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Original Article

Mutations in circulating tumor DNA detected in the postoperative period predict poor survival in patients with ovarian cancer

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ARTICLE INFO

Article history:
Received 23 April 2021
Accepted 30 September 2022
Available online 5 October 2022

Key words:
Circulating tumor DNA
Ovarian cancer
High-grade serous carcinoma
Clear cell carcinoma

ABSTRACT

Background: We investigated whether mutations in plasma circulating tumor DNA (ctDNA) can provide prognostic insight in patients with different histological types of ovarian carcinoma. We also examined the concordance of mutations detected in ctDNA samples with those identified in the corresponding formalin-fixed paraffin-embedded (FFPE) tumor specimens.

Methods: Between July 2016 and December 2017, 29 patients with ovarian carcinoma were prospectively enrolled. FFPE tumor specimens were obtained from all participants. A total of 187 blood samples for ctDNA analysis were collected before surgery (C0), immediate after surgery before adjuvant chemotherapy (C1), and at six-month intervals. Progression-free survival (PFS) and overall survival (OS) served as the main outcome measures.

Results: The study cohort consisted of 13 (44.8%) patients with high-grade serous carcinomas (HGSC), 9 (31.0%) with clear cell carcinoma, 2 (6.9%) with mucinous carcinomas, 4 (13.8%) with low-grade serous carcinomas, and 1 (3.4%) with endometrioid carcinoma.

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Peer review under responsibility of Chang Gung University.
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https://doi.org/10.1016/j.bj.2022.09.004

Twenty-four (82.8%) patients had at least one detectable ctDNA variant. The concordance rate between mutations identified in pretreatment ctDNA and corresponding FFPE tumor specimens was 92.3% for patients with HGSC and 58.6% for the entire cohort. The median follow-up time was 33.15 months (range: 0.79–46.13 months). Patients with an advanced stage disease more likely had detectable ctDNA mutations before surgery (C0) and after surgery at C1, while those with HGSC more likely had ctDNA mutations detected before surgery. The presence of ctDNA mutations at C1 was an independent predictor of worse OS with a hazard ratio of 6.56 (95% confidence interval, (1.07–40.17) for detectable *versus* undetectable C1 ctDNA variants, $p = 0.042$).

Conclusions: ctDNA mutations are common in patients with ovarian carcinoma. The presence of ctDNA mutations after surgery was an independent predictor of less favorable PFS and OS.

At a glance commentary

Scientific background on the subject

The presence and potential prognostic value of mutations identified by next-generation sequencing in patients with ovarian cancer were generally limited. The analysis of circulating tumor DNA (ctDNA) has recently become a field of intensive research aiming at identifying genetic biomarkers relevant for improving prognostication.

What this study adds to the field

The present study – conducted in different histological types of ovarian carcinoma – demonstrate that the concordance rate between mutations identified in pretreatment ctDNA samples and formalin-fixed paraffin-embedded tumor specimens was 58.6% for the entire cohort and 92.3% in patients with high-grade serous carcinoma (HGSC), suggesting that the concordance in non-HGSC was low (31.3%). Furthermore, the presence of ctDNA mutations in the first post-operative sample was identified as an independent predictor of less favorable outcomes.

Ovarian carcinoma is a heterogeneous malignancy comprising multiple histological types [1,2]. Albeit rare in western countries, ovarian clear cell carcinoma (CCC) is the second most common form of ovarian malignancy in Asia after high-grade serous adenocarcinoma (HGSC) [3–6]. Serum levels of cancer antigen-125 (CA125) are commonly increased in patients with ovarian carcinoma [7,8] and their measurements are widely used for surveillance in the postoperative period [1,9,10]. However, CA125 has a low specificity as a diagnostic test [10]. Thus, there is an urgent need to identify novel biochemical markers of ovarian carcinoma with sufficient sensitivity and specificity for prognostic applications.

The analysis of circulating tumor DNA (ctDNA) has recently become a field of intensive research aiming at identifying genetic biomarkers relevant for improved prognostication [11,12]. Several factors may have an impact on ctDNA shedding rates – including tumor type, size and anatomic location of the lesion, mitotic rate, and vascularization extent [13,14]. Compared with archival formalin-fixed paraffin-embedded (FFPE) tumor specimens [15], ctDNA testing has the ability to capture the cancer mutational landscape in a real-time fashion.

Published reports on ctDNA in patients with ovarian carcinoma were aimed at investigating the presence and potential prognostic value of mutations identified by next-generation sequencing (NGS) in patients with HGSC [16–22] and were generally limited – with a few exceptions [12,17,18,20,21] – by small sample sizes. Patients with less common subtypes of ovarian carcinoma such as CCC were rarely enrolled in previous ctDNA investigations [23,24]. We therefore designed the current study to investigate whether the mutational landscape of plasma ctDNA can have prognostic value in patients with different histological types of ovarian carcinoma. We also examined the concordance of mutations detected in ctDNA samples with those identified in the corresponding FFPE tumor specimens.

Materials and methods

Patients

The study followed the tenets of the Helsinki Declaration and was approved by the Institutional Review Board of the Chang Gung Memorial Hospital. The inclusion criteria were: (1) patients with epithelial ovarian cancer, tubal or peritoneal primary cancer; (2) age 18–85 years old; (3) patients underwent primary or interval debulking surgery after neoadjuvant therapy; (4) International Federation of Gynecology and Obstetrics (FIGO) stage I-IV, and (5) signed informed consent. For advanced ovarian cancer (stage III or IV), treatment option was based on physician's discretion to remove all macroscopic disease at primary or interval surgery. Neoadjuvant chemotherapy was indicated if there were metastases precluded optimal cytoreduction [25]. The exclusion criteria were (1) non-epithelial ovarian cancer (n = 4); (2) borderline tumors of ovary (n = 12); (3) tumor percentage <15% (n = 3); (4) plasma not available before treatment and after 7–10 days after surgery (n = 4), and (5) patients with pregnancy. Of the 52 patients, 29 patients were finally included. Venous blood samples for ctDNA extraction were collected into Cell-Free DNA BCT® tubes (Streck, La Vista, NE, USA) at the following time points: before surgery (C0), after surgery before adjuvant chemotherapy (C1, at 7–10 post-surgical days), as well as at 6 (C2), 12 (C3), and 18 (C4) months after surgery. The presence of residual disease after surgery was determined using the

cytoreduction (CC) score [26], as follows: CC-0, no macroscopically visible tumor; CC-1, largest residual tumor ≤2.5 mm; CC-2, largest residual tumor >2.5 mm and ≤2.5 cm; and CC-3, largest residual tumor >2.5 cm.

DNA extraction and next-generation sequencing

DNA from FFPE tumor specimens was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Circulating ctDNA was isolated from plasma (4–8 mL) using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's protocol. The amount of ctDNA used for NGS ranged from 5 to 20 ng. Ultra-deep NGS analysis was performed using the Ion PGM Sequencer with the Ion 318 Chip (Thermo Fisher Scientific, Waltham, MA, USA) [27] covering 50 hotspot regions of oncogenes and tumor suppressor genes (ACT Monitor®+, Supplementary file 1). Sequencing coverage for ctDNA and DNA from FFPE tumor specimens was set at 30,000 × and 6000 × , respectively (mean uniformity: 95% and 93%, respectively).

Mutation analysis

Screening was performed for single nucleotide variants and indels. The Torrent Suite Software (version 4.4) was used for base calling and alignment to the human genome reference hg19. Among tumor variants, single-nucleotide variants (SNV) with at least 20 reads and an allele frequency greater than 0.5% were considered as true variants. Indels present in at least 20 reads and an allele frequency greater than 0.1% were considered true variants. The following repositories were used for annotation: COSMIC (v74 release), dbSNP Build 138, and 1000 Genomes Project (phase 1 variant release).

Definitions

In patients with detectable mutations, the concordance rate was calculated by taking into account the detection of the same variant in both ctDNA and FFPE tumor specimens. The absence of mutations in both specimen types was also considered to calculate complete concordance. Progression-

Table 1 General characteristics of the study patients.

Number	Age, years	Histology	FIGO stage	Residual disease	Primary treatment	Status
1	41	HGSC	IIB	CC2	Debulking + TPx6	NED
3	62	HGSC	IIIC	CC3	Debulking + TPx6+Avastin	DOD
7	53	HGSC	IVB	CC1	Debulking + TPx6	AWD
10	67	HGSC	IC1	CC0	Debulking + TPx6	NED
12	48	HGSC	IIIC	CC3	Debulking + TPx6+Avastin	DOD
15	58	HGSC	IIIC	CC3	Debulking + dTPx1	DOD
16	64	HGSC	IIIC	CC1	Debulking + dTPx6+Avastin	NED
23	58	HGSC	IIIC	CC0	Debulking + TPx6	AWD
25	82	HGSC	IIIC	CC0	Debulking + TPx6	AWD
27	50	HGSC	IIIC	CC0	Debulking + HIPEC + dTPx6	AWD
28	59	HGSC	IA	CC0	Debulking + TPx6	NED
24	28	HGSC (with contralateral LGSC)	IVB	CC1	Debulking + TPx6	DOD
5	57	HGSC	IIIC	CC0	Debulking + TPx6	NED
30	55	CCC	IIIC	CC2	Debulking + TPx6	DOD
26	50	CCC	IC1	CC0	Debulking + TPx6	NED
17	60	CCC	IC2	CC0	Debulking + TPx6	NED
22	38	CCC	IIIC	CC2	Debulking + TPx2→Topotecan + carbo	AWD
2	38	CCC	IC1	CC0	Debulking + TPx6	NED
8	50	CCC	IIB	CC0	Debulking + TPx6	AWD
11	49	CCC	IC	CC0	Debulking + TPx6	NED
21	83	CCC	IC3	CC0	LAVH + BSO + dTPx2	DOD
29	49	CCC	IA	CC0	Debulking + TPx6	NED
13	21	mucinous carcinoma	X	CC3	Right ovarian tumor enucleation →debulking	DOD
14	62	mucinous carcinoma	IA	CC0	Debulking	NED
4	58	LGSC	IC1	CC0	Debulking + TPx6	NED
19	47	LGSC	IC1	CC0	Debulking + PCx2→letrozole	NED
20	34	LGSC	IIIC	CC2	Debulking + TPx6	AWD
9	59	LGSC	IIIC	CC2	Neoadjuvant CT→Debulking	DOD
18	35	endometrioid carcinoma	IC2	CC0	Debulking + TPx6	NED

Abbreviations: AWD: alive with disease, CCC: clear cell carcinoma, CC: completeness of cytoreduction, CT: chemotherapy, dTP: dose-dense platinum (cisplatin or carboplatin) plus paclitaxel, DOD: died of disease, FIGO: International Federation of Gynecology and Obstetrics, HGSC: high-grade serous carcinoma, LAVH + BSO: laparoscopic-assisted vaginal hysterectomy plus bilateral salpingo-oophorectomy, LGSC: low-grade serous carcinoma, NED: no evidence of disease, PC: cisplatin plus cyclophosphamide, TP: platinum (cisplatin or carboplatin) plus paclitaxel.
Note: Debulking: total abdominal hysterectomy + bilateral salpingo-oophorectomy + pelvic lymph node dissection.

free survival (PFS) was defined as the time from diagnosis to disease progression – which was established in accordance with the RECIST criteria based on imaging findings (computed tomography, magnetic resonance imaging, and positron emission tomography) or CA125 levels. Overall survival (OS) was calculated as the time from diagnosis to death from any cause.

Statistical analysis

Fisher's exact test was used for the comparison of patients with detected and undetected ctDNA variants before surgery and after surgery. Kaplan–Meier estimate curves for PFS and OS were generated and differences in survival endpoints were analyzed with the log-rank test. Multivariable Cox proportional hazards regression analyses with a forward stepwise selection procedure were applied to identify independent predictors of survival endpoints. Results are expressed as hazard ratios (HR) with their 95% confidence intervals (CIs). Statistical calculations were performed using SPSS version 20.0 (IBM, Armonk, NY, USA), with all tests two-sided at a 5% level of significance.

Results

Patient characteristics

The general characteristics of the 29 study patients are summarized in Table 1. Between July 2016 and December 2017, a total of 29 patients with ovarian carcinoma were prospectively enrolled and FFPE tumor specimens were obtained for all participants. The distribution of histological types was as follows: HGSC (n = 13; 44.8%), CCC (n = 9; 31.0%), mucinous carcinomas (n = 2; 6.9%), low-grade serous carcinomas (LGSC, n = 4; 13.8%), and endometrioid carcinoma (n = 1; 3.4%), Patient #24, who had HGSC, also harbored a LGSC in the contralateral ovary. Fourteen of the 29 patients were diagnosed at an FIGO advanced stage, among which 76.9% (10/13) were HGSC and 25.0% (4/16) were non-HGSC. Thirteen patients received primary cytoreductive surgeries and one patient (Patient #9) received neoadjuvant chemotherapy followed by interval debulking. Two patients with stage IV (Patient #7 had umbilical metastasis and #24 with lung metastasis) underwent primary cytoreductive surgery had residual disease of CC-1. Meanwhile, five of the 12 patients with stage III patients had residual disease of CC-1. Patient #13 underwent ovarian enucleation at a local hospital and was subsequently transferred to our facility for debulking surgery.

Variant detection

We sequenced 187 ctDNA samples extracted from plasma and 30 FFPE tumor specimens (one specimen for each of the 29 study participants, the only exception being patient #24 who had two ovarian carcinomas of distinct histological types). As for ctDNA, we collected a preoperative sample and at least one postoperative specimen for all participants. All variants detected in FFPE tumor specimens and ctDNA were depicted in Fig. 1. Of the 29 patients, 24 (82.8%) harbored at least one variant in ctDNA detectable during the follow-up period. Mutations in

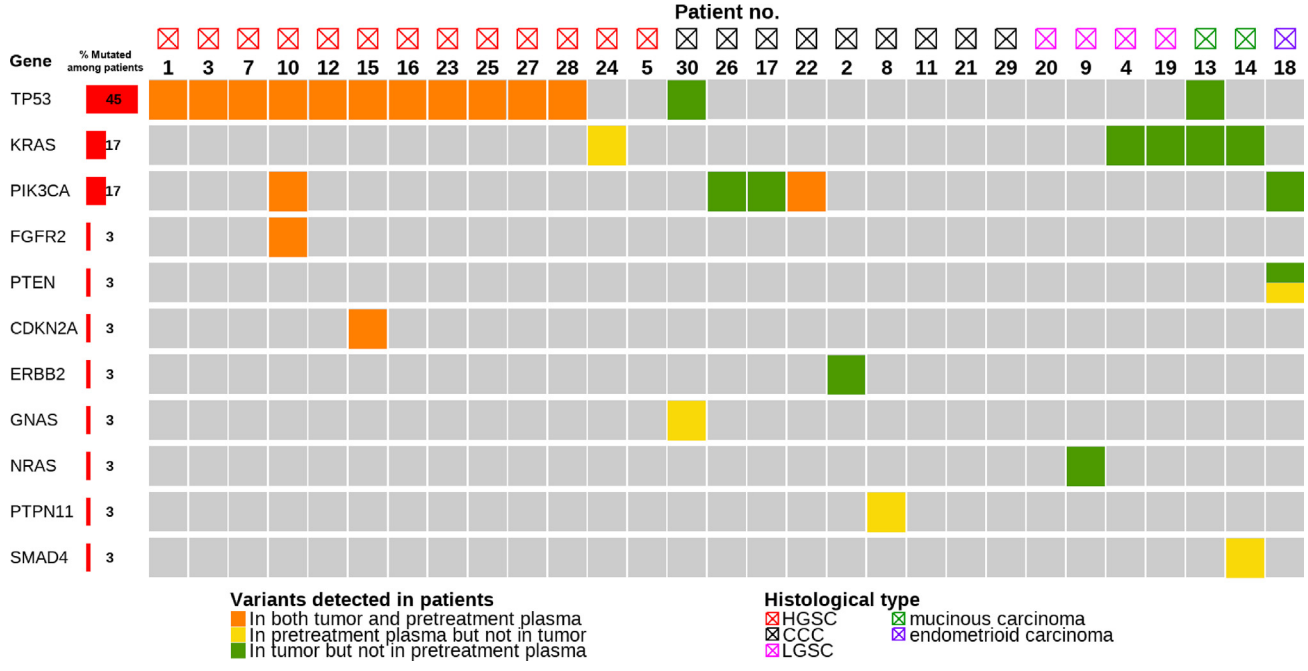

Fig. 1 Genes mutated in ctDNA samples and FFPE tumor specimens and their distribution in 29 patients with ovarian cancer. Abbreviations: HGSC: high-grade serous carcinoma; CCC: clear cell carcinoma; LGSC: low-grade serous carcinoma.

ctDNA were identifiable before surgery in 17 (58.6%) patients within the entire study cohort and in 12 (92.3%) with HGSC. We were unable to identify any variant in ctDNA or FFPE specimens in five patients (cases number #5, 11, 20, 21, and 29).

Comparison between variants in FFPE tumor specimens and ctDNA samples collected before surgery

We subsequently compared the variants identified in FFPE tumor specimens and ctDNA samples at C0 (before surgery). Twenty-two and 17 patients had detectable variants in FFPE tumor specimens and ctDNA samples, respectively. Four (case #10, #13, #15, and #18) and two patients (case #10 and #15) carried more than one variant in FFPE tumor specimens and ctDNA samples, respectively. The complete concordance rates for FFPE tumor specimens and ctDNA samples at C0 were 58.6% (17/29) in the entire study cohort and 92.3% (12/13) in patients with HGSC. After exclusion of patients without any detectable variant in both FFPE tumor specimens and ctDNA samples at C0, the concordance rates in the entire study cohort and patients with HGSC were 50.0% (12/24) and 91.7% (11/12), respectively.

Mutated genes

Eleven of the 13 patients (84.6%) with HGSC harbored *TP53* variants in both FFPE tumor specimens and ctDNA samples at C0 [Figs. 1 and 2] *PIK3CA* mutations were identified in three patients with CCC (two in FFPE tumor specimens only). *KRAS* variants were detectable in FFPE tumor specimens of LGSC and mucinous carcinoma. *PIK3CA* and *PTEN* variants were identified in FFPE specimens of endometrioid carcinoma. As for ctDNA, certain samples collected at follow-up were found to carry mutations not identifiable at C0 [Figs. 1 and 2] as follows: HGSC, patient #27 (*SMO* V392G) and patient #23 (new *TP53* splice-acceptor variant); LGSC, patient #20 (*FGFR2* N546S). In patients with CCC, case #26 had five new variants (*ABL1* E316G, *EGFR* Y869C, *IDH2* N136Y, *TP53* W91*, and *TP53* Y220C) detected during follow-up and case #17 showed a single new variant (*APC* D1512 fs).

ctDNA mutations and survival endpoints

The median follow-up time was 33.15 months (range: 0.79–46.13 months). Patients with an advanced stage disease

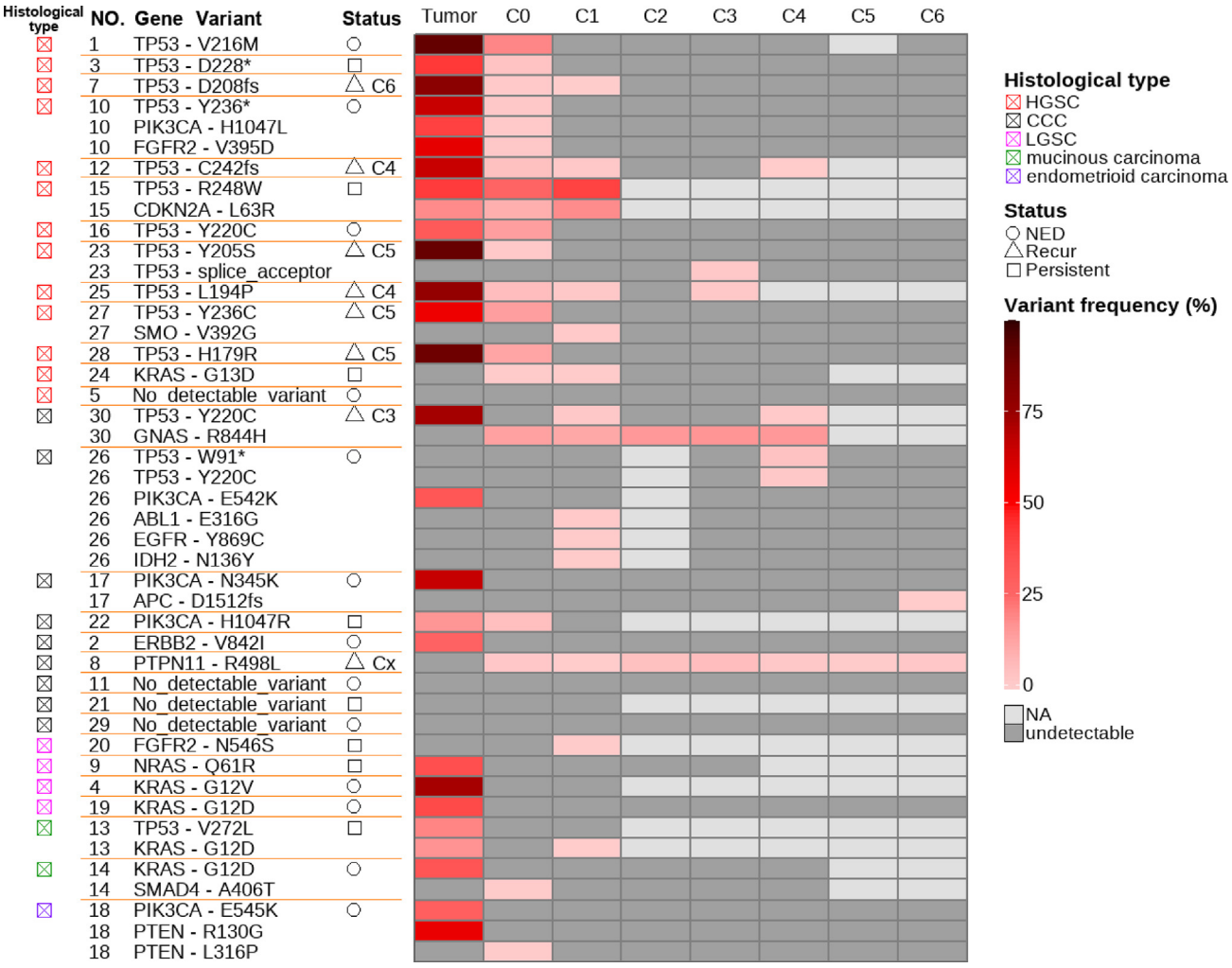

Fig. 2 Variants detected in FFPE tumor specimens and ctDNA samples obtained during the entire course of follow-up. Abbreviation: NED: no evidence of disease.

Table 2 Comparison of patients with detected and undetected ctDNA variants before surgery (C0) and after surgery (C1).

	ctDNA_C1 undetected (N = 18)		ctDNA_C1 detected (N = 11)			ctDNA_C0 undetected (N = 12)		ctDNA_C0 detected (N = 17)		
	N	(%)	N	(%)	*p*	N	(%)	N	(%)	*p*
FIGO stage					0.016					0.019
I	11	(91.7)	1	(8.3)		8	(66.7)	4	(33.3)	
II, III, IV	7	(43.8)	9	(56.2)		3	(18.8)	13	(81.2)	
Residual disease					0.237					0.694
CC-0/1	14	(70.0)	6	(30.0)		9	(45.0)	11	(55.0)	
CC-2/3	4	(44.4)	5	(55.6)		3	(33.3)	6	(66.7)	
Histology					0.466					0.001
Non-HGSC	11	(68.8)	5	(31.2)		11	(68.8)	5	(31.2)	
HGSC	7	(53.8)	6	(46.2)		1	(7.7)	12	(92.3)	

Abbreviations: CC: completeness of cytoreduction, FIGO: International Federation of Gynecology and Obstetrics, HGSC: high-grade serous carcinoma.

more likely had detectable ctDNA mutations before surgery (C0) and after surgery at C1 (Table 2), while those with HGSC more likely had ctDNA mutations detected before surgery. The detectable ctDNA in patients' blood at C0, C1 and C2 were 58.6%, 37.9% and 9.1%. Patients who did not carry any ctDNA mutation at C1 (after surgery before chemotherapy) showed significantly better 2-year PFS [77.8% *versus* 27.3%, respectively, $p = 0.001$, Fig. 3A and OS [94.4% *versus* 51.1%, respectively, $p = 0.039$; Fig. 3B compared with mutation carriers. The presence of ctDNA mutations at C1 was an independent predictor of worse OS with a hazard ratio of 4.18 (95% confidence interval, 0.97–18.06) for detectable *versus* undetectable C1 ctDNA variants. The results of univariable and multivariable analyses for the predictors of PFS and OS are shown in Tables 3 and 4, respectively. After adjustment for potential confounders in multivariable analysis, the presence of ctDNA mutations at C1 and residual disease were identified as independent predictors of less favorable OS [HR: 6.56 (95% CI: 1.07–40.17) for detectable *versus* undetectable C1 ctDNA mutations, $p = 0.042$; HR: 17.12, (95% CI: 2.71–108.08) for residual disease CC-2/3 *versus* CC-0/1, $p = 0.003$; Table 4]. For PFS and OS for ctDNA at C0 and C2 have been added as Fig. 3C and D and Fig. 3E and F, respectively. Nevertheless, detected ctDNA posed no significance at C0 and C2.

Discussion

The results of the present study conducted in a series of 29 patients with different histological types of ovarian carcinoma demonstrate that ctDNA mutations are frequently detectable. In addition, the concordance rate between ctDNA mutations identified in pretreatment plasma samples and FFPE tumor specimens was 58.6% – a figure chiefly driven by HGSC (92.3% concordance rate). Finally, the absence of ctDNA mutations in the first post-operative sample predicted more favorable PFS and OS figures.

Table 3 Univariable and multivariable analyses of progression-free survival.

		Univariable			Multivariable		
	N	HR	95% CI	*p*	HR	95% CI	*p*
ctDNA_C1							
undetected	18	Ref.			Ref.		
detected	11	5.34	1.87–15.27	0.002	8.41	2.49–28.39	0.001
CA125_C1							
<35	2	Ref.					
≥35	27	1.12	0.15–8.58	0.913			
Stage							
I	12	Ref.					
II, III, IV	16	6.89	1.55–30.7	0.011			
Residual disease							
CC-0/1	20	Ref.			Ref.		
CC-2/3	9	4.21	1.55–11.4	0.005	7.00	2.14–22.89	0.001
Histology							
Non-HGSC	16	Ref.					
HGSC	13	1.64	0.61–4.44	0.327			
Variant							
Non-*TP53*	15	Ref.					
TP53	14	2.39	0.86–6.64	0.094			

Abbreviations: CI: confidence interval, HGSC: high-grade serous carcinoma, HR: hazard ratio, Ref: reference.

Table 4 Univariable and multivariable analyses of overall survival.

		Univariable			Multivariable		
	N	HR	95% CI	*p*	HR	95% CI	*p*
ctDNA_C1							
undetected	18	Ref.			Ref.		
detected	11	4.18	0.97–18.06	0.055	6.56	1.07–40.17	0.042
CA125_C1							
<35	2	Ref.					
≥35	27	22.98	0–3190306.49	0.604			
Stage							
I	12	Ref.					
II, III, IV	16	5.49	0.66–45.73	0.115			
Residual disease							
CC-0/1	20	Ref.			Ref.		
CC-2/3	9	12.04	2.37–61.03	0.003	17.12	2.71–108.08	0.003
Histology							
Non-HGSC	16	Ref.					
HGSC	13	1.15	0.29–4.61	0.843			
Variant							
Non-*TP53*	15	Ref.					
TP53	14	1.93	0.46–8.09	0.369			

Abbreviations: CI: confidence interval, HGSC: high-grade serous carcinoma, HR: hazard ratio, Ref: reference.

The published prevalence of ctDNA mutations in ovarian cancer ranged between 70% and 100% [12,16,20,28]. The most commonly mutated gene in the current investigation was *TP53*, whose variants were detected in 84.6% of pretreatment ctDNA specimens in patients with HGSC. These results confirm previous observations that *TP53* ctDNA mutations are commonly detected in patients with HGSC (range: 66–85%) [12,16,28–30]. While the overall concordance rate between ctDNA and FFPE tumor specimens was moderate (58.6%), a high concordance (92.3%) was observed in patients with HGSC. Concordance rates (58–85%) for mutations in ctDNA and FFPE tumor specimens have been previously reported for HGSC [12,20,30,31]. In this malignancy, Forshew et al. [30] demonstrated a 85% concordance rate between tumor and ctDNA specimens. Additionally, the CancerSEEK study (number of patients with ovarian cancer: 54) reported a

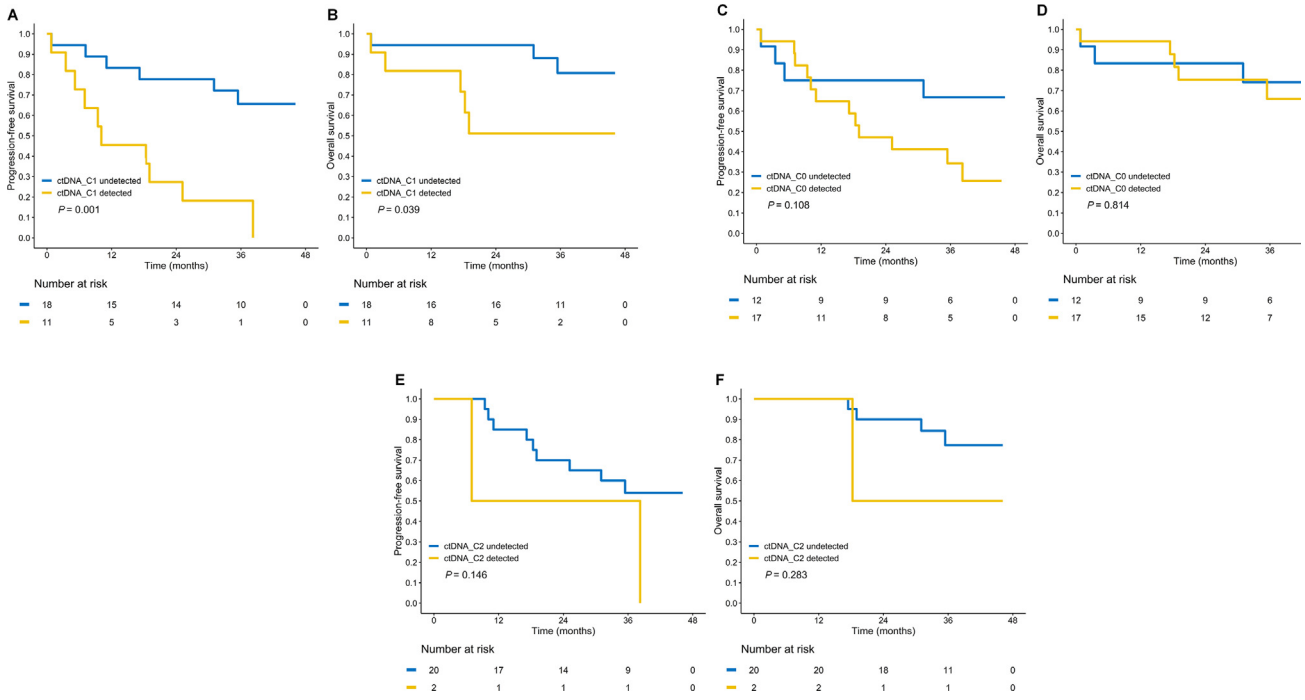

Fig. 3 Kaplan–Meier plots of progression-free survival (A) and overall survival (B) in patients stratified according to the presence or absence of ctDNA mutations detected at C1 (C) and (D) represents progression-free survival and overall survival at C0, respectively while (E) and (F) represents progression-free survival and overall survival at C2, respectively.

concordance rate of 85% for seven pairs [12]. Furthermore, Phallen et al. [20] have shown concordance rates of 77% and 68% for patients with advanced stage and stage I–II, respectively. The high detection and concordance rates in HGSC can be explained by the fact that most of these tumors harbor *TP53* mutations that are readily detectable; additionally, they tend to be diagnosed at an advanced stage when larger amounts of ctDNA are released.

The distribution of gene mutations observed in our study was found to be histology-dependent and is in line with the published literature [20,30,32–36]. *TP53* mutations were predominantly identified in HGSC [20,30,32], whereas *KRAS* was typically mutated in LGSC [33]. *PIK3CA* mutations have been commonly identified in CCC [34] and endometrioid ovarian carcinoma [35], respectively. In addition, *KRAS* mutations may drive the progression of mucinous carcinoma of ovary [37].

The presence of residual disease after primary surgery is strongly associated with overall survival [38]. After a median follow-up of 33 months, we found that residual disease and the presence of ctDNA mutations immediately after surgery were independent predictors of PFS and OS. Our findings confirm and expand previous data on the potential prognostic significance of ctDNA mutations in post-surgical samples from patients with ovarian malignancies. On analyzing the prognostic significance of liquid biopsies in ovarian cancer, Parkinson et al. [28] demonstrated that a minor decrease in the *TP53* mutant allele fraction after one cycle of chemotherapy was a predictor of poor response to treatment after a median follow-up of 59 months. In addition, Pereira et al. [29] have shown that the absence of ctDNA mutations at six months from the initial treatment is an independent predictor of PFS and OS in newly diagnosed patients. Notably, the independent association between ctDNA mutations and PFS may have implications for treatment monitoring.

In the current study, we identified ctDNA mutations as an independent predictor of OS irrespective of tumor histology. While early-stage disease is more prevalent in CCC than in HGSC, the prognosis of the former malignancy is less favorable than that of the latter – even in patients with stage I disease [5,6,39,40]. Besides the presence of minimal residual disease, the peculiar features of CCC highlight the need for early identification of recurrences. Furthermore, neither CA125 nor HE4 are reliable diagnostic or prognostic biomarkers in patients with CCC. Morikawa et al. [41] identified the presence of *PIK3CA*-H1047R and *KRAS*-G12D mutations in patients with CCC and analyzed their impact on response to treatment using droplet digital PCR (ddPCR) [41]. Using the same approach, Ogasawara et al. [24] investigated 12 patients with CCC and demonstrated that the presence of ctDNA mutations was associated with less favorable PFS and OS. In the current study, a patient with CCC (case #30) who had a persistently detectable ctDNA mutation (*GNAS*-R844H) died of disease (Fig. 2). Another patient who had recurrent CCC (case #8) harbored a persistent *PTPN11*-R498L mutation.

The technical aspects pertaining to the detection of ctDNA merit comment; accordingly, its concentration may be sporadically low, ultimately limiting the identification of clinically relevant genomic alterations. The most commonly used technologies utilized include targeted NGS [12,17,20,21] and ddPCR [41,42]. Tagged-amplicon deep sequencing is capable of detecting ctDNA at concentrations as low as 2% [28,30]. Conversely, a potential limitation inherent in the use of ddPCR lies in the necessity to identify potential target genes using an *a priori* approach. In the present study, annotated tumor variants with at least 20 variant read counts and an allele frequency greater than 0.5% for SNV and 0.1% for indel were considered as true variants after raw data analysis.

In 7 patients (#13, 17, 20, 23, 26, 27, 30), ctDNA mutations were identified at recurrence but not identified at C0. In five of these patients (#17, 20, 23, 26, 27), ctDNA mutations absent at C0 were also not identified in tumor FFPE tissue. It is likely for these five patients that the ctDNA mutations identified at recurrence were due to clonal hematopoiesis (mutations in while blood cells that were released into plasma) [43,44]. On the other hand, patients #13 and 30 had ctDNA mutations identified at follow-up and in tumor tissue but not at C0. Actually, very low levels of mutated ctDNA were identified at C0 for both cases (mutation allele frequency 0.11% and 0.27%). They were deemed negative at C0 as we set an allele frequency cutoff at 0.5%. Both patients had gross residual disease after debulking surgery. The biological factors that may affect ctDNA release are complex, including necrosis, apoptosis, tumor location, and tumor vascularity. Possible speculation for the fluctuation of ctDNA mutation allele frequency in these two patients include (1) an increased rate of necrosis/apoptosis of residual tumor cells immediately after surgery, and (2) an increase of vascular permeability due to post-surgical inflammation.

Eight genes (*BRAF*, *CTNNB1*, *FBXW7*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, and *TP53*) in our sequencing panel are known to be frequently mutated in major types of ovarian cancer: *BRAF*, *KRAS*, and *NRAS* are commonly mutated in low-grade serous carcinoma [45]; *TP53* in high-grade serous carcinoma [32]; *KRAS* and *PIK3CA* in clear cell carcinoma [46]; *KRAS* and *TP53* in mucinous carcinoma [37]; and *CTNNB1*, *FBXW7*, *KRAS*, *PIK3CA*, *PTEN*, and *TP53* in endometrioid carcinoma [47]. Including these recurrently mutated genes in our panel allowed us to detect at least one mutation in 21 of 29 tumor samples. Post-operative ctDNA monitoring would not have been possible in our cohort of ovarian cancer patients if these genes had not been included in the panel.

There are limitations to our research. First, this was a single-center investigation with a limited sample size. This poses a caveat regarding the generalizability of our conclusions, and replication in independent samples is paramount for ensuring external validity. Second, the panel used for ultra-deep NGS analysis did not include the *BRCA* and *ARID1A* genes. *BRCA1/2* mutations have been previously shown to predict resistance to PARP inhibitors in ovarian cancer [17,21,48], whereas *ARID1A* mutations are common in CCC.

Conclusions

The results of the present study – conducted in a series of 29 patients with different histological types of ovarian carcinoma – demonstrate that ctDNA mutations were common (82.8% in

the entire cohort throughout the entire study period). The concordance rate between mutations identified in pretreatment ctDNA samples and FFPE tumor specimens was 58.6% for the entire cohort and 92.3% in patients with HGSC, suggesting that the concordance in non-HGSC was low (31.3%). Finally, the presence of ctDNA mutations in the first postoperative sample was identified as an independent predictor of less favorable PFS and OS.

Conflict of interest

Shu-Jen Chen, Hua-Chien Chen, Wen Hsiao, and Kien-Thiam Tan are employees of ACT Genomics, Co. Ltd.

Acknowledgements

This work was supported by ACT Genomics, Co. Ltd. We also received grants from Chang Gung Medical Foundation (CRRPG3F0043, CMRPG3H1152), Ministry of Science and Technology (MOST 108-2320-B-182-036, MOST 109-2629-B-182-003, and MOST 109-2314-B-182-034). We thank Jung-Erh Yang, Chun-Yu Yeh, Wei-Yang Chang, and Shih-Sin Huang for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2022.09.004.

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