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Comprehensive genomic sequencing of paired ovarian cancers reveals discordance in genes that determine clinical trial eligibility

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HIGHLIGHTS

- Ovarian cancer samples collected during clinical care were analyzed using a diagnostic next-generation sequencing gene panel.
- For most patients, the detected genomic alterations were stably maintained over time.
- No discordant alterations were found in genes currently used for therapy selection.
- 30% had discordant alterations in genes used for clinical trial eligibility; 23% were detected by repeat tumor testing.
- Low discordance was detected in assays for tumor mutation burden and loss of heterozygosity.

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ABSTRACT

Objective: We analyzed comprehensive genomic sequencing results from paired ovarian cancer samples to identify changes in mutational events over time.
Methods: DNA from paired FFPE tumor samples from 50 ovarian cancer patients in the Clearity Foundation Data Repository was analyzed for genomic mutations (GM), copy number alterations (CNA), microsatellite status (MS), tumor mutation burden (TMB), and loss of heterozygosity (LOH) by hybrid-capture, next-generation sequencing of up to 315 genes. Genomic profiles were compared between samples from the same patient. Poor quality results excluded 6 pairs from all analyses and 9 from CNA or LOH.
Results: Forty-four patients with predominantly advanced stage disease (34, 77%) and serous histology (31, 70%) received a median of 3 intervening treatment regimens (range 1–13). Analysis of 22 primary and recurrent sample pairs and 22 recurrent tumor pairs detected a median of 2 GM (range 0–5) and 1 CNA (range 0–6)/sample. TMB, MS, and LOH results were mostly concordant across paired samples. GM were consistent across most pairs [32/44 (73%) concordant], while CNA concordance was less [18/35 (51%)]. No changes were detected in therapeutically relevant GM, but 23% of patients had GM or CNA in the second sample that affect clinical trial eligibility.
Conclusions: Paired ovarian cancer samples demonstrate stable genomic alterations across time. However, discordance was observed for some genes used as eligibility criteria for molecularly targeted clinical trials. Repeat tumor testing may be useful in cases where eligibility for such trials is deemed important after consideration of testing costs and potential clinical benefit.

1. Introduction

Advanced stage ovarian cancer patients are typically treated with cytoreductive surgery and platinum-based chemotherapy at the time of diagnosis and are initially platinum-sensitive, but the majority will experience disease recurrence [1,2]. Prior studies have demonstrated that ovarian tumors are characterized by a high prevalence of TP53 mutations, copy number alterations and low mutation burdens, but can be clonally heterogeneous [3–5]. Genomic events that are associated with improved patient prognosis have been identified, such as defects in homologous recombination DNA repair (e.g., BRCA1 and BRCA2), as well as with poor prognosis, such as *CCNE1* amplification [3,6–8]. Genomic

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alterations can also be associated with opportunities for therapeutic intervention (e.g., PARP inhibitors for patients with BRCA 1 and BRCA2 mutations).

Relatively few studies have investigated changes in genomic profiles over time and these studies are largely limited to patients with high grade serous carcinomas [9–11]. In whole exome analyses of tumors sampled at diagnosis and first recurrence after platinum-based chemotherapy, significant genomic variability between samples was observed. Despite observing frequent loss of heterozygosity (LOH) in genes associated with homologous recombination repair deficiency, these studies did not detect any genetic alterations that would restore such repair processes in the recurrent specimens [11,12]. Among patients whose tumors have acquired resistance to chemotherapy, higher mutational burdens and an increased frequency of structural variants were observed in relapse compared to primary samples [10].

Some literature suggests specific genomic changes occur in response to treatment, particularly *BRCA1* or *BRCA2* reversion mutations leading to resistance to platinum-based chemotherapy and PARP inhibitors [10,13–15]. For non-platinum chemotherapy, less is known about the relationship between somatic mutations and specific treatments [16]. Promoter fusion of *ABCB1*, which encodes the MDR1 protein, a rapid efflux pump for many chemotherapy agents used to treat ovarian cancer, including paclitaxel, etoposide, and doxorubicin, has been noted in 8% of ovarian cancer patients with resistant disease [10,17].

Tumor molecular analysis is becoming increasingly common to inform treatment decisions for ovarian cancer patients in order to determine eligibility for targeted therapies or clinical trial participation. National Comprehensive Cancer Network (NCCN) guidelines now recommend tumor molecular testing prior to initiating therapy for persistent or recurrent disease, using the most recent available tumor tissue. Historically it has not been standard practice to perform repeat tumor sampling at the time of recurrence outside of secondary cytoreduction or clinical trial participation [18], so tissue sent for molecular analysis is often from surgery at the time of diagnosis. Unless repeat tumor sampling is performed at the time of recurrence, the tissue tested reflects the tumor's mutations prior to exposure to chemotherapy rather than capturing any additional genomic alterations accumulated in response to treatment or through selection of a resistant clone from the parental population. Furthermore, since many ovarian cancer patients experience multiple recurrences and are exposed to multiple different therapies, it is unclear how an individual's tumor might change over time or how accurately the profile of the primary tumor sample may reflect the disease later in the clinical course.

The objective of this study was to analyze paired recurrent ovarian cancer tumor samples by comprehensive genomic profiling (CGP) using a multi-gene panel test that interrogates ovarian cancer driver genes and genomic biomarkers [e.g., *BRCA1/2*, tumor mutation burden (TMB), microsatellite instability (MSI), loss of heterozygosity (LOH)] to identify any changes in genomic alterations and multi-gene biomarker scores over time and to assess the potential impact on therapy options and patient management.

2. Materials and Methods

2.1. Patient samples and clinical histories

50 ovarian cancer patients with two or more genomically profiled samples collected serially during the course of routine clinical care (2012–2018) were identified in the Clearity Foundation Data Repository of more than 600 ovarian cancer patients. This repository contains de-identified data from clinical histories and various tumor profiles, including those from next generation sequencing gene panels. Patient informed consent was obtained for the use of this data for research purposes under a Western IRB-approved protocol. Clinical data for treatment dates was extracted from medical records or provided in patient communications and records. Primary platinum response was annotated as sensitive if recurrence was greater than or equal to 6 months following the end of first-line platinum-based chemotherapy, resistant if less than 6 months, and refractory if progression occurred on platinum treatment. Paired specimens were either collected at the time of diagnosis and again at recurrence (primary-recurrent) or at multiple time points for recurrent disease (recurrent-recurrent).

2.2. Genomic analyses

Comprehensive genomic profiling (CGP) was performed on DNA extracted from two or more formalin-fixed paraffin-embedded (FFPE) samples (FoundationOne™, Cambridge, MA). The sequencing methods used for CGP have been validated and reported previously [19]. Sample processing and sequencing was performed in a Clinical Laboratory Improvement Amendments (CLIA)- and College of American Pathologists (CAP)-accredited laboratory. Briefly, following pathologic review to confirm sufficient tumor nuclei (minimum 20%) and mitigate pathologic inconsistencies, at least 50 ng DNA was extracted from 40 μm of tumor samples provided as FFPE tissue blocks. The samples were assayed using adaptor-ligation and hybrid capture next-generation sequencing (FoundationOne®) for all coding exons from 182 (version 1), 287 (version 2), or 315 (version 3) cancer related genes, plus select introns from 14 (version 1), 19 (version 2), or 28 (version 3) genes frequently rearranged in cancer [20]. Sequencing of captured libraries was performed using Illumina HiSeq technology to a mean exon coverage depth of >500X, and resultant sequences were analyzed using both an algorithmic pipeline and manual curation for base substitutions, small insertions or deletions, copy number alterations (focal amplifications and homozygous deletions), and select gene fusions, as previously described [20]. Variants identified by the dbSNP database (https://www.ncbi.nlm.nih.gov/snp) or with two or more counts in the ExAC database (http://exac.broadinstitute.org/) were designated as germline and removed. Recurrent variants of unknown significance predicted as germline by an internally developed algorithm were also excluded. Inactivating events of known tumor suppressor genes and known, confirmed somatic alterations identified from the Catalog of Somatic Mutations in Cancer (COSMIC v62) were considered biologically significant [21]. Tumor mutational burden was assessed on 1.1 megabases (Mb) of sequenced DNA for each case based on the number of somatic base substitution or indel alterations per Mb after filtering to remove likely or known to be germline polymorphisms or bona fide oncogenic drivers utilizing a validated algorithm as previously described [22]. TMB was measured in mutations per megabase (mb) and were grouped into three bins: TMB-low (1–5 mutations/mb), TMB-intermediate (6–19 mutations/mb), and TMB-high (>20 mutations/mb). The cut off of 20 coding mutations per megabase is approximately equal to 400 nonsynonymous mutations per exome [23]. Principal components analysis (PCA) for a set of optimized loci containing microsatellites is used to produce an NGS-based "MSI score" [20]. Ranges of the MSI score were assigned MSI-High (MSI-H) or microsatellite stable (MSS). The genomic loss of heterozygosity (LOH) score was determined by leveraging SNPs spaced evenly at 1 Mb intervals across the genome and extrapolating a percentage of genome with LOH, excluding arm- and chromosome-wide LOH segments [12]. The genomic LOH score results were reported as "LOH-High" (score > 16%), "LOH-Low" (score < 14%), or "Borderline" (score 14%–16%).

Because copy number determination for individual genes is affected by a specimen's tumor content, a re-review of all prior

CNAs by a single computational biology curator (LJ) was performed to confirm the computational purity, the copy number model selected, and presence of each amplification and deletion in the pair. Sample pairs where at least one of the specimens had a copy number plot that could not be definitively interpreted were excluded from the final CNA comparison.

2.3. Paired sample analyses

We compared genomic mutations or rearrangements, copy number alterations (CNA), and genomic biomarker scores in paired samples from the same patient. We excluded discordant mutations and CNAs if the gene was not assessed in the versions of the assay used for the sample pair (i.e. discordance due to baitset difference), if an alteration was detected but not reported (i.e. discordance due to changes in reporting rules), or if the alteration was designated as subclonal (detected in less than 10% mutant allele fraction). We additionally excluded discordant CNAs if the CNA was categorized as equivocal (discordance due to amplification at limit of detection). In order to focus on the most clinically relevant findings for CNAs, we limited our final analysis to genes with copy number amplification or loss that have or are being used as enrollment criteria for clinical trials: amplifications of *AKT1, AKT2, AKT3, BRAF, CCND1, CCND2, CCND3*, *CDK4*, *CDK6*, *MYC*, *CCNE1*, *EGFR*, *ERBB2*, *FGFR1, FGFR2, FGFR3, FGFR4, KRAS*, *PIK3CA*, *MET, and RICTOR* and loss of *ATM*, *CDKN2A*, *PTEN*, and *RB1* (Supplementary Table 1). Six ovarian cancer sample pairs were excluded due to poor quality metrics. Fig. 1 illustrates the evaluation workflow used to identify patients with discordant mutations and/or CNA in evaluable genes.

2.4. Statistical analyses

We compared clinical and treatment characteristics between patients based on the presence or absence of discordant mutations/rearrangements or CNA using Fisher's exact and Kruskal-Wallis tests. We compared tumor collection techniques, adjusted for pair type using a Cochran-Mantel-Haenszel test. All statistical analyses were performed using R v3.6.0. Statistically significant results were pre-defined as $p \leq 0.05$.

3. Results

Of 44 patients with evaluable sample pairs, 22 had primary-recurrent specimens and 22 had multiple recurrent tissue specimens (Table 1). Three patients had three specimens available for analysis. Ovarian cancer was diagnosed at a median age of 58.7 years (range 34.5–76.9 years). The majority of patients had stage III/IV (34/77%) and primary platinum-sensitive disease (32/73%). Most tumors demonstrated serous histology (31/70%); there were four cases (9%) with clear cell and three cases (7%) with endometrioid histology. The median time between sample collections was 25.2 months (range 8.0–77.4 months). Patients received a median of three treatment regimens between tumor samplings (range 1–13). Treatment regimens were relatively heterogeneous, but a majority of patients received platinum-based chemotherapy (35/80%), taxanes (30/68%), liposomal doxorubicin (23/52%), or VEGF inhibitors (25/57%) between sample collections. Median overall survival from the time of diagnosis was 55.7 (range 19.7–204.9) months. The most frequent sites of disease for the first specimen were ovary (21%) and omentum (23%) and for the second specimen, liver (16%), peritoneal fluid (14%) and bowel (14%). Tumor specimens were procured by surgical resection, core needle or other biopsy, or by paracentesis or thoracentesis (Supplementary Tables 2 and 3).

A median of two genomic mutations/rearrangements (range 0–5)/sample were detected in the 44 pairs of tumor samples (Table 2, Supplementary Table 4). Twelve (27%) had discordant mutations/rearrangements; 9 new and 6 not detected in the later sample. Because copy number determination for individual genes can be affected by a specimen's tumor content, a re-review of all the CNAs previously reported for each sample pair was performed to

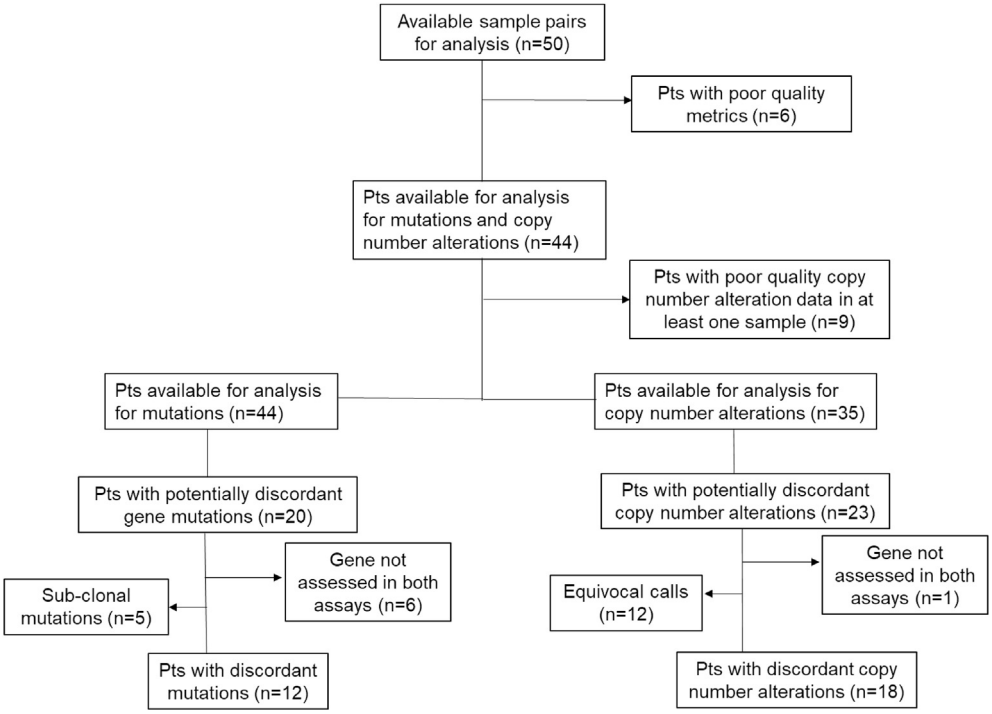

Fig. 1. Identification of patient samples with discordant mutations and copy number alterations. Flow diagram depicts number of patient sample pairs and genes affected by applying quality metrics (see Materials and Methods). Numbers may not sum to the total due to patients with discordant mutations/CNA that were excluded for more than one reason or with both reported and excluded discordant mutations/CNA.

Table 1
Clinical, pathologic, and treatment characteristics of patients in this study.

	Patients, n (%)
Patients	44 (100%)
Median age at diagnosis, years (range)	58.7 (34.5–76.9)
Stage[a]	
I	3 (7%)
II	5 (11%)
III	27 (61%)
IV	7 (16%)
Primary disease site	
Ovary	34 (77%)
Fallopian tube	7 (16%)
Peritoneum	3 (7%)
Tumor histology	
Serous	31 (70%)
Clear cell	4 (9%)
Endometrioid	3 (7%)
Adenocarcinoma, NOS	2 (5%)
Mixed	2 (5%)
Carcinosarcoma	1 (2%)
Not otherwise specified	1 (2%)
Tumor grade[a]	
G1	1 (2%)
G2	3 (7%)
G3	37 (84%)
G4/undifferentiated	1 (2%)
Primary platinum response	
Sensitive	32 (73%)
Resistant	10 (22%)
Refractory	2 (5%)
Pair type	
Primary-recurrent	22 (50%)
Recurrent-recurrent	22 (50%)
Median time between specimen collections, months (range)	25.2 (8.0–77.4)
Median survival following diagnosis, months (range)	54.8 (19.7–137.9)
Median number of treatment regimens in between samples (range)	3 (1–13)

[a] Missing for two patients.

confirm the computational purity, the copy number model selected, and presence of each amplification and deletion. A median of 1 CNA (range 0–6)/sample was detected in the 35 pairs of tumor samples that met the quality criteria for this analysis and CNA were discordant in 17 (49%); 11 new and 7 not detected in the later specimen.

Fig. 2 illustrates the prevalence and trends over time of mutation/rearrangement and CNA events by gene. 36 of 44 pairs (82%) had *TP53* mutations and all were conserved across samples. All other genomic alterations were significantly less common, but the majority of mutations were conserved between sample pairs, including *BRCA1* (5 patients, 11%), *KRAS* (7 patients, 16%), and *TERT* (4 patients, 9%). The *CCNE1* gene was frequently amplified (10 patients out of 35, 29%) and those CNA were conserved in 8 of the 10 patients.

Table 3 summarizes the clinicopathologic characteristics and treatment histories between samples for patients with discordant mutation/rearrangements and CNA. Of these 21 patients, 16 (76%) had primary platinum sensitive disease and 14 (67%) were pairs of primary and recurrent tumor samples. *ARID1A* mutations were detected in 4 patients and for 3 of them only in the subsequent recurrences, including patient TCFr-R18Y, who had a second *ARID1A* mutation that was detected in both samples. Three of these patients had clear cell histology where *ARID1A* alterations are frequently detected [24]. In one of the patients who had three samples available for analysis (TCFr-TOF6), a frameshift mutation in *MLL2* was detected in the primary ovarian tumor specimen and in the most recent liver sample but not in the liver specimen collected in the intervening period. This patient's tumor had an *RB1* mutation detected in that sample that was not found in the others in addition to mutations in *ATRX* and *TP53* that were present in all three samples, suggesting the existence of clonal heterogeneity.

We identified discordant CNAs in 17 of the 35 copy number-evaluable sample pairs (Table 3). Eleven (31%) patients had new CNAs detected in the later sample, while 7 patients had a CNA in the first specimen that was not detected in the later specimen (20%). Two patients had a *CCNE1* amplification detected only in the second sample. All patients with *CCNE1* amplifications in at least one specimen had grade 2/3 tumors with serous histology. In six of the 17 discordant CNA cases, 6–8 copies of the amplified gene, which is at the CNA calling threshold for the assay, were detected in one of the paired samples (Table 3). This differentially detected low level amplification may suggest that a sub-population of tumor cells with the amplified gene could have been present in the other specimen but in a ratio below the limit of detection of the assay or that a subclone of higher amplification was admixed with cells with lower level CNAs. Thus, some cases of discordance may reflect the sensitivity around the calling threshold. In most cases, however, 9 or more gene copies were detected in one sample and no amplification was detected in the paired sample. Some of these cases had high level focal gene amplifications (e.g., *CCNE1* in TCFr-XVE3, *MYC* in TCFr-X43W, *FGFR1* in TCFr-FCCV, and *RICTOR* in TCFr-79KK, 11, 15, 20, and 21 copies, respectively).

In two of the patients with discordant gene alterations, similar signaling pathways would be predicted to be activated in both samples, suggesting evolutionarily convergent clonal populations (Table 3): in patient TCFr-FCI2 an *NF1* mutation was detected only in sample one, while sample two displayed *KRAS* amplification, consistent with possible activation of the RAS-MAPK pathway in both specimens. Sample one from patient TCFr-OIPC contained an

Table 2
Genomic alteration and biomarker frequencies for paired samples.

Number of paired samples	44
Number of mutations per sample, median (range)	
Sample one	2 (0–4)
Sample two	2 (0–5)
Discordant mutations between samples, n (%)	
Sample one only	6 (14%)
Sample two only	9 (20%)
Number of concordant mutations between samples, median (range)	2 (0–4)
Number of CNA per sample, median (range)[a]	
Sample one	1 (0–6)
Sample two	1 (0–4)
Discordant CNA between samples, n (%)[a]	
Sample one only	7 (20%)
Sample two only	10 (29%)
Number of concordant CNA between samples, median (range)[a]	1 (0–4)
Tumor mutation burden, n (%)	
Sample one	
Low	37 (84%)
Intermediate	4 (9%)
Missing	3 (7%)
Sample two	
Low	37 (84%)
Intermediate	6 (14%)
High	1 (2%)
Microsatellite Status, n (%)	
Sample one	
Stable	23 (52%)
High	0 (0%)
Missing	21 (48%)
Sample two	
Stable	38 (86%)
High	0 (0%)
Missing	6 (14%)
Loss of heterozygosity, n (%)	
Sample one	
Low	27 (61%)
High	11 (25%)
Missing	6 (14%)
Sample two	
Low	25 (57%)
Borderline	2 (4%)
High	13 (30%)
Missing	4 (9%)

[a] Includes only the 35 sample pairs for which CNA data was evaluable.

AKT3 amplification while *PIK3CA* amplification was observed in sample two only, consistent with activation of the PI3K-AKT-mTOR pathway across specimens.

While none of the observed discordant genetic alterations are associated with FDA approved therapies in ovarian cancer, 13 patients (30%) had discordant samples that involved genes (8 with amplifications or losses and 2 with mutations/rearrangements) that have been used as enrollment criteria for clinical trials (Table 3 and Supplementary Table 1). Four patients had such alterations detected in the first but not the second specimen, while 10 patients (23%) had alterations detected only in the second sample and would not have been considered eligible for those clinical trials if a repeat biopsy was not performed.

In addition to genomic alterations, we also analyzed results from multi-gene scores for TMB, microsatellite status, and LOH (Table 2, Supplementary Table 4). All samples were microsatellite stable and only one of 41 patients (TCFr-GUR0) had a sample with high TMB (25.2 muts/Mb). That patient's earlier tumor sample had an intermediate TMB (7.9 muts/Mb) and is the only case of TMB discordance detected. TMB was relatively stable between the other paired samples; the median change in TMB between samples was 0.11 (range −4.35 to 17.32) muts/Mb. LOH scores were determined for 35 paired samples. Approximately 40% of those samples had high LOH scores, which is lower than, yet generally consistent with, the 54% observed in a large phase 2 study of rucaparib [12]. The results were concordant in all but 3 pairs, which were from primary and recurrent tumors. For TCFr-OIPC, the primary tumor had a high score and the recurrent sample a low score, while the opposite was true for TCFr-TOF6 and TCFr-GUR0.

We performed statistical analyses to explore clinicopathologic and specimen characteristics that could be associated with the observed discordance in mutation/rearrangement or CNA detection (Table 4). Patients with primary-recurrent samples were more likely to have discordant CNA than those with recurrent-recurrent sample pairs ($p = 0.004$). There was also a significant association with higher frequency of discordant mutations in samples from patients with early stage disease ($p = 0.03$), but this may be influenced by the small number of samples from those patients ($n = 8$). We found no statistically significant clinical associations for patients with discordant mutations or CNA based on age at

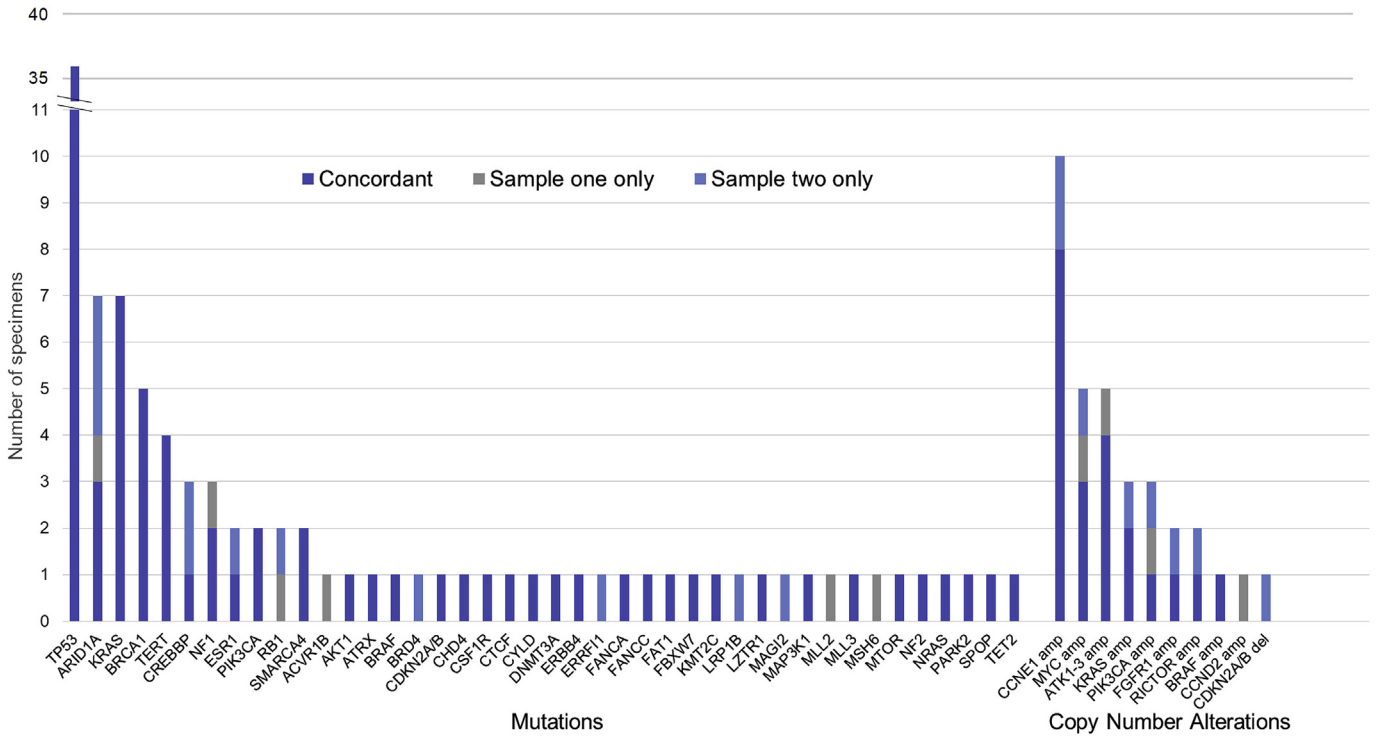

Fig. 2. Frequency of mutations and copy number alterations in paired samples. Graphical representation of the number of alterations detected that were concordant (dark blue bars) or discordant in either sample one (gray bars) or sample two (light blue bars). Only genes used as clinical trial eligibility criteria are shown for CNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diagnosis, histology, platinum sensitivity, number of intervening treatments, or time between sample collections. Because different collection methods were used for many of the first and second specimens (i.e., 84% of the first samples were procured by tumor resection and 64% of the second ones were obtained by biopsy or paracenteses/thoracenteses; Supplementary Tables 2 and 3), we also explored the potential relationship between discordance and collection method, as well as tumor purity/content for the paired samples. No statistically significant association between discordance and collection method was found (Table 4). Differences in tumor purity and tumor content between samples were also compared and no significant relationship was identified for pairs with discordant mutations or CNA.

4. Discussion

In a cohort of patients with recurrent ovarian cancer, the majority of mutation events were preserved between different samples over time, but nearly half had at least one discordant mutation or copy number alteration event. No discordant alterations were clinically actionable, but approximately 30% of patient samples demonstrated variable detection of mutations or copy number alterations that could affect clinical trial eligibility. In 23 % (10/44), a new alteration was detected in the second sample that that may have made the patient eligible for a molecularly targeted clinical trial. We also compared the TMB, MSI, and LOH multi-gene scores in these paired specimens and found concordance in these measures for the majority of patients. Discordant mutations or CNAs were not related to the time between sample collections for testing, but discordance in CNAs was more frequently observed in the paired primary and recurrent specimens than in paired recurrences, suggesting that initial therapy is more likely to result in genomic changes reflected in the tumor mutation profile than intervening treatments for recurrent disease. This difference suggests that obtaining a new tissue sample for molecular profiling at the time of recurrence instead of sequencing the primary specimen at this point in the patient's care can potentially add value regardless of the time to recurrence.

Our results are largely consistent with other studies that have investigated changes in the genomic profile of ovarian cancers after treatment. Patch et al. [10] performed whole genome sequencing (WGS) of primary and recurrent tumor specimens from chemoresistant ovarian cancer patients and also did not identify any currently actionable recurrent point mutations in specimens obtained from patients with recurrent disease. Unlike Patch and colleagues, we did not identify *BRCA1/2* reversion events in the samples from 5 patients with *BRCA1* mutations despite intervening treatment and progression/recurrence on PARP inhibitor therapy. We also did not observe an increased frequency of *NF1* or *RB1* mutations, regardless of the timing of sample collection relative to diagnosis. Lambrecht et al. [11] performed whole-exome sequencing and single nucleotide polymorphism profiling of 31 platinum-sensitive ovarian cancer tumors at diagnosis and following adjuvant platinum-based chemotherapy. 58% of the detected mutations and 41% of the copy number alterations were conserved between samples which is less than the 73% concordance in mutations and 51% in copy number alterations detected in our study.

In contrast to these other studies, our analysis interrogated a selected and smaller number of genes that are known oncogenes, tumor suppressors, and modulators of tumor growth. Many are considered "actionable" because their mutation or alteration is correlated with the sensitivity of tumor cells to targeted therapies in some cancers. Compared to WGS, this targeted approach may be somewhat less likely to identify passenger mutations which have less impact on tumor biology accounting for the lower discordance

Table 3
Mutations, Copy Number Alterations and biomarkers across paired samples with discordant genomic alterations.

Patient ID	Concordant Alterations	Discordant Alterations		Trial Elig.	TMB	LOH	Pair Type	Stage, Hist, Grade	1° Pt Resp	# Tx	Treatments between samples
		Sample one only	Sample two only								
TCFr-TWAU	TERT, TP53, **CCNE1, PIK3CA, PRKCI, TERC**	ACVR1B	**SOX2(7)**		L, L	L, L	P	IIIC, S, G3	S	1	C-P-B
TCFr-TOF6	ATRX, TP53, **AXL**	MLL2	MLL2*, RB1, **SOX2(9)***	X	L, L, L	L. H, B	P	IIIC, S, G3	S	3	C-P, C-D-B, C; nab-P, G
TCFr-OIPC	NF1, TP53	ARID1A, **AKT3(7), MCL1(7)**	CREBBP, *LRP1B(0),* **PIK3CA(6), MYCN(7), SOX2(6)**	X	L, I	H, L	P	IIC, S, G3	S	1	C-P
TCFr-FCI2	**ERBB4,** TP53, **CCNE1**	NF1	**KRAS(17), PRKCI(12), TERC(12)**	X	L, I	L, L	P	IIIC, S, G3	S	5	C-Cis-P-B, C-G-B, Nivo-Cabi, C-D-B, Ola-Adav
TCFr-PDK9	KRAS, PIK3CA, TERT		ARID1A, MAGI2, **CDKN2A/B(0)**	X	L, L	N/A, L	P	IIC, CC, G3	S	1	C-P-B
TCFr-QKYE	TP53		LRP1B, **MYC(10)**	X	N/A, L	N/A, H	P	IIIC, S, G3	R	6	C-P, G, Pem, Pem-B, D, E
TCFr-XVE3	TP53		**CCNE1(11), MAP2K2(10)**	X	L, L	L, L	P	IIIC, S, G3	R	2	C-P, D
TCFr-FCCV	TP53, **CCNE1**		**FGFR1(20), IKBKE(8)**	X	L, L	L, L	P	IV, S, G3	S	3	C-P, D, B
TCFr-INK5	FAT1, PARK2, TP53, **CCNE1**		**MYST3(10),** *CIC(0)*		L, L	L, L	P	IIIC, S, G2	S	4	C-P-B-V, C-D, Mirv, Def-Avel
TCFr-79KK	TP53, SMARCA4, TET2		**FGF10(9), RICTOR(21)**	X	L, L	H, H	P	IIIC, Ad, G3	R	1	C-P
TCFr-X43W	TP53, **LYN, MYCL1**	**MYC(15)**		X	L, L	L, L	P	IIC, S, G3	S	2	C-P, C-D
TCFr-GUR0	BRCA1, CREBBP, FBXW7, TP53, KDM5A, **FGF12**	**PIK3CA(8),** *SLIT2(0)*		X	I, H	L, H	P	IVB, S, G3	R	6	C-P-B, Olap-Durv, D-B, T-B, P-B, Ev
TCFr-MAQT	CYLD, TP53, **AKT2**	**PRSS8(8)**			L, L	L, L	P	IIIC, S, G3	S	4	C-P-B, C-G, D, Lurb
TCFr-6ESZ	AKT1, ARID1A, CHD4, KRAS	**AURKA(9), GNAS(9), ZNF217(9)**			L, L	L, L	P	IIIC, CC, G3	R	1	C-P-B
TCFr-FC54	NRAS, TP53, **RICTOR**	RB1		X	L, L, L	L, L, L	R	IIIC, SE, G3	S	6	C-P-Cis-Dt, G, Dt, B-D, Pem-B, O
TCFr-L6ON	TP53	**ARFRP1(10)**	ERFFI		L, L	L, L	R	IV, S, G3	S	7	T-Cy, Cy, Vino-Cap, Cy-NYESO, ONT, ABT, Def-Avel
TCFr-20YJ	BRCA1, TP53	MSH6	ESR1		L, L	H, H	R	IIC, S, G3	S	7	C-G, V, P, D, B, C-B, T-B
TCFr-ANNE	TP53	**CCND2(8), FGF23(8), FGF6(8), KDM5A(8)**	ARID1A	X	L, L	H, H	R	IIIC, S, G3	S	1	C-D
TCFr-NN7F	TP53, **AKT2, MYC**		**CCNE1(8), ARFRP1(8)**	X	L, L	L, B	R	IIIC, S, G3	S	2	C-P, C-G
TCFr-R18Y**	ARID1A, TERT, TP53		ARID1A***, BRD4		I, L	L, L	R	IC, CC, G3	S	2	C-G, D-B
TCFr-W1GK**	KRAS		CREBBP		L, L	L, L	R	IA, E, 2	S	4	Pem, nabP, Tr, Tr-E

CN amplifications denoted in bold font; CN deletions denoted in italics; (Number of copies); Underlined genes denote those used for clinical trial eligibility.
* Alterations detected in sample three, not sample two.
** Paired samples used only for copy number analysis.
*** Additional mutation in ARID1A detected in second sample.
Trial Elig., X indicates discordant genomic alteration used as eligibility criterion for clinical trial.
TMB, Tumor Mutation Burden; LOH, Loss of Heterozygosity; low, I, intermediate; H, high; B, borderline; N/A, not available.
Pair Type: P, Primary-recurrent R, Recurrent-recurrent S, Serous; CC, Clear cell; SE, Serous endometrioid; C, Carcinosarcoma; Ad, Adenocarcinoma; NS, Not specified.
1° Pt Resp, primary platinum response; S, sensitive; R, resistant.
Tx, number of treatments between samples.
A, anastrozole; ABT, ABT165; Avel, avelumab; Adav, adavosertib; B, bevacizumab; C, carboplatin; Cabi, cabiralizumab; Cap, capecitabine; Cet, cetuximab; Cis, cisplatin; Cy, cyclophosphamide; D, liposomal doxorubicin; Def, defactinib; Dt, docetaxel; Durv, durvalumab; E, erlotinib; Ev, everolimus; G, gemcitabine; L, letrozole; Mirv, mirvetuxumab, nabP, nab-paclitaxel; Nivo, nivolumab; NYESO, NY-ESO-1c259 T cells; O, oxaliplatin; Olap, olaparib; ONT; ONT-10; P, paclitaxel; Pem, pemetrexate; V, veliparib; Vino, vinorelbine; T, topotecan; Tr, trametinib.

Table 4
Association between clinicopathologic variables and specimen characteristics and discordant mutation or copy number alterations.

	Mutations (n = 44)			Copy number alterations (n = 35)		
	Discordant mutations	**No discordant mutations**	***p* value**	**Discordant copy number alterations**	**No discordant copy number alterations**	***p* value**
Age at diagnosis, median (range)	59.7 (39.5–76.9)	58.7 (34.5–74.1)	0.71	58.7 (34.6–76.9)	57.5 (39.5–74.1)	0.31
Primary tumor site			0.14			0.22
Ovary	12	7		13	12	
Fallopian tube	0	22		4	3	
Primary peritoneal	0	3		0	3	
Stage			**0.03**			1
I-II	5	3		3	2	
III-IV	7	27		14	14	
Unknown	0	2		0	2	
Grade			1			1
1	0	1		0	1	
2	1	2		1	0	
3 or 4	11	27		16	16	
Unknown	0	2		0	1	
Histology			1			1
Serous	8	23		14	14	
Non-serous	4	9		3	4	
Primary platinum response			0.13			0.71
Sensitive	11	21		11	13	
Resistant	1	9		6	3	
Refractory	0	2		0	2	
Pair type			1			**0.004**
Primary-recurrent	6	16		14	5	
Recurrent-recurrent	6	16		3	13	
Number of treatments between samples, median (range)	4 (1–7)	2 (1–13)	0.968	3 (1–6)	2.5 (1–7)	0.332
Time between sample collection, months, median (range)	25.1 (16.0–52.9)	25.5 (8.0–77.4)	0.46	25.3 (10.9–35.3)	23.7 (10.0–77.4)	0.8
Sample collection method by pair			0.794			0.923
Tumor resection for both	5	10		6	5	
Tumor resection and biopsy/other	6	17		10	9	
Biopsy/other for both	1	5		1	4	
Change in % tumor purity between specimens			0.734			0.068
<25%	7	20		13	9	
25–49%	3	8		2	6	
≥50%	0	4		0	3	
Not available	2	0		2	0	
Change in % tumor content between specimens			0.615			1
<25%	6	13		7	8	
25–49%	2	8		3	5	
≥50%	0	4		1	2	
Not available	4	7		6	3	
One sample with minimal tumor purity			0.404			0.068
≤20% tumor purity	1	9		1	5	
>20% tumor purity	9	23		14	13	
Not available	2	0		2	0	
One sample with minimal tumor content			0.649			0.214
≤20% tumor content	3	6		1	3	
>20% tumor content	5	20		10	12	
Not available	4	6		6	3	

Categorical variables compared with Fisher's exact test.
Age, number of treatments, and time between sample collection compared with Kruskall Wallis test.
Sample collection method stratified by pair type, compared with Cochran-Mantel-Haenszel test.

rate observed herein. *TP53* mutations were present in 80% of patient tumors and conserved in all. *BRCA1/2* mutations were detected and conserved in 5 patient tumors. *CCNE1* amplifications were concordant in most paired samples, with 2 of 10 samples demonstrating amplifications in the second specimen. Of the genes assessed, *MYC* appears to be most frequently discordant (3/5 discordant).

CCNE1 amplifications have been associated with poor prognosis as well as platinum-resistant disease [3]. Conservation or new detection of *CCNE1* amplifications in specimens obtained following treatment is consistent with the putative role for *CCNE1* in tumor survival and progression. When *MYC* amplification was discordant, it was detected in either earlier or later specimens. This suggests clonal heterogeneity for this gene or chromosomal region and indicates that *MYC* aberrations may not always be associated with patient outcome. Of note, within our cohort, 31 (70%) of patient tumors had serous histology. Nearly 70% of these patients had primary platinum-sensitive disease and the frequency of gene mutation/alterations detected for these specimens was similar to that reported by The Cancer Genome Atlas (TCGA) Project [3] for *TP53* (90% vs 96% in TCGA) and *BRCA1/2* (16% vs 22%), suggesting that our cohort is representative of the typical ovarian cancer population. However, we detected different frequencies of *MYC* (15% vs 30%) and *CCNE1* amplifications (29% vs 21%) which may reflect the inclusion of recurrent tumor samples in our analysis, innate self-selection bias, or a different methodology for measuring copy number alterations.

The observation of discordant mutation or copy number alterations in specimens obtained from nearly half of the patients is consistent with reports of clonal heterogeneity in ovarian cancers

[5]. Both intra-and inter-tumoral heterogeneity may account for the discordance detected in our study. Mixtures of molecularly distinct clones may be present as early as diagnosis. The selective expansion of one or more of these clones can occur during tumor progression. Our study suggests that this selection is more pronounced between the primary tumor and recurrence than between recurrences. Specimen tumor content and sampling procedure may also impact these results since detection of genomic alterations, particularly CNA, can be compromised in specimens with low tumor content. The majority of the first specimens analyzed from the primary-recurrent sample pairs were collected by tumor resection while most specimens analyzed in the recurrent-recurrent pairs were procured by biopsy or para/thoracentesis. However, we did not find a significant association between discordance and the method of sample collection or differences in tumor purity or content of the paired specimens.

Some of the genetic alterations that were variably detected in our analyses have been used for determining clinical trial eligibility. It is possible that patient responses and the interpretation of the ability of biomarkers to predict responses in such cases may be influenced by clonal heterogeneity. For example, two patients whose paired samples exhibited characteristics of convergent evolution with unique RAS-MAPK (NF1 deleterious mutation and KRAS amplification) or AKT-PI3K-mTOR (PIK3CA and AKT3 amplifications) pathway alterations were detected in different specimens from each patient [25]. Despite similar predicted pathway activities in each specimen, different targeted therapies could be selected based on the specimen profiled. For example, PI3K inhibitors might be more appropriate for treating the tumor with the PIK3CA amplification than the AKT3 amplification, while an AKT or mTOR inhibitor might provide equivalent benefit for tumors with either alteration. Incorporating evaluation of multiple tissue or liquid biopsies into clinical trial design may resolve these issues.

This study also reports results for paired sample analysis of LOH scores. High LOH scores have been correlated with a greater likelihood of benefit from treatment with rucaparib in both the maintenance and recurrent settings [12,26]. Of 35 patients with LOH scores available for both samples, only three had discordant LOH scores: two low to high and one high to low. These results suggest that some degree of clonal heterogeneity exists in the extent that impaired double strand break repair manifests as the level of genomic LOH present in tumors at different sites. LOH is an indicator of homologous recombination DNA repair deficiency that can be driven by loss of function in multiple components of this pathway, including *BRCA1/2*, *RAD51* and *PALB2*, as a result of mutations or epigenetic silencing. One of these three patients (TCFr-GUR0) had a germline *BRCA1* mutation; although the majority of tumors in patients with germline *BRCA1/2* mutations display increased genomic LOH scores, it is not uniformly present [27].

Consistent with other studies, all of the patient samples in this study were microsatellite stable and all but one had low or intermediate TMB [3,28]. The detection of a high TMB in a recurrent tumor from one patient is noteworthy as this patient also had a low LOH score in her primary specimen but a high score in her recurrent tumor, suggesting accumulation of both additional mutations increasing TMB and focal segmental loss increasing the genomic LOH score. She was diagnosed with stage IVB fallopian tube cancer and received six lines of therapy over the course of 25 months, including the combination of olaparib and the PD-L1 inhibitor durvalumab as second line in a clinical trial, before this specimen was collected and analyzed. Precise causation and the timing for the emergence of a clone with these characteristics cannot be deduced. This patient's tumor was resistant to primary platinum therapy despite having a germline *BRCA1* mutation, which may be explained by the initially low LOH score. The patient had progressive disease on olaparib-durvalumab therapy after three months.

Only one patient sample displayed a mutational event that is known to be potentially related to a specific prior treatment. A *TP53* mutation was detected in the initial sample for patient TCFr-QKYE, but after six intervening treatment regimens (liposomal doxorubicin, carboplatin-paclitaxel, gemcitabine, pemetrexed, pemetrexed-bevacizumab, liposomal doxorubicin, and erlotinib), repeat testing also detected a *LRP1B* deletion. Downregulation of *LRP1B* is associated with resistance to liposomal doxorubicin in patients with high grade serous ovarian cancer, potentially preventing uptake of anionic liposomes and drugs such as liposomal doxorubicin [13]. Thus, it is possible that the *LRP1B* alteration may have contributed to liposomal doxorubicin failure in this patient.

One strength of this study is the detailed clinicopathologic and treatment data available for our patient cohort. Although we do not know if treatment-related decisions were made based on the results of molecular profiling, our data allows us to investigate correlations between specific clinical characteristics and sequencing results. The use of a commercially available sequencing platform makes our results relevant for providers ordering tumor sequencing in the clinic, but does not consider epigenetic events, such as methylation, that may influence tumor biology or influence sensitivity to specific therapies. Because the panel of genes tested by this assay has grown over time and the multi-gene test scores for microsatellite status, TMB, and LOH have also been added, up to 14% of patients have missing data for LOH and TMB and nearly half are missing data for microsatellite status in sample one.

In this cohort of 44 ovarian cancer patients whose tumors underwent sequential molecular profiling, changes in mutation events or copy number alterations between paired specimens were found in almost half of the cohort, but these changes are not in currently clinically actionable genes with FDA-approved therapies. Approximately 30% of patient samples demonstrated molecular changes that could potentially modify their clinical trial eligibility; 23% had alterations detected in the later specimen that would have been missed without subsequent testing. Discordant mutations or CNAs were not related to the time between sample collections for testing, but discordance in CNAs was more frequently observed in the paired primary and recurrent specimens than in paired recurrences. Repeated molecular testing may reveal genomic alterations which provide additional opportunities for clinical trial enrollment, but the potential clinical benefit for each patient should be weighed against the risk of the sampling procedure, likelihood of identifying such alterations, and the additional cost of testing to the patient and/or the health care system.

Author contributions

Conception and design: JEF, DAL, DAZ.
Collection and assembly of data: JEF, LJ, JE, DAZ.
Data analysis and interpretation: JEF, AAB, LJ, JE, DAL, DAZ.
Manuscript writing: All authors.
Final approval of manuscript: All authors.

5. Conflict of interest disclosures

Drs. Fehniger, Berger, and Zajchowski have no disclosures.

Dr. Elvin reports employment and stock from Foundation Medicine, Inc.

Dr. Juckett reports employment by Foundation Medicine, Inc.

Dr. Levine reports personal fees from Tesaro, personal fees from Merck, grants from Splash Pharmaceuticals, outside the submitted work; In addition, Dr. Levine has a Patent Application US20130078319A1 - Detection of ovarian cancer pending, and a Patent application WO2015103431A9 - Compositions and methods for the diagnosis and treatment of ovarian cancers that are

associated with reduced SMARCA4 gene expression or protein function pending.

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Appendix A. Supplementary data

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