TH, Lim M, Park J, et al. FAST: Size-Selective, Clog-Free Isolation of Rare Cancer Cells from Whole Blood at a Liquid-Liquid Interface. *Anal Chem*. 2017;89(2):1155-1162.

22. Kim J, Weber JA, Jho S, et al. KoVariome: Korean National Standard Reference Variome database of whole genomes with comprehensive SNV, indel, CNV, and SV analyses. *Sci Rep*. 2018;8(1):5677.

23. Cho YS, Kim H, Kim HM, et al. An ethnically relevant consensus Korean reference genome is a step towards personal reference genomes. *Nat Commun*. 2016;7:13637.

24. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7(4):248-249.

25. Boeva V, Popova T, Lienard M, et al. Multi-factor data normalization enables the detection of copy number aberrations in amplicon sequencing data. *Bioinformatics*. 2014;30(24):3443-3450.

26. Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc*. 2016;11(1):1-9.

27. Laes JF, Aftimos P, Barthelemy P, et al. The clinical impact of using complex molecular profiling strategies in routine oncology practice. *Oncotarget*. 2018;9(29):20282-20293.

28. Chakravarty D, Gao J, Phillips SM, et al. OncoKB: A Precision Oncology Knowledge Base. *JCO Precis Oncol*. 2017;2017.

29. Martinez JS, von Nicolai C, Kim T, et al. BRCA2 regulates DMC1-mediated recombination through the BRC repeats. *Proc Natl Acad Sci U S A*. 2016;113(13):3515-3520.

30. Follis AV, Llambi F, Ou L, Baran K, Green DR, Kriwacki RW. The DNA-binding domain mediates both nuclear and cytosolic functions of p53. *Nat Struct Mol Biol.* 2014;21(6):535-543.

31. Shirole NH, Pal D, Kastenhuber ER, et al. TP53 exon-6 truncating mutations produce separation of function isoforms with pro-tumorigenic functions. *ELife*. 2016;5.

32. Calabuig-Farinas S, Jantus-Lewintre E, Herreros-Pomares A, Camps C. Circulating tumor cells versus circulating tumor DNA in lung cancer-which one will win? *Transl Lung Cancer Res*. 2016;5(5):466-482.

33. Iyevleva AG, Imyanitov EN. Cytotoxic and targeted therapy for hereditary cancers. *Hered Cancer Clin Pract*. 2016;14(1):17.

34. Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol*. 2003;21(21):3902-3908.

35. Traver S, Assou S, Scalici E, et al. Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy. *Hum Reprod Update* 2014;20(6):905-23.

36. Shu Y, Wu X, Tong X, et al. Circulating Tumor DNA Mutation Profiling by Targeted Next Generation Sequencing Provides Guidance for Personalized Treatments in Multiple Cancer Types. *Sci Rep*. 2017;7(1):583.

37. Chebouti I, Kasimir-Bauer S, Buderath P, et al. EMT-like circulating tumor cells in ovarian cancer patients are enriched by platinum-based chemotherapy. *Oncotarget*. 2017;8(30):48820-48831.

38. Muinelo-Romay L, Casas-Arozamena C, Abal M. Liquid Biopsy in Endometrial Cancer: New Opportunities for Personalized Oncology. *Int J Mol Sci*. 2018;19(8).

39. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426-437.

40. Lowes LE, Lock M, Rodrigues G, et al. The significance of circulating tumor cells in prostate

cancer patients undergoing adjuvant or salvage radiation therapy. *Prostate Cancer Prostatic Dis*. 2015;18(4):358-364.

41. MacArthur DG, Balasubramanian S, Frankish A, et al. A systematic survey of loss-of-function variants in human protein-coding genes. *Science*. 2012;335(6070):823-828.

42. Pagel KA, Pejaver V, Lin GN, et al. When loss-of-function is loss of function: assessing mutational signatures and impact of loss-of-function genetic variants. *Bioinformatics*. 2017;33(14):i389-i398.

43. Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. *Proc Natl Acad Sci U S A*. 2001;98(11):6086-6091.

44. Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol cell*. 2001;7(2):263-272.

45. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer*. 2011;12(1):68-78.

46. Murai J, Huang SY, Renaud A, et al. Stereospecific PARP trapping by BMN 673 and comparison with olaparib and rucaparib. *Mol Cancer Ther*. 2014;13(2):433-443.

47. Dal Molin GZ, Omatsu K, Sood AK, Coleman RL. Rucaparib in ovarian cancer: an update on safety, efficacy and place in therapy. *Ther Adv Med Oncol*. 2018;10:1758835918778483.

48. Turk AA, Wisinski KB. PARP inhibitors in breast cancer: Bringing synthetic lethality to the bedside. *Cancer*. 2018;124(12):2498-2506.

Supporting information