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Comprehensive genomic profiling of epithelial ovarian cancer by next generation sequencing-based diagnostic assay reveals new routes to targeted therapies

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HIGHLIGHTS

- Using targeted NGS, 141 genomic alterations were identified in 48 ovarian epithelial carcinomas 67of which were actionable.
- Most common alterations were in TP53 (79%); MYC (25%); BRCA1/2 (23%); KRAS (16.6%) and NF1 (14.5%).
- NGS identifies an unexpectedly high frequency of genomic alterations that could influence targeted therapy selection for ovarian carcinoma.

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ABSTRACT

Objective. Targeted next generation sequencing (NGS) was evaluated for its ability to identify unanticipated targetable genomic alterations (GA) for patients with relapsed ovarian epithelial carcinoma (OC).

Methods. DNA sequencing was performed for 3320 exons of 182 cancer-related genes and 37 introns of 14 genes frequently rearranged in cancer on indexed, adaptor ligated, hybridization-captured libraries using DNA isolated from FFPE sections from 48 histologically verified relapsed OC specimens. The original primary tumor was sequenced in 26 (54%) of the cases and recurrent/metastatic tumor site biopsies were sequenced in 22 (46%) of the cases. Actionability was defined as: GA that predict sensitivity or resistance to approved or standard therapies or are inclusion or exclusion criteria for specific experimental therapies in NCI registered clinical trials.

Results. There were 38 (80%) serous, 5 (10%) endometrioid, 3 (6%) clear cell, 1 mucinous (2%) and 1 (2%) undifferentiated carcinomas. 141 GA were identified with an average of 2.9 GA (range 0–8) per tumor, of which 67 were actionable for an average of 1.4 actionable GA per patient (range 0–5). 33/48 (69%) of OC patient samples harbored at least one actionable GA. Most common GA were *TP53* (79%); *MYC* (25%); *BRCA1/2* (23%); *KRAS* (16.6%) and *NF1* (14.5%). One tumor featured an *ERBB2* point mutation. One of 3 (33%) of clear cell tumors featured *cMET* amplification validated by both FISH and IHC.

Conclusions. NGS assessment of therapy resistant OC identifies an unexpectedly high frequency of GA that could influence targeted therapy selection for the disease.

Introduction

Adenocarcinoma of the ovarian surface epithelium encompasses 90% of all ovarian malignant tumors and is the second most frequent gynecologic malignancy [1–3]. The incidence of ovarian carcinoma exceeded 22,000 new cases in the United States in 2012 and was responsible for approximately 14,000 cancer-related deaths [2,3]. Despite many attempts to develop methods and tests to detect the disease at an early stage, 85% of patients diagnosed with epithelial ovarian cancer present with advanced stage disease [1]. Although the use of radical surgery and cytotoxic chemotherapy in the last 3 decades has achieved a significant improvement in overall survival from 37% to 46% [3], ovarian cancer remains a major cause of morbidity and mortality for women both in the United States and around the world. After several decades of clinical trials in the 1990's, the

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Gynecologic Oncology Group (GOG) and other international study groups formally recommended a combination of platinum and taxane cytotoxic chemotherapy as the standard of care for epithelial ovarian cancer [4,5]. However, despite this regimen helping to improve the survival for this disease, the mortality rate for ovarian cancer patients with stages III and IV disease continues to be greater than 50% [1]. The inability to cure and, in many cases slow the progression of ovarian carcinoma has prompted investigators to search for potential new targets of systemic therapy that might improve the progression free and overall survival for the disease. In the following study, whose design contrasts with The Cancer Genome Atlas study [6] which was based on primary tumor assessment at the time of diagnosis, this comprehensive NGS-based test interrogating 182 cancer-related genes and 14 genes frequently rearranged in cancer was applied to 48 chemo-refractory relapsed/metastatic ovarian epithelial carcinomas to identify known and novel drug targets with the aim to personalize the therapy for patients with the advanced form of this life-threatening disease.

Methods

Next generation sequencing (NGS) was performed on hybridization-captured, adaptor ligation based libraries using DNA extracted from 4 FFPE sections cut at 10 μm from 26 (54%) primary OC and from 22 (46%) recurrent and metastatic tumor sites. The pathologic diagnosis of each case was confirmed on routine hematoxylin and eosin stained slides and all samples forwarded for DNA extraction contained a minimum of 20% tumor cells. Based on the submitted pathology reports that accompanied the tissue samples, of the 22 cases where a metastatic site was sequenced, 8 (36%) were obtained at the time of first surgical exploration, 10 (45%) were obtained at re-operation and 4 (18%) cases could not be established as to whether the metastatic site sample was synchronous with primary tumor surgery or obtained at a second operation or biopsy procedure. DNA sequencing was performed for 3320 exons of 182 cancer-related genes and 37 introns of 14 genes frequently rearranged in cancer (1.14 million total bps) on indexed, adaptor ligated, hybridization-captured (Agilent SureSelect custom kit) and fully sequenced using 49 bp paired reads on the Illumina HiSeq 2000 to at an average depth of 877× and evaluated for genomic alterations including base substitutions, insertions, deletions, copy number alterations (amplifications and homozygous deletions), and select gene fusions/rearrangements as previously described [7]. The bioinformatics processes used in this study included Bayesian algorithms to detect base substitutions, local assembly algorithms to detect short insertions and deletions, a comparison with process-matched normal control samples to detect gene copy number alterations and an analysis of chimeric read pairs to identify gene fusions.

Actionability classification

The genomic alterations identified were further divided into two main classes of actionability: 1) Genomic alterations that predict sensitivity or resistance to approved or standard therapies and 2) genomic alterations that are inclusion or exclusion criteria for specific experimental therapies in NCI registered clinical trials.

Results

Patients and tumors

The 48 ovarian cancer patients included in this study had a mean age at the time of genomic profiling of 55.7 years with a range of 23 to 76 years. There were a range of histologic phenotypes, with 38 (79%) papillary serous carcinomas, 5 (10%) endometrioid carcinomas, 3 (6%) clear cell carcinomas, 1 (2%) mucinous carcinoma and 1 (2%) undifferentiated carcinoma (Table 1). The majority of these tumors were high grade lesions, with only 2 (4%) FIGO grade 1 tumors, 6 (12.5%) FIGO grade II tumors and 40 (83%) FIGO grade III tumors. All of the patients had advanced stage disease at the time of genomic profiling (36 (71%) were stage III and 15 (29%) were stage IV.) In these 48 relapsed OC cases, the original primary tumor was sequenced in 26 (54%) of the cases and recurrent and metastatic tumor site biopsies were sequenced in 22 (46%) of the cases. Local site permissions to use clinical samples were used for this study.

A total of 141 genomic alterations were identified in the 48 OC with an average of 2.9 alterations per tumor (range 0–8) (Fig. 1). The most common alterations were *TP53* mutation (79% of tumors); *MYC* amplification (25% of tumors); *BRCA1/2* truncation (23%); *KRAS* mutation/amplification (16.6%) and *NF1* mutation truncating alterations (14.5%) (Supplementary Table 1). The *TP53* mutations were identified in many loci within the *TP53* gene and there was no significant recurrent locus or base substitution seen in this series of cases, as consistent with previous large-scale sequencing studies. The calculated *MYC* gene copy number gains in the 12 cases with amplifications varied from 6 to 16 with an average of copy number 9. When the alterations identified in the primary tumor specimens (which ultimately relapsed) are compared with the alterations identified in metastatic tumor samples, the findings were quite similar. Alterations in the *ARID1A* (5 patients); *PIK3CA* (4 patients) and *BRAF* (2 patients) genes were uniquely detected in the primary tumors and alterations in the *CCND2* (3 patients); *BRCA2* (3 patients); and *CCND1*; *ESR1*; *ERBB2*; *ERBB3*; and *ERBB4* (1 patient each) were restricted to the metastatic tumor samples. A larger follow up series that includes both primary and relapsed tumors from the same patient is required to determine whether these differences are biologically relevant.

Actionable genomic alterations

Sixty-seven genomic alterations identified in this series of 48 ovarian epithelial carcinomas (1.4 alterations per tumor) were potentially associated with clinical benefit of targeted therapies (Supplementary Table 2). Noteworthy genomic alterations potentially impacting the use of targeted therapy included: 11 (22.9%) tumors with either *BRCA1* or *BRCA2* mutations potentially sensitive to PARP inhibitors and DNA damaging agents. Eight of 8 (100%) of the alterations in *BRCA1* were frame shift truncations including 3 (38%) E23fs*17, 2 (25%) Q1756fs*74, 1 (13%) A17fs*14, 1 (13%) M1775fs*54 and 1 (13%) Y1522* mutation. Three (6%) of the tumors featured a *BRCA2* frame shift deletion. Of the 8 cases with *KRAS* genomic alterations potentially predicting resistance to anti-EGFR targeted therapies and sensitivity to MEK inhibitors, 6 (75%) were gene amplifications and 2 (25%) were point mutations including one G12D and one G13D mutation. Alterations in *NF1* that potentially predict responsiveness to mTOR inhibitors such as everolimus and temsirolimus included 2 nonsense mutations, 2 frame shift deletions, 1(14%) genomic truncation, 1 (14%) splice site modification mutation and 1 (14%) partial gene duplication predicted to be destructive. There were 4 (8%) tumors with two H1047R and two E545K *PIK3CA* mutations, and 1 (2%) *AKT3* mutation, also evoking the potential use of mTOR inhibitors (temsirolimus/everolimus). There was 1 (2%) tumor with a V842I *ERBB2* mutation raising potential for the use of multiple on the market anti-ERBB2 (HER2) targeted therapies and 1 (2%) tumor with a V104M *ERBB3* mutation also potentially treatable with anti-ERBB2 targeting agents. One (2%) tumor had an *ATM* mutation potentially sensitive to PARP inhibitors. In a single endometrioid case, an alteration in intron 11 leading to truncation in *PTCH1* was identified, suggesting potential treatment with a hedgehog pathway inhibitor such as vismodegib. A total of five OC harbored the amplifications of cell cycle regulatory genes including 1 case each for *CCND1*, *CCNE1* and *CDK4* and 3 cases with amplification of *CCND2*. The alterations in the cell cycle regulatory genes raise the potential for use of pazopanib, FGFR inhibitors and CDK4/6 inhibitors. Finally, 2 cases of ovarian carcinoma (one clear cell and one papillary serous) had *c-MET*

Table 1
Clinico-pathologic features and summary of genomic alterations in 51 cases of ovarian epithelial carcinoma assessed by tumor type.

	Serous carcinoma	Endometrioid carcinoma	Clear cell carcinoma	Mucinous carcinoma	Undifferentiated carcinoma
Number of cases	41	5	3	1	1
Mean age	54	57	51	38	67
Low grade cases (grades 1 and 2)	7	1	0	1	0
High grade cases (grade 3)	34	4	3	0	1
Stage III cases	30	1	3	1	1
Stage IV cases	11	4	0	0	0
Mean number of genomic alterations	3.5	2.4	5.7	5	2
Mean number of actionable genomic alterations	0.6	0.8	3.0	2	0
Mean number of available clinical trials	5.1	2.0	9.0	10.0	4
Most frequently altered genes	*TP53* *MYCC* *NF1* *KRAS* *BRCA1*	*KRAS* *TP53*	*MET*	*AURKA* *NOTCH1* *FGF1R*	*BRCA2* *MYCC*

amplification raising the potential for responsiveness to MET inhibitors including crizotinib.

Genomic alterations and pathologic characterizations

Although *TP53* mutations were seen in 79% of OC, these mutations were more common in papillary serous carcinomas (83%) than in non-papillary serous tumors (50%). Only 40% of the endometrioid and 33% of the clear cell carcinomas featured *TP53* mutations. Three of 5 (60%) of *ARID1A* mutations also occurred in non-serous tumors. One of 3 (33%) of CC featured a copy number gain in the *MET* gene, which was a high copy number that was also validated by both FISH and IHC (Fig. 2). The number of genomic alterations and the histologic grade of the ovarian epithelial carcinoma were positively correlated, with 8 grades 1 and 2 tumors averaging 2.1 genomic alterations per tumor and 40 grade 3 tumors averaging 3.0 alterations per tumor ($p = 0.0003$). In contrast, there was no apparent correlation between the tumor stage and the number of genomic abnormalities in this series of ovarian carcinomas with the stage III tumors averaging 3.0 alterations per tumor and the stage IV tumors averaging 2.8 alterations per tumor.

Discussion

The complexity of genomic alterations found in ovarian epithelial carcinomas was initially elucidated by traditional cytogenetic and DNA sequencing techniques, [8]. The large numbers of chromosomal abnormalities found by cytogenetic and comparative genomic hybridization studies was difficult to decipher, suggesting the need for a technology platform such as next generation sequencing that could

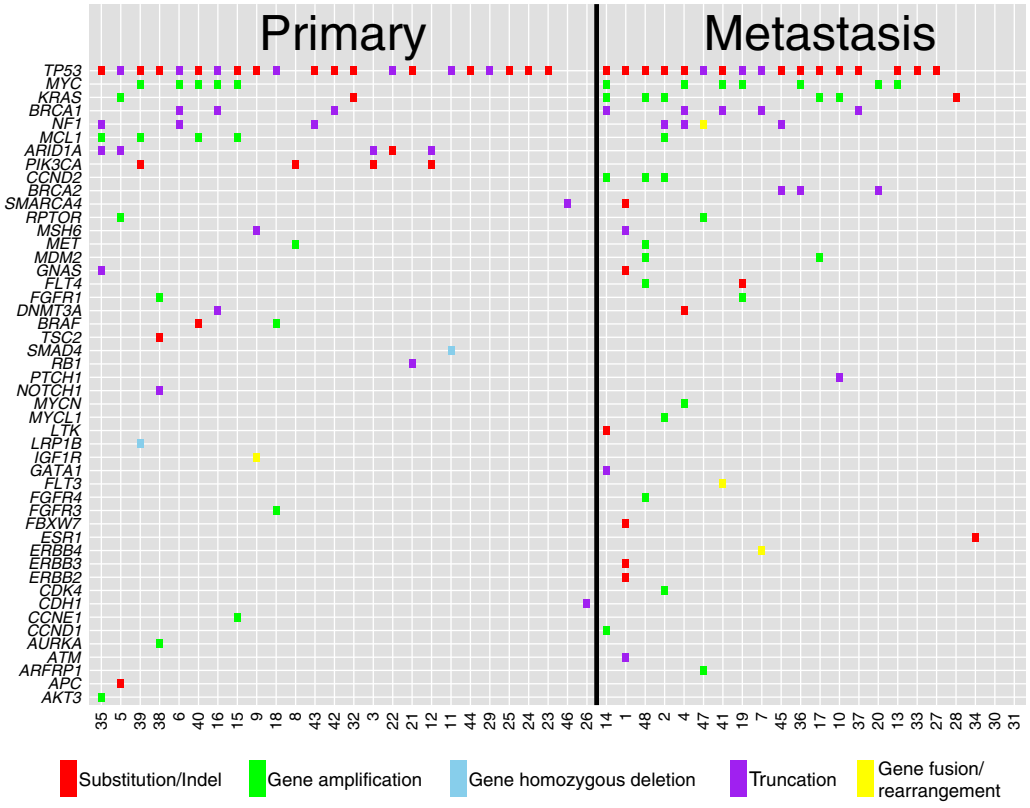

Fig. 1. Tile plot of genomic alterations in 48 cases of ovarian epithelial malignancy separated into "Primary" when a primary tumor sample was sequenced and "Metastatic" when a metastatic tumor sample was sequenced.

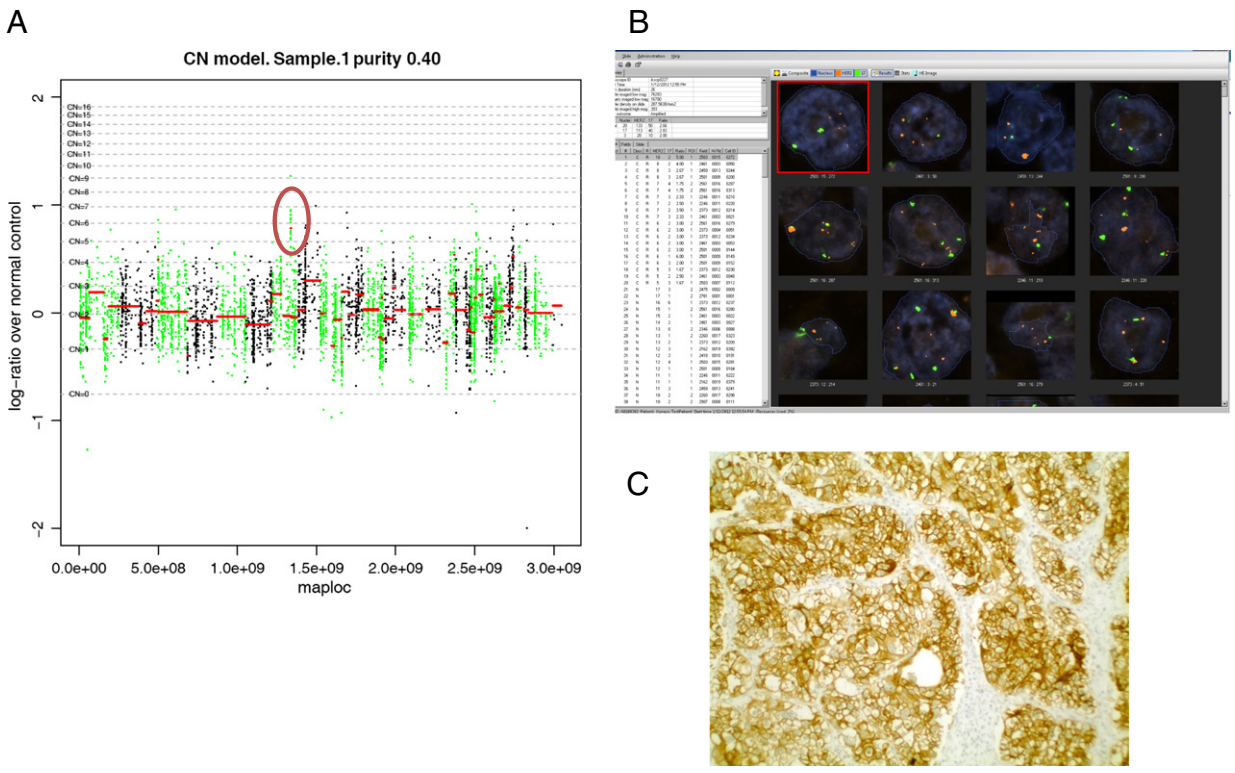

Fig. 2. *MET* amplification in clear cell ovarian carcinoma. The panel on the left (A) shows the NGS gene copy number model in the region of chromosome 7. The red circle indicates an increase in gene copy number to 6 copies at the cMET gene locus measured in a background of DNA estimated to be comprised of 40% tumor cell derived DNA. The panel to the upper right (B) is the digital image of the fluorescence in situ hybridization analysis of the same tumor using cMET gene copy and chromosome 7 centromere region probes (Abbott-Vysis, Inc., Downers Grove, IL.) using the Ikoniscope Digital Fluorescence Image Analysis System (Ikonisys, Inc., New Haven, CT.). The cMET copy number detected by FISH in the same tumor was 6.6 copies/nucleus. The panel to the lower right (C) is the immunohistochemical staining of the same tumor using the Ventana Inform anti-cMET antibody (Ventana Medical Systems, Inc., Tucson, AZ.) showing uniform membranous staining in 100% of the tumor cells (IHC 3+; H score 300).

evaluate multiple genes for genomic alteration in a single tumor with high accuracy [9]. Of particular importance was the critical advantage of next generation sequencing is the capability to simultaneously characterize all classes of genomic alterations (base substitutions, short insertions and deletions, fusions/translocations and copy number alterations including both homozygous deletions and amplifications) in a single assay. In contrast, the Sanger sequencing methods initially used are much less sensitive in detecting the former three alteration classes [9]. The comprehensive NGS-based genomic profiling assay used in the present study was capable of detecting all four classes of genomic alterations in routine formalin fixed paraffin embedded at high sensitivity and specificity based on high degree of sequencing depth and uniformity averaging greater than 800 fold with >99% exons covered at least 100 fold, and provided an in depth evaluation of such alterations in individual ovarian carcinomas. Given that fresh or frozen samples were not available for this study, DNA extraction methodologies were optimized for traditional FFPE samples [7]. The results in the current study confirmed the predominant finding of previous studies demonstrating a high frequency of *TP53* mutations in ovarian carcinoma [10–12], although such mutations are not known to sensitize cancer cells to current therapies, whether cytotoxic or targeted. Specifically, alterations detected by NGS-based genomic profiling in this study correlate extensively with the results of the Cancer Genome Atlas project which employed focused DNA sequencing and, DNA copy number assessment along with assays for mRNA, microRNA and DNA promoter methylation status assessment [6]. Using a PCR-based sequencing method, *TP53* mutations are found at a high frequency, especially in high grade serous carcinomas where they are present in greater than 90% of tumors thus negating their potential prognostic significance for this disease [10]. In the current NGS-based study, *TP53* mutations were present in greater than 85% of high grade serous carcinomas. In the National Cancer Genome Atlas study, the DNA sequences of exons from coding genes in 316 high grade serous carcinomas were determined and *TP53* was mutated in 96% of the tumors [9]. In contrast, the *NF1* mutation rate in the current series is significantly higher than in the previous studies [9] and COSMIC database. In the current NGS-based study, there were 7 (14% of cases) tumors with *NF1* mutations exclusively found in tumors that also were positive for *TP53* mutation. This association of *NF1* mutation with *TP53* mutation in ovarian cancer has been previously reported [13]. Although this pairing may suggest a possible deployment of targeted therapy for such *NF1* mutant tumors as in vitro work has demonstrated the sensitivity of *NF1* null cells to mTOR inhibitors, definitive proof awaits a well-designed clinical study with appropriate molecular diagnostics gating entry of an appropriately population [14].

Other alterations that suggest a possible deployment of targeted therapy included alterations in the PI3K/mTOR pathway and in members of the EGFR family. Beyond the previously mentioned *NF1* alterations, the 8% of OC with *PIK3CA* mutation also highlights potential clinical utility for inhibitors of the mTOR/PI3K pathway. In particular, recent findings in breast cancer have suggested a possible synergy for ovarian cancer patients between PI3K inhibitors and poly-adenylate ribose polymerase (PARP) inhibitors [15]. There are ongoing clinical studies in both breast and ovarian carcinoma to assess the efficacy of this combination in patients. Would mutations in *NF1* or *PIK3CA* potentiate the effects of this therapeutic combination? Such studies are gated solely on tumor type, but could benefit from molecular assessments such as in this study.

Although alterations in the *ERBB2* (*HER2*) gene and overexpression of HER2 protein have driven scientific interest and launched clinical

trials of anti-HER2 targeted therapy for ovarian epithelial carcinomas, the current study of 48 tumors was noteworthy for the paucity of *ERBB2* alterations, as there were no cases with *ERBB2* copy number gain (≥6 copies) despite copy gain being identified in many cell cycle regulatory genes and *cMET*. The incidence of *ERBB2* amplification and/or HER2 protein expression in ovarian epithelial carcinoma has an extremely wide estimated range of 1.8% to 76% in the published literature likely reflecting the lack of standardization on interpretation of the slide based assays [16]. The lack of an approved HER2-targeted therapy for the disease may reflect this difficulty in identifying the correct patients for treatment in clinical trials. However, a single (2%) case featured an *ERBB2* mutation, and such mutations have been previously detected in ovarian carcinomas. The V842I *ERBB2* mutation in this study was identified in a patient with an advanced clear cell carcinoma of the ovary within the kinase domain of the HER2 receptor (amino acids 720 − 987) (UniProt.org, Jul 2012). In general, such kinase domain mutations in ERBB2 have been described as activating [17]. The V842I mutation may therefore be an activating mutation although this has not been directly demonstrated in functional assays. Finally, overexpression of HER2 in ovarian cancer is potentially associated with a poor prognosis, but additional studies to formally demonstrate this are required [18].

Interestingly, the current study identified only 1 tumor with a *BRAF* mutation and 1 tumor with a*BRAF* amplification (case 40). *BRAF* mutation has been linked to favorable prognosis in one study of ovarian cancer [19], although that mutation was the most common V600E *BRAF* mutation and not the G469R mutation found in a high grade serous carcinoma in the current series. *BRAF* mutations, predominantly V600E, have been reported in 10–35% of ovarian cancers [19], and such mutations are seen more commonly in low grade ovarian tumors rather than in high grade tumors such as the *BRAF* mutated case in the current series [20]. The G469R *BRAF* mutation is located in the well-characterized G loop domain of Braf and has not been functionally characterized. Although BRAF inhibitors are effective in the in vitro context of BRAF-activating mutations, their use can be paradoxically detrimental by inducing activation of the downstream MAPK kinase pathway [21,22] and further supporting oncogenic signaling. The *BRAF* G469R mutation may predict sensitivity to MEK pathway inhibitors, which have not been approved for ovarian cancer, but are under investigation in multiple clinical trials in ovarian and other solid tumors.

BRCA1/2 mutations are known to confer some sensitivity to DNA damaging agents, such platinum, or possibly agents that inhibit DNA repair pathways, i.e. PARP inhibitors. In the present study, 8 (18%) of tumors had a *BRCA1* mutation and 3 (6%) had a *BRCA2* mutation. All 8 (100%) of the ovarian tumors that featured *BRCA1* mutations were serous carcinomas and 7 (88%) had a high histologic grade. Two of the three tumors with *BRCA2* mutations were both high grade serous carcinomas with stage IV relapses beyond the abdominal cavity. The National Genome Atlas study also identified somatic *BRCA1* and *BRCA2* mutations as a significant feature of high grade serous ovarian carcinoma [9].

One (33%) of the ovarian clear cell carcinomas in this study featured copy number increase of the *MET* gene with >6 MET copies per cell was confirmed by both FISH and immunohistochemical assessment of the MET protein expression level. *MET* amplification has emerged as a potential biomarker of the clear cell tumor subtype [23]. Thus, ovarian carcinoma patients with *MET* amplifications may be candidates for referrals to registered clinical trials designed to test the efficacy of MET inhibitors in this disease. The ALK-targeted therapeutic used in non-small cell lung cancer, crizotinib, was originally developed as a MET inhibitor, and correspondingly appears to be also effective in malignancies with high level *MET* amplification [24].

A wide variety of other genomic alterations were identified in this study that is not currently targetable by either commercially available therapies or therapies being tested in registered clinical trials. Amplification of the *MYC* gene was the second most frequent genomic alteration identified at 25% incidence. This frequency is comparable to previous studies that used fluorescence in situ hybridization to detect *MYC* copy number [25]. *MYC* amplification has not been linked with clinico-pathologic features, prognosis or response to cytotoxic therapy in ovarian carcinomas [25]. The 16.6% incidence of *KRAS* genomic alterations is similar to previous reports [26], but the current study featured 6 tumors with *KRAS* amplification and only 2 tumors, 1 serous and 1 endometrioid, with *KRAS* point mutations. Amplification of the *KRAS* gene in ovarian carcinomas has not been widely reported, and the prognostic and therapeutic implications are of yet unknown. The absence of a *KRAS* mutation in the one mucinous carcinoma included in the current study is of some interest given the recent report that *KRAS* wild type mucinous ovarian carcinomas may be sensitive to anti-EGFR antibody-based therapy with cetuximab [27]. *ARID1A* alterations were identified in 5 (10%) of the ovarian carcinoma cases. *ARID1A* encodes a protein which is a member of the SWI/SNF chromatin remodeling complex and is believed to function as a tumor suppressor [28,29]. *ARID1A* mutations have been previously reported in 29% of ovarian tumors, with the highest prevalence in clear cell carcinomas (up to 57%) [29,30]. In the current study only 1 (33%) of the clear cell carcinomas featured an *ARID1A* mutation. Loss of ARID1A expression in ovarian clear cell carcinoma has been correlated with advanced tumor stage, and also with chemoresistance to platinum-based therapy [31]. However, there are no current therapies that target the inactivation of ARID1A. In this study, the next generation sequencing of 48 relapsed ovarian carcinomas generated potential entry into a total of 267 NCI-registered clinical trials of established and experimental targeted anti-cancer drugs. The number of available clinical trials of targeted agent available averaged 5.2 per patient.

A number of recent reviews have described the growing interest in the development of a targeted approach to the treatment of relapsed and refractory ovarian carcinoma [32–37]. The overwhelming consensus of these studies is that identification of therapeutic vulnerabilities such as genomic alterations are critical to the development of a clinical targeted approach to treatment of this disease. Furthermore, a recently published study using the same method as in the current study confirmed that, for non-small cell lung cancer, the genomic alterations identified in primary tumors are maintained in patient matched metastatic lesions [38]. The current study demonstrates that deep sequencing of hundreds of cancer-related gene from DNA extracted from clinical grade tumor samples of conventional therapy resistant ovarian carcinomas by NGS uncovers an unexpectedly high frequency actionable genomic events that may inform targeted treatment decisions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2013.06.019.

Conflict of interest statement

Authors JS Ross, SM Ali, K Wang, G Palmer, R Yelensky, D Lipson, VA Miller are employees of and own stock in Foundation Medicine, Inc.

Authors D Zajchowski, LK Shawver have no conflicts of interest to disclose.

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