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Genes associated with bowel metastases in ovarian cancer

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HIGHLIGHTS

- We identified 21 genes overexpressed in ovarian cancer (OC) bowel metastases compared to primary tumors.
- High expression of these genes in primary OCs is associated with a need of complex bowel surgery and poor prognosis.
- These genes may help identify potential therapeutic targets of malignant bowel obstruction in OC.

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ABSTRACT

Objective. This study is designed to identify genes and pathways that could promote metastasis to the bowel in high-grade serous ovarian cancer (OC) and evaluate their associations with clinical outcomes.

Methods. We performed RNA sequencing of OC primary tumors (PTs) and their corresponding bowel metastases (n = 21 discovery set; n = 18 replication set). Differentially expressed genes (DEGs) were those expressed at least 2-fold higher in bowel metastases (BMets) than PTs in at least 30% of patients ($P < .05$) with no increased expression in paired benign bowel tissue and were validated with quantitative reverse transcription PCR. Using an independent OC cohort (n = 333), associations between DEGs in PTs and surgical and clinical outcomes were performed. Immunohistochemistry and mouse xenograft studies were performed to confirm the role of LRRC15 in promoting metastasis.

Results. Among 27 DEGs in the discovery set, 21 were confirmed in the replication set: *SFRP2*, *Col11A1*, *LRRC15*, *ADAM12*, *ADAMTS12*, *MFAP5*, *LUM*, *PLPP4*, *FAP*, *POSTN*, *GRP*, *MMP11*, *MMP13*, *C1QTNF3*, *EPYC*, *DIO2*, *KCNA1*, *NETO1*, *NTM*, *MYH13*, and *PVALB*. Higher expression of more than half of the genes in the PT was associated with an increased requirement for bowel resection at primary surgery and an inability to achieve complete cytoreduction. Increased expression of *LRRC15* in BMets was confirmed by immunohistochemistry and knockdown of LRRC15 significantly inhibited tumor progression in mice.

Conclusions. We identified 21 genes that are overexpressed in bowel metastases among patients with OC. Our findings will help select potential molecular targets for the prevention and treatment of malignant bowel obstruction in OC.

Abbreviations: BMets, bowel metastases; DEGs, differentially expressed genes; ECM, extracellular matrix; IHC, immunohistochemistry; sh-LRRC15, *LRRC15* shRNA; sh-NTC, nontargeted control shRNA; OC, ovarian cancer; PTs, primary tumors; qRT-PCR, quantitative reverse transcription PCR; RNAseq, RNA sequencing; TBST, TBS with 0.025%Triton X-100.

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1. Introduction

Ovarian cancer (OC) is lethal and spreads to the abdominal cavity and peritoneum early in its natural history. The most common sites of intra-abdominal metastases are the omentum and peritoneum [1]. Although somewhat less common at initial diagnosis, metastases to the intestinal wall, which can lead to impaired bowel function and obstruction, are a major cause of poor quality of life in patients with OC [2]. Specifically, this intramural invasion can be associated with progressive fibrosis and obliteration of the intestinal wall involving a substantial portion of the gastrointestinal tract. Given this specific phenotype in OC and its high clinical impact, a better understanding of the genes that promote metastasis of cancer cells to the bowel could potentially lead to novel treatment options to improve quality of life and survival for women with OC.

Previous studies of gene expression in metastatic OC have focused extensively on omental metastases [3–7]. Although these studies have provided some information on the biology of metastasis, the omentum (like the primary tumor) is usually removed almost entirely at the time of the primary surgery. Thus, omental metastases are not a common cause of morbidity and death in OC. In contrast, bowel metastases (BMets), which most likely represent growth of occult microscopic disease on the bowel serosa and peritoneum, contribute to bowel obstruction and death, even in patients who undergo optimal surgical debulking [8,9]. These considerations highlight the potential importance of studying BMets.

Although differentially expressed genes (DEGs) associated with metastasis to the omentum have been reported [3–7], currently there are no reports of genes associated with OC metastasis to the bowel. Identified genes and pathways associated with omental metastasis are involved in collagen remodeling, adhesion, invasion, and proteolytic and immune-modulating pathways [10]. It is not known whether the DEGs identified in omental metastasis studies are also aberrantly expressed in tumors that metastasize to the bowel.

To identify genes that are overexpressed in BMets compared with their matched primary tumors (PTs), we performed RNA sequencing (RNAseq) of PTs and their corresponding BMets from 21 patients with high-grade serous ovarian, fallopian tube, and primary peritoneal cancer. Genes identified by RNAseq were validated with quantitative reverse transcription PCR (qRT-PCR) in a technical cohort and in an independent replication PT cohort. We also performed a secondary analysis of an independent set of PTs to identify whether the BMet-upregulated DEGs were correlated with the need for bowel resection. The genes identified may serve as important candidate targets for preventing or mitigating BMet.

2. Methods

2.1. Population and sample selection

From our patient database, we identified patients who underwent primary cytoreductive surgery for advanced-stage OC in the Department of Obstetrics and Gynecology at Mayo Clinic, Rochester, Minnesota, between August 1, 2010, and April 30, 2012. We further identified patients with stage III–IV high-grade serous epithelial ovarian, fallopian tube, or primary peritoneal cancer who had documented BMet and tissue available for analysis from both the primary and the metastatic site. One cohort of patients was used for identifying genes (*discovery set*) and another cohort was used for replicating results (*replication set*). The presence of BMets was defined as gross cancer (≥2 cm) deeply involving the wall of the small or large bowel, causing symptoms of intestinal obstruction or impairment of bowel function, and requiring major bowel surgery for complete removal. All patients underwent systematic surgical staging, and none received chemotherapy before surgery. Fresh frozen samples were reviewed by a pathologist (D.W.V.), who confirmed the presence of at least 70% tumor content. The histologic subtype and grade of the tumors were evaluated according to World Health Organization criteria [11,12]. Among the discovery set (n = 21), 4 patients also had samples of "normal" bowel tissue, which was confirmed by a pathologist (D.W.V.).

An independent set of PTs included 333 previously studied Mayo Clinic patients with stage III/IV high-grade serous OC [13]. Four patients of the replication set (n = 18) are also represented in this independent set. Debulking status was categorized as 0 cm of residual disease at the end of surgery (RD0, *complete cytoreduction*) vs >0 cm residual disease at the end of surgery (non-RD0). Of the 333 patients, a subset of 322 patients had complete data on surgical debulking status (100 RD0; 222 non-RD0), and a subset of 317 patients had complete data on bowel resection procedures (99 resected; 218 not resected). Details of the study populations are given in Table S1 and summarized in Fig. 1A. The study was approved by the Mayo Clinic Institutional Review Board.

2.2. Tissue preparation, mRNA analysis, and RNAseq data processing

Tissue from patients in the discovery and replication sets was obtained as follows: Samples varying from 0.5 to 3 cm collected intraoperatively were snap-frozen in liquid nitrogen within 30 min of removal. From each sample, hematoxylin-eosin-stained slides were obtained at the beginning and at the end of cutting sections. All tumors were between 5 and 10 mm in diameter, and 4 10-μm sections of each sample were used for RNA extraction. The TruSeq method and TruSeq RNA Sample Prep Kit v2 (Illumina) were used to isolate total RNA, followed by preparation of RNA libraries according to the manufacturer's instructions. RNA loading, sequencing of the paired-end reads, and final base-calling were performed as previously described [14]. The RNAseq data were analyzed with MAP-RSeq v.1.2.1 [15] in the Mayo Clinic Bioinformatics Core pipeline. MAP-RSeq consists of alignment with TopHat2 [16] and resulting mRNA expression quantifications. We analyzed 23,398 genes, excluding 49 genes mapping to multiple chromosomes and 922 with no transcript counts for any sample. The remaining 22,427 genes were distributed across all the chromosomes. The quality control of the raw counts was assessed using box plots, mean vs average residual plots, counts vs gene length, counts vs GC content, and total count plots. No samples were excluded after the quality control of the raw counts. All samples were normalized with CQN [17], which adjusts for library size, gene length, and GC content. The normalized count data were reviewed for quality control in the same manner as the raw counts. Again, no suspicious samples were found after review.

2.3. Differential expression analysis and metastatic gene selection

The discovery set consisted of 21 patients with available samples of paired BMets and PTs and an additional 18 unmatched primary tumors. RNAseq data from all the samples were analyzed to choose genes as follows: 1) the gene was upregulated at least 2-fold in the BMets relative to PTs in at least 30% of patients (Fig. 1A) and 2) using the RNAseq data from the 4 patients with paired PT and benign bowel, the selected genes were either downregulated in the benign bowel, or the upregulation of the specific gene in the benign bowel was significantly different than in the PT. That is, genes with expression that was significantly upregulated in 4 benign bowel tissues were excluded.

2.4. Survival analysis and association with bowel resection and debulking outcomes

For BMet-upregulated genes identified by RNAseq analyses of the discovery and replication sets, an independent secondary analysis was performed using Cox regression to evaluate associations between gene expression and overall survival, for all the patients (n = 333), patients with no macroscopic disease after surgery (i.e., RD0), and patients with visible disease after surgery (i.e. non-RD0). Student *t*-tests were used to evaluate the associations between gene expression and

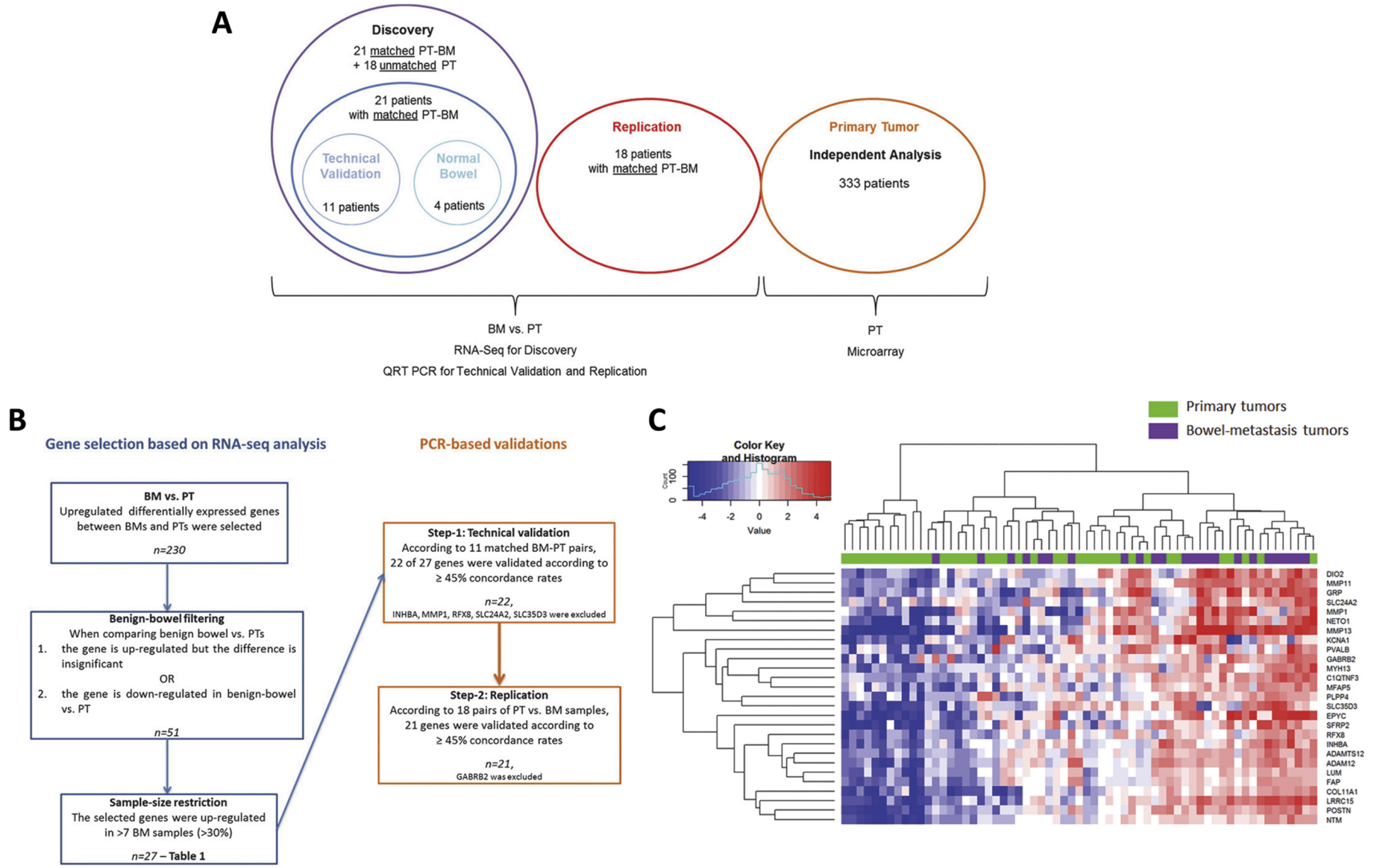


Fig. 1. Summary of patient populations used and the gene selection and validation process. A, Population cohorts of the study. In the discovery set, we identified 27 genes upregulated in BMets compared with PTs using whole-transcriptome RNAseq. Only genes that were not significantly upregulated in normal bowel compared with PT were included (normal bowel set). Expression of 22 of the 27 upregulated genes was verified in the technical validation set using a different approach (qRT-PCR). qRT-PCR was used to validate the genes in an independent set of 18 matched PTs-BMets (replication set); 21 of the 22 tested genes were validated. To investigate the importance of these upregulated genes, we evaluated both their gene expression in 333 PTs and the association between gene expression and surgical outcomes. B, Overall scheme for identifying and validating BMet-upregulated genes. C, Standardized RNAseq expression heat map of 27 genes selected for PCR validation.

debulking status. For patients with complete bowel resection information, *t*-tests were used to evaluate whether expression levels of each gene were associated with bowel resection status. R statistical software was used for analysis, and $P < .05$ was considered significant.

2.5. Ingenuity pathway analysis

To gain additional biological insight, genes differentially expressed between BMets and PTs were investigated using Ingenuity Pathway Analysis (Qiagen) for network enrichment analysis, according to canonical pathways and biological processes.

2.6. Immunohistochemistry

Immunohistochemistry (IHC) was carried out essentially as described previously [18]. Briefly, sections were deparaffinized in xylene, rehydrated through a graded alcohol series, and washed in TBS with 0.025% Triton X-100 (TBST). TBST was used for all subsequent washes. Tissue sections of matched PTs and BMets from 16 patients (10 from the discovery set and 6 additional pairs (4 from the replication set and 2 new samples used in neither the discovery or the replication set)) were quenched in 3% H_2O_2 and blocked with TBST-1% bovine serum albumin with 10% horse serum for 1 h at room temperature. Slides were then incubated at 4 °C overnight with rabbit polyclonal immune serum raised against LRRC15 (Abcam; Ab157484) at a 1:50 dilution, followed by 1:200 anti-rabbit biotinylated antibody (Vector Laboratories) for 1 h. All slides then were processed by the ABC method (Vector Laboratories) for 30 min at room temperature. DAB Peroxidase (Vector Laboratories) was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Slides were scanned at ×40 magnification on the Aperio ScanScope AT Turbo brightfield instrument (Leica Biosystems) at a resolution of 0.25 μm/pixel. The expression level of LRRC15 was scored for intensity and compared in different specimens by 3 separate observers (V.P.P., V.S., and J.K.S.) in a double-blind fashion and described as absent (0), very low to low [1], moderate [2], or high [3]. Noticeable quantitative variations in LRRC15 expression (staining intensity) were observed, with less variation in the total number of stained cells.

2.7. Pathology scoring

Archived paraffin sections of the PTs and BMets from the discovery set were evaluated by a pathologist (D.W.V.), and each section was graded (0–3) for the degree of matrix deposition, inflammation, and fibroblast proliferation, described in Table S4. A score of 0 corresponds to the absence of inflammation, matrix deposition, or fibroblast proliferation.

2.8. Tumor xenograft experiments

All experimental use of animals conformed with the guidelines of the Mayo Clinic Institutional Animal Care and Use Committee. Animals were 4- to 6-week-old female athymic nude mice (nu/nu strain) (NCr *nu/nu*; Animal Production Area, National Cancer Institute, Frederick Cancer Research and Development Center); the mice were handled as stated in the Guide for the Care and Use of Laboratory Animals. For analysis of in vivo tumor growth, 5×10^6 OVCAR5 transfectants stably expressing sh-NTC or sh-LRRC15 were injected intraperitoneally (10 mice/group). When OVCAR5 tumors were palpable, control sh-NTC-bearing mice were humanely euthanized on day 27, and sh-LRRC15-bearing mice were euthanized on day 39.

Supplemental Methods: The methods describing qRT-PCR, Gene Expression by High-Throughput qRT-PCR, Western Immunoblot Analysis and Generation of LRRC15-Downregulated Stable Clones.

3. Results

3.1. Gene expression analysis

Results of RNAseq of the PT and paired BMets from 21 patients revealed 230 upregulated DEGs that showed a >2-fold change and a false discovery rate <0.2. Among these 230 genes, we chose the top 27 genes for further validation and analysis on the basis of the criteria stated in the Methods section (Fig. 1B). Fig. 1C shows the heat map of these genes. Table 1 shows the fold change in expression in the BMets compared with the PTs for each gene and level of expression in the benign bowel vs PT in the discovery set.

3.2. Validation of gene expression by qRT-PCR

To verify the RNAseq results, we assessed the expression of the 27 BMet-upregulated genes in 11 matched PTs and BMets from the discovery set by qRT-PCR on the high-throughput platform. If the direction of fold change (up or down) in a specific sample was the same, then these samples were considered concordant for that specific gene and indicated as % concordance (technical validation) (Table 1). We further applied a more stringent criterion that at least a 2-fold change assessed by this method for any specific gene should be present in at least 45% of the BMets, leaving 22 of the 27 genes for testing in the replication cohort.

In a replication study, high-throughput qRT-PCR was performed on an independent set of paired PTs and BMets from 18 additional patients (replication set). In this group, expression of 21 of the 22 tested genes was again upregulated at least 2-fold in BMets relative to PTs in at least 45% of the tumors (Table 2). According to Ingenuity Pathway Analysis, the 2 most highly enriched functional networks were centered around extracellular matrix (ECM) and collagen (Fig. S1).

3.3. Pathologic and clinical associations of genes upregulated in the BMets

Examination of the correlation between expression of these 21 genes and histologic quantitation of fibroblast proliferation, fibrosis, and inflammation in the discovery set showed that 2 genes, *ADAM12* and *FAP*, had significant positive correlations with increased fibroblast proliferation scores (Fig. S1; Table S5).

To investigate the importance of high expression of these DEGs, we evaluated the association between gene expression in the PTs and surgical outcomes in an independent set of 333 previously described patients with OC [13]. Of the genes upregulated in BMets, 14 had significantly higher expression in PTs of patients who required bowel resection compared with those who did not ($P < .05$) (Fig. 2; Table S6). In addition, 19 of the 21 genes were significantly associated with the success of surgical resection; expression of these genes was associated with a lower likelihood of achieving complete cytoreduction (RD0 vs non-RD0 debulking) (Fig. 2; Table S6). Seven of the genes were significantly associated with poor survival ($P < .05$) on univariate analysis: *DIO2*, *EPYC*, *FAP*, *LUM*, *MMP11*, *PVALB*, and *SFRP2* (Table S7). After selecting patients with RD0 debulking, however, only the *EPYC* gene remained significantly associated with poor survival (Fig. S3). In contrast, among patients with non-RD0 debulking, no genes were significantly associated with survival (Table S7).

3.4. Validation of protein expression of selected BMet-associated proteins by immunoblot analysis

We assessed the expression of ADAM12, Col11A1, EPYC, FAP, LRRC15, Lumican, Periostin, and SFRP2 at the protein level by Western blot analysis in 15 matched PTs and BMets. Of the 8 proteins analyzed, the leucine-rich repeat-containing protein LRRC15 showed the highest frequency of upregulation in the BMets compared with their matched PTs (13 of 15) (Fig. 3A–C).

Table 1
RNAseq results for 27 identified candidate BMet-upregulated genes[a].

Upregulated genes in BMet vs PT	Unmatched analysis	Matched analysis			
		Discovery set			Technical validation set
	RNAseq based	RNAseq based			High-throughput analysis
	P value[b]	BMet-PT pairs with > 2-fold change, %	Fold-change, benign bowel vs PT	*P* value	Bowel concordance with RNAseq, %[c]
SFRP2	0.00049	62	1.46	0.32	82
ADAM12	0.00022	43	−2.77	0.01	73
ADAMTS12	0.00012	38	−1.34	0.10	73
Col11A1	0.00163	57	−5.21	<0.001	73
LRRC15	0.00054	62	−0.75	0.59	73
GABRB2	0.00837	48	0.03	0.97	64
LUM	0.00029	38	−0.02	0.98	64
MFAP5	0.00449	48	1.82	0.06	64
GRP	0.00773	62	1.14	0.29	60
FAP	0.00031	48	−1.92	0.04	55
KCNA1	0.00705	57	2.41	0.052	55
NETO1	0.00749	57	−0.62	0.62	55
PLPP4	0.00318	43	−1.81	0.07	55
C1QTNF3	0.00056	43	0.5	0.54	45
DIO2	0.00778	52	−0.35	0.73	45
EPYC	2.82e−06	81	−5.26	<0.001	45
MMP11	0.00036	71	−3.98	<0.001	45
MMP13	0.00067	76	−4.56	0.02	45
MYH13	0.00162	43	−0.15	0.87	45
NTM	7.45e−05	57	1.39	0.19	45
POSTN	2.37e−05	76	0.92	0.49	45
PVALB	0.0342	38	−2.58	0.02	45
SLC35D3	0.00305	52	1.99	0.07	38
INHBA	0.00018	52	−2.23	0.03	36
MMP1	0.0149	52	−1.33	0.31	36
RFX8	0.00169	43	−1.98	0.03	36
SLC24A2	0.00814	52	−0.2	0.79	18

Abbreviations: BMets, bowel metastases; PTs, primary tumors; RNAseq, RNA sequencing.
[a] For RNAseq differential results, $\log_2$-based expression fold-changes and *P* values are shown for BMet vs PT, and 4 pairs of benign bowel samples vs PTs, respectively.
[b] For the change between 24 BMets vs 39 PTs.
[c] 11 Matched tumor sets.

Table 2
Genes with 2-fold upregulation in BMets vs PT in at least 45% of the tumors[a].

Upregulated genes in BMet vs PT	Replication set
	High-throughput analysis
	Tumors with 2-fold upregulation, %[b]
SFRP2	67
ADAM12	67
ADAMTS12	67
Col11A1	55
LRRC15	55
LUM	50
MFAP5	50
GRP	61
FAP	50
KCNA1	55
NETO1	50
PLPP4	55
C1QTNF3	61
DIO2	55
EPYC	67
MMP11	55
MMP13	67
MYH13	50
NTM	50
POSTN	61
PVALB	61

Abbreviations: BMets, bowel metastases; PT, primary tumors.
[a] 21 differentially expressed genes in BMets vs PTs, validated in the replication set. *GABRB2* was excluded (44% upregulation).
[b] 18 matched tumor sets.

3.5. IHC of LRRC15 in 16 sets of matched PTs and BMets

To examine the expression of LRRC15, we selected pairs of samples (10 from the technical validation set, 4 from the replication set, and 2 new samples, OV1548 and OV7110) for staining with anti-LRRC15 antibody. Higher LRRC15 expression was seen in BMets than PTs in 87.5% (14/16) of the matched samples (Fig. 3D and E); only 2 BMet samples (OV4243 and OV8990) showed lower levels than their matched PTs.

3.6. LRRC15 knockdown inhibits tumorigenesis in vivo

The effect of *LRRC15* knockdown on primary tumor growth and metastasis was evaluated in nude mice bearing intraperitoneal OVCAR5 xenografts stably expressing sh-NTC or sh-LRRC15. Sustained downregulation of LRRC15 was confirmed by immunoblotting (Fig. 4A). The sh-NTC-bearing cells formed extensive peritoneal nodules and metastasis to the ovary, bowel, and other organs; all the mice had to be euthanized on day 27 (Fig. 4B). In contrast, sh-LRRC15-bearing xenografts had no visible peritoneal or metastatic nodules on any of the organs through day 39 when they were euthanized (Fig. 4C). The average tumor weight and ascites volume were significantly lower in sh-LRRC15 than sh-NTC xenografts (Fig. 4D and E). No significant difference in body weight loss was observed between the 2 groups (data not shown).

4. Discussion

BMets are the major cause of morbidity and death in OC [19,20]. In the current study we identified and validated 21 genes that are

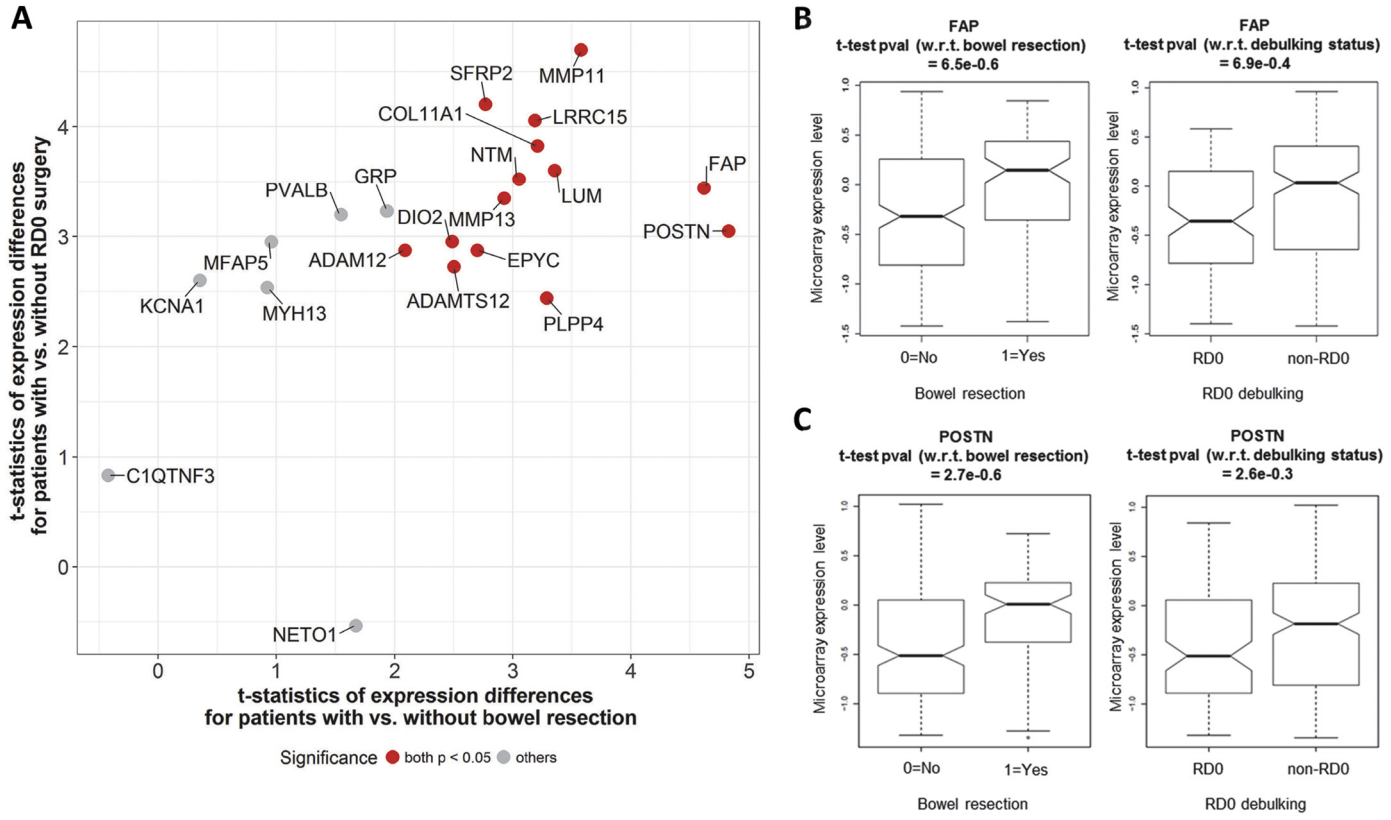


Fig. 2. Correlation between gene expression and need for bowel resection in the "primary tumor" ovarian cancer population. A, Scatterplot of *t*-statistics evaluating expression differences for patients with vs without bowel resection against patients with vs without RD0 debulking status. Genes with significantly different expression ($P < .05$) in both comparisons are red. B and C, Box plots showing differences in expression of *FAP* (B) and *POSTN* (C) between patients with and without bowel resection and RD0 debulking status (all *t*-test $P < .001$).

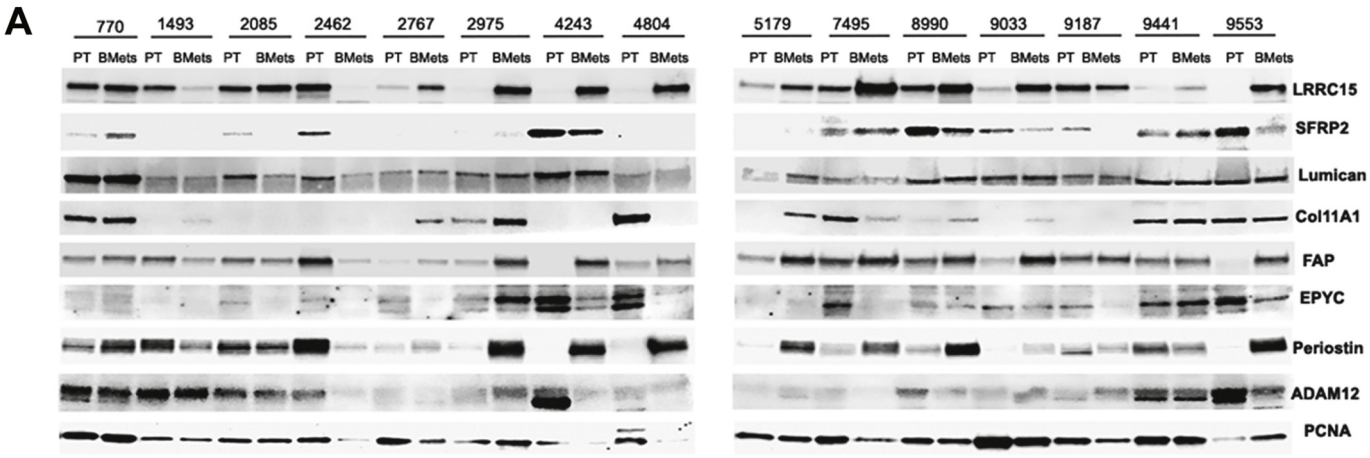
differentially upregulated in BMets relative to PTs in advanced high-grade serous OC. These genes are likely to be involved in promoting intestinal metastases in OC and may encode targets for future novel therapeutic approaches aimed at preventing bowel obstruction and death. As additional validation of our findings, we observed that overexpression of some of these genes in BMets is associated with increased fibroblast proliferation and that high expression in the PT is associated with suboptimal debulking and requirement for complex bowel surgery.

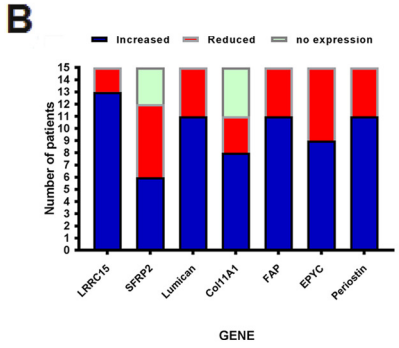
Several DEGs have been shown to be associated with metastasis to the omentum in OC. Using expression microarrays, Lancaster et al. [6] identified 56 DEGs between PTs and omental metastatic deposits from the same patients, whereas Malek et al. [7] and Brodsky et al. [4] described genes with prognostic significance. There was no overlap in the genes identified in the 3 reports. In contrast, Bignotti et al. [3] identified DEGs in omental metastases relative to matched PTs. Likewise, using publicly available gene expression databases, Cheon et al. [5] identified 10 genes, including several ECM components, that correlate with metastasis to the omentum as well as poor survival in OC. It was not known, however, whether any of the omental metastasis–associated genes identified in any of these studies are also more highly expressed in BMets.

In contrast to those studies, ours is, to our knowledge, the first designed specifically to identify genes that are differentially expressed in BMets. We also excluded genes with higher expression in benign bowel disease to identify 21 DEGs upregulated in BMets compared with PTs. These 21 genes show minimal overlap with the 10-gene signature for omental metastases reported by Cheon et al. [5]; *Col11A1* and *POSTN1* are the only 2 common upregulated transcripts. In contrast, of the 120 genes reported to be upregulated in omental metastases by Bignotti et al. [3], *Col11A1*, *POSTN*, *EPYC*, *LUM*, *FAP*, *MMP11*, and *LRRC15* were also upregulated in BMets in the current study. The knowledge that several of the genes upregulated in BMets are also expressed at a higher level in omental metastases provides further evidence that these genes are truly associated with the metastatic phenotype and should be studied as potential therapeutic targets to decrease implantation or growth of OC cells on the bowel wall. The above observation also emphasizes the fact that our signature is representative (but not specific) of bowel metastases.

Given previous evidence that, compared with PTs, metastatic tumors are associated with increased fibrosis, inflammation, and fibroblast proliferation [10], we examined the correlation between expression of these 21 genes and histologic quantitation of fibroblast proliferation, fibrosis, and inflammation in the discovery set (Table S4). Two genes, *ADAM12* and *FAP*, had significant positive correlations with increased fibroblast proliferation scores (Fig. S2; Table S5), which suggests that these genes may facilitate the production of fibrosis and increased stromal proliferation in OC BMets.

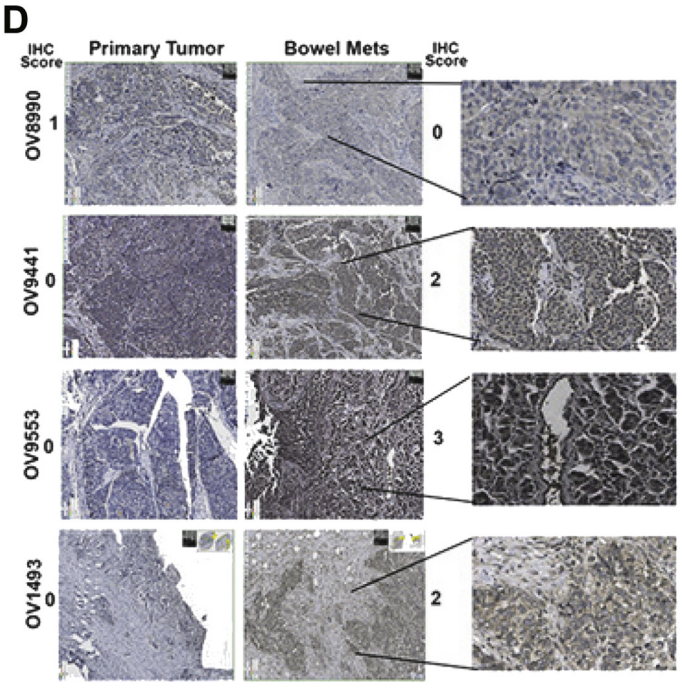
Fifteen of the 21 DEGs in BMets are ECM-related genes (Table S8). Whereas 13 of the 21 genes are reportedly expressed exclusively in the stroma [5,21–32], we have determined that LRRC15 is expressed in both the stroma and the epithelial compartment (Fig. 3). Additionally, 11 of the 15 ECM-related proteins are secreted into the extracellular space. Increasing evidence supports the idea that these genes also contribute to epithelial mesenchymal transition, fibrosis, and metastasis. Both the stroma and malignant cells secrete ECM components, which makes it difficult to identify the source of these ECM proteins. However, *POSTN1*, *FAP*, *Col11A1*, *MFAP5*, and, more recently, *SFRP2*, have been shown to have increased expression in the stroma of metastatic or recurrent tumors, which implicates them in OC progression. Thus, it is becoming evident that the tumor microenvironment has a major role in mediating OC progression. By Ingenuity Pathway Analysis, the 2 most highly enriched functional networks involved ECM and collagen. In





C

Protein	Expression level changes (BMets/PT) No.of patients		
	Increased	Decreased	No expression
LRRC15	13	2	
SFRP2	6	6	3
Lumican	11	4	
Col11a1	8	3	4
FAP	11	4	
EPYC	9	6	
Periostin	11	4	
ADAM12	10	5	



E

Sample ID	LRRC15 IHC Score Primary Tumor	Bowel Mets
OV5179	1	3
OV4804	1	3
OV4243	2	1
OV2085	1	2
OV7110	1	2
OV1548	1	2
OV9441	0	2
OV8990	1	0
OV9033	0	1
OV2975	0	1
OV1493	0	2
OV7495	1	3
OV9187	0	1
OV9553	0	3
OV2462	0	3
OV2767	0	2

Fig. 3. Expression analysis of BMet-associated proteins by Western blot and IHC analysis of LRRC15. A, Western blot of matched PTs and BMets with indicated antibodies. B, Densitometric analysis of the intensity of LRRC15 expression in PTs and BMets normalized to expression of control PCNA was used to determine higher (blue) or lower (red) level of expression in BMets compared with matched PTs. Lack of expression in both PTs and BMets is indicated in green. C, Expression level changes (BMet/PT) of specific proteins. D, Representative IHC analysis of LRRC15 expression in matched ovarian PTs and BMets showing low to moderate (0–1) and high [2,3] levels of LRRC15 staining. Small sections of tumors with 4 representative LRRC15 expression levels are shown. Scale bars represent approximately 100 μm. E, IHC scores in PTs and their paired BMets.

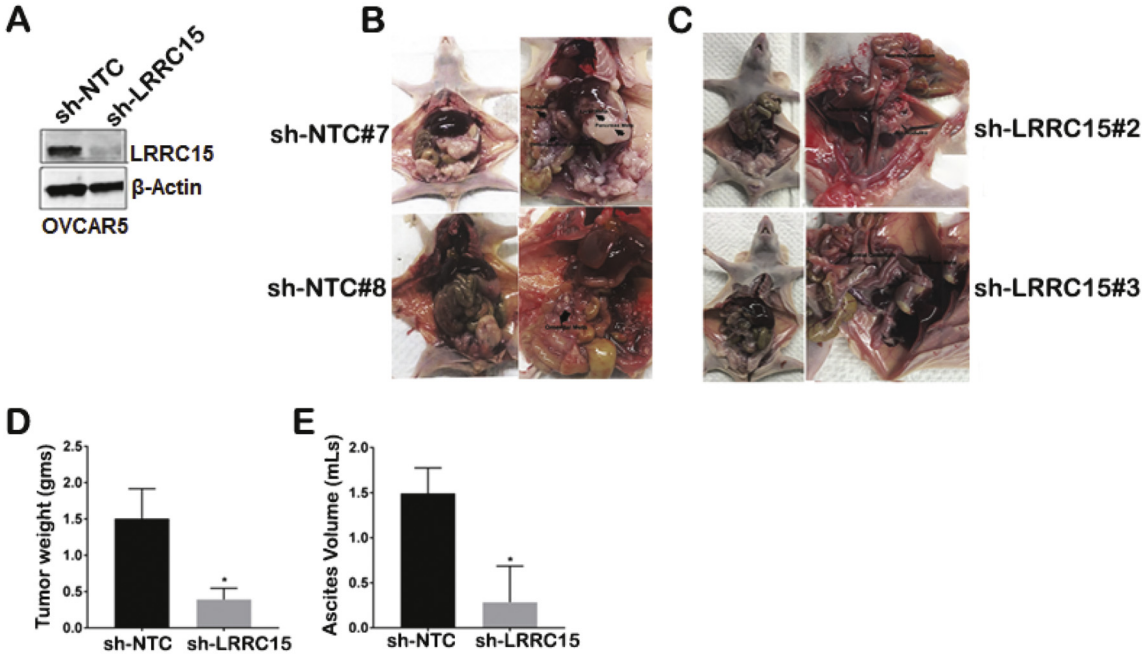


Fig. 4. Inhibition of tumorigenesis and metastatic spread in LRRC15-knockdown xenografts. A, Western blot analysis showing downregulation of LRRC15 in sh-LRRC15-transfected compared with sh-NTC-transfected OVCAR5 cells. B and C, Representative photographs of nude mice with tumors that formed 27 days after intraperitoneal injection of OVCAR5 cells transduced with nontargeted sh-NTC (B) or sh-LRRC15 (day 39) (C). Arrows indicate large tumor nodules. D and E, Quantification of wet tumor weight (D) and ascites volumes (E) of sh-NTC and sh-LRRC15 xenografts. Data are presented as mean ± SEM. *$P < .05$.

addition, these functional networks include growth factor signaling pathways that have been implicated in mediating tumor progression, including the TGFβ and NFκB pathways. Six of the 21 genes (*PLPP4*, *NTM*, *DIO2*, *MYH13*, *NETO1*, and *KCNA1*) encode non-ECM proteins. Little information is currently available about the specific roles of these genes in metastasis. Further studies are required to determine whether these genes truly have a role in OC metastasis.

In a recent study from our group [33], we showed that COL11A1 is specifically expressed in the stroma displayed a higher level in metastatic sites (but not in cancer), when compared to the primary tumor Also, COL11A1, FAP and POSTN are most commonly (or exclusively) expressed in the stroma. These findings emphasize the fact that the 21-gene signature described in the current study is representative of the tumor as a whole (i.e. both the cellular and the stromal components). However, the fact that many of these genes are overexpressed in the stroma underscores the importance of the stromal component in the metastatic process.

On microarray analysis of the gene expression levels in PTs of 333 patients with OC, those who underwent bowel resection had significantly higher expression of 14 of the BMet-upregulated genes than did patients who did not require bowel resection (Fig. 2; Table S6). This observation supports that increased expression of these genes in PTs might predict metastasis to the bowel and association with aggressive cancers. In further analysis, one striking finding was the increased expression of 19 of the 21 genes in PTs of patients who had visible residual disease after the surgical procedure. Residual disease after surgery is generally associated with more aggressive cancer and poorer outcomes, when compared with no macroscopic residual disease after surgery (i.e., RD0). Invasion of the bowel by cancer is a sign of an aggressive tumor, which requires more complex surgery and is less likely to be completely removed at primary surgery [34]. This finding indicates that some of these genes may also be used for preoperative identification of patients who require either complex surgery or neoadjuvant chemotherapy. However, this is a secondary finding of our analysis that requires further validation.

Although our list includes several genes previously reported to be associated with suboptimal surgical outcome, we also identified 4 novel genes, *PLPP4*, *NTM*, *DIO2*, and *PVALB*, that have not previously been associated with this phenotype. With the exception of *PVALB*, high expression of the other 3 genes was associated with the need for bowel resection. The protein encoded by *PVALB* is thought to be involved in muscle relaxation and is structurally and functionally similar to calmodulin and troponin C [35]. In our study, the expression of *PVALB* was slightly higher in cases with suboptimal surgical resection. In contrast, *PVALB* expression has been reported to be higher in benign vs malignant thyroid lesions and in normal glial cells vs glioma [36,37].

As the most common OC histologic subtype, high-grade serous OCs have been extensively studied and can be further stratified, based on high-throughput mRNA measurements, into 4 distinct molecular subtypes: mesenchymal, immune-reactive, proliferative, and differentiated [38]. However, all the previous subtype studies were generated on the basis of gene expression in the PTs, with little investigation into their relationship with gene expression in metastases. In the current study, 10 of the 21 BMet genes were significantly upregulated in the mesenchymal subtype vs other subtypes of tumors ($P < .001$).

The 7 genes significantly associated with poor survival by univariate analysis were also associated with suboptimal debulking. However, only the *EPYC* gene remained significantly associated with poor survival after selecting patients with optimal (i.e., RD0) debulking. This may suggest that BMet-upregulated genes are more immediately associated with surgical outcomes and intra-abdominal extension of cancer, whereas survival associations are most likely mediated by surgical results (RD0 vs non-RD0). Interestingly, Lisowska et al. [24], reported *EPYC* (*DSPG3*) as 1 of 2 genes (along with *LOX*) significantly associated with overall survival and disease-free survival in OC.

The leucine-rich repeat-containing protein LRRC15—a member of a family of proteins involved in cell-cell and cell-ECM interactions, including adhesion, and receptor-ligand binding—has previously been implicated in adherence to fibronectin [39] and in breast cancer progression [18,40]. Expression of LRRC15 in 16 matched PTs and BMets by immunoblot analysis and IHC validated observations at the RNA level. In particular, the expression of LRRC15 was increased in the BMets in 87.5% of the samples compared with their matched PTs, which indicates that LRRC15 could serve as a marker of OC progression.

Of note, LRRC15 showed the highest frequency of upregulation at the protein level of any of the 8 markers tested by immunoblotting. This is the first report of LRRC15 overexpression at the protein level in the BMets of patients with OC.

Understanding the molecular mechanisms that control tumor growth and metastatic spread can aid in the development of improved chemotherapeutic options. Our study demonstrated that blocking LRRC15 expression may decrease both tumorigenicity and metastatic dissemination. This finding suggests that the LRRC15 protein may be a potential candidate as an antitumor target, a concept given further support by ongoing development of ABBV085 [41] an LRRC15–antibody-targeted drug conjugate.

In summary, BMets and bowel obstruction represent the most common cause of morbidity and death in OC. We identified 21 genes associated with BMets in OC. These genes and cellular pathways may guide future attempts to develop therapies aimed at prevention and treatment of malignant bowel dysfunction in OC. Our results with LRRC15 knockdown raise the possibility of future use of BMet-prevention agents targeting these genes as long-term "maintenance therapy" to decrease metastatic dissemination, fibrosis, and fibroblast proliferation, thereby averting bowel obstruction and improving quality of life and survival in patients with OC.

Author contributions

Concept and design: A.M., S.W., K.K., L.H., and A.O.
Data collection: C.W., V.S., E.G., F.M., T.G., M.T., S.R., and A.M.
Execution of experiments: D.J., G.S., D.R., L.J., J.S., and V.S.
Data and statistical analysis: C.W., A.O., and S.R.
Data interpretation: C.W., J.K., E.G., S.K., A.M., and V.S.
Drafting and revising the manuscript: V.S., C.W., A.M., E.G., W.C., and S.K.
Pathology review: D.W., V.P., and J.S.

Declaration of Competing Interest

S.J.W declares research funding from Novartis, Genetech, Tesaro, and KIVATEC and an Ovarian PDX patent with royalties. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.06.010.

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