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14. ABSTRACT The recent addition of bevacizumab to the treatment of primary ovarian cancer and its FDA approval for this disease follow from the AURELIA and GOG/NRG 218 trials. From 1873 enrolled patients on GOG/NRG 218, it was found that the addition of bevacizumab to chemotherapy and its inclusion in the extended period resulted in a significant improvement in progression free survival (PFS) of 4 months compared to chemotherapy alone, with a hazard ratio of 0.717. The critical questions that must now be answered are 1) what are the molecular markers that predict for response on this landmark ovarian cancer trial? and 2) what are alternative treatment strategies for non-responders? Our central hypothesis is that targeted agents including bevacizumab, chosen based upon the knowledge of the TP53 mutational status, synergize with chemotherapy and/or the PARP inhibitor olaparib and promote catastrophic tumor cell death. These studies are anticipated to have a significant positive impact on the field by enhancing the design and choice of therapy for ovarian cancer based on TP53 mutations.					
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1. Introduction

The recent addition of bevacizumab to the treatment of primary ovarian cancer and its FDA approval for this disease follow from the AURELIA and GOG/NRG 218 trials. From 1873 enrolled patients on GOG/NRG 218, it was found that the addition of bevacizumab to chemotherapy and its inclusion in the extended period resulted in a significant improvement in progression free survival (PFS) of 4 months compared to chemotherapy alone, with a hazard ratio of 0.717. The critical questions that must now be answered are 1) what are the molecular markers that predict for response on this landmark ovarian cancer trial? and 2) what are alternative treatment strategies for non-responders? Our central ***hypothesis*** is that targeted agents including bevacizumab, chosen based upon the knowledge of the *TP53* mutational status, synergize with chemotherapy and promote catastrophic tumor cell death. The rationale for our hypothesis is that *TP53*, the ‘guardian of the genome’, is mutated in 95% of all high grade serous ovarian cancers and is considered the fundamental early genomic alteration associated with this disease. However, it is critically important to note that varying types of p53 mutant proteins exist, with different implications for cell cycle regulation and for chemosensitivity. Extensive preclinical data from our team demonstrate that the *TP53* mutations alter the master regulators of cell cycle checkpoints in predictable *yet distinct* ways that can be capitalized upon to overcome resistance to chemotherapy using targeted agents that block compensatory survival pathways. We term this principle “molecularly enhanced chemotherapy.” Our specific aims are to 1) in a retrospective study of GOG/NRG 218 clinical samples, determine the functional class of *TP53* mutation for each case and correlate with response; 2) assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations; and 3) interrogate the function of *TP53* mutations that are *somatic variants of unknown function* (sVUF) to define the best therapy for patients with these mutations. These studies are anticipated to have a significant positive impact on the field by enhancing the design and choice of therapy for ovarian cancer based on specific *TP53* mutations. Moreover, our findings have the potential for immediate clinical application since we are performing retrospective analyses of a positive trial to better define a subgroup of patients that are more likely to respond. We will also prioritize drug choice in Aims 2 and 3 to use agents that have proven safety profiles and efficacy in other cancer types. Hence, our findings can be quickly implemented to provide personalized precision treatment options and improve outcomes for women with ovarian cancer using currently defined and FDA-approved treatments.

2. Keywords

Ovarian cancer; p53; bevacizumab; chemotherapy

3. Accomplishments

What were the major goals of the project?

Project/Grant Period: 09/30/2020-08/31/2023	Target Date (Month(s) relative to start date)	Actual Completion Date or % of Completion
Specific Aim 1: From GOG/NRG 218 clinical samples, determine the functional class of TP53 mutation for each case and correlate with response		
Major Task 1: Categorize cases in GOG/NRG 218 based upon TP53 status.		
Subtask 1: Submit institution’s IRB approval and related material for DoD’s HRPO approval	1	03/2020
Subtask 2: Receive HRPO approval or exempt finding before initiating human subjects/HAS related studies.	1	03/2020
Subtask 3: Extract de-identified TP53 sequencing data from GOG/NRG 281 BROCA sequencing files *To be carried out at Site 2.	1-4	90%
Subtask 4: Bin TP53 mutations into function categories (LOF, GOF, sVUF, WT)	5-6	90%
Milestone Achieved: Bin TP53 mutations	6	90%
Major Task 2: Correlate binned TP53 subclass designations with response, PFS and OS		
Subtask 1: Provide NRG Statistical Office with a table containing de-	6	50%

identified patient identifiers and TP53 functional category (LOF, GOF, sVUF, WT)		
Subtask 2: Receive a report of the correlation between <i>TP53</i> mutational status and outcomes on GOG/NRG 218 *25% to be carried out at Site 2.	9	First report on GOF mutations completed; second report on all mutations pending.
Subtask 3: Assemble data from Major Tasks 1-2 for publication *25% to be carried out at Site 2.	9-12	Projected to be completed in the next 9 months
Milestone Achieved: Determine the correlation of TP53 subclass designations with PFS, OS and response overall and on each arm of GOG/NRG 218	12	Projected to be completed in the next 9 months
Specific Aim 2: Assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations.		
Major Task 3: Determine the functional impact of GOF mutants.		
Subtask 1: Generate cell models engineered to express all GOF mutants in GOG/NRG 218 dataset	5-8	50% completed
Subtask 2: Assess the impact of each GOF mutant on canonical p53 WT function	8-12	50% completed
Subtask 3: Assess the impact of each GOF mutant on cell cycle checkpoint signaling	8-12	50% completed
Subtask 4: Define the impact of each GOF mutant on the transcriptome using RNAseq	13-16	25%
Milestone(s) Achieved: Assign function to each TP53 GOF mutant	16	Projected to be completed in the next 6 months
Major Task 4: Identify the most effective molecular inhibitor to combine with chemotherapy in order to overcome the impact of each GOF mutant		
Subtask 1: Test the impact of select agents on cell viability, cell cycle progression, percent of mitotic cells and activation of cell cycle checkpoint	17-24	75% completed
Milestone Achieved: Identify appropriate molecular agent to use in studies in Major Task 5 (Aim 2C)	24	75% completed
Major Task 5: Determine the <i>in vivo</i> efficacy of the best combinations from Aim 2B		
Subtask 1: Obtain local IACUC Approval for proposed studies in PDX mice	1	02/21/2020
Subtask 1: Submission of institution approved animal protocols and related material for DoD's ACURO approval	1	03/06/2020
Subtask 2: Receive ACURO approval before initiating animal experiments.	1	05/2020
Subtask 2: Perform drug testing studies in PDX mice	25-36	100% completed
Subtask 3: Analyze post-treatment tumor specimens from PDX mice	28-36	100% completed
Milestone Achieved: IACUC approval for animal studies	3	100% completed
Milestone Achieved: Assemble final data for Major Tasks 3-5	36	100% completed
Milestone Achieved: Manuscript preparation for data generated in Major Tasks 3-5	36	Manuscript published in <i>Cell Death & Disease</i> 01/2022;
Specific Aim 3: Interrogate the function of TP53 mutations that are somatic variants of unknown function to define the best therapy for patients with these mutations		
Major Task 6: Develop a predictive algorithm to bin sVUF in GOG/NRG 218 into functional categories		
Subtask 1: Estimate mutation effect of each sVUF in the GOG/NRG 218 cohort <i>in silico</i>	6-8	50% completed
Subtask 2: Predict functional effect of mutations	6-8	50% completed
Subtask 3: Obtain FFPE slides from NRG Oncology for immunohistochemistry (IHC)	9	Projected to be completed in next 6 months
Subtask 4: Perform IHC for p53 protein expression and bin cases	10-18	Projected to be completed in next 12 months
Milestone Achieved: Functionally bin cases as WT, LOF or GOG		Projected to be completed in next 18

		months
Major Task 7: Binning based on <i>in vitro</i> assessment of mutant p53 activity.		
Subtask 1: Generate cell models engineered to express recurrent sVUFs in GOG/NRG 218 dataset	5-8	50% completed
Subtask 2: Assess the impact of each sVUF mutant on canonical p53 WT function, cell cycle and transcriptome	8-16	50% completed
Milestone(s) Achieved: Assign function to each TP53 sVUF mutant	16	Projected to be completed in 18 months
Major Task 8: Determine the impact of TP53 sVUF on sensitivity to chemotherapy		
Subtask 1: Test the impact of select agents on cell viability, cell cycle progression, percent of mitotic cells and activation of cell cycle checkpoint	17-24	50% completed
Subtask 2: Drug testing studies in PDX mice	25-36	Completed, published in <i>Cell Death and Disease</i> , Bi J et al. 2022
Milestone Achieved: Manuscript preparation for data generated in Major Tasks 6-7	36	Projected to be completed in next 24 months

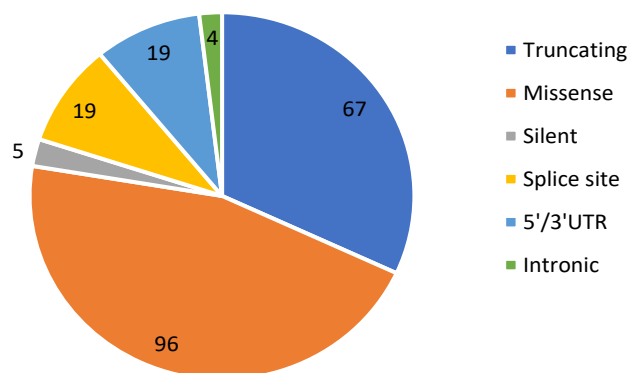
What was accomplished under these goals? Here we detail the accomplishments by Specific Aim and Subaim.

Specific Aim 1 From GOG/NRG 218 clinical samples, determine the functional class of TP53 mutation for each case and correlate with response.

Aim 1A: Categorize cases in GOG/NRG 218 based upon TP53 status.

- We have received approval from the NRG Oncology Ancillary Projects Committee approval for TR studies.
- Our collaborators at the University of Washington have provided a list of mutations in TP53 that occurred as single events among the 713 patients with available sequencing data enrolled on Arms 1 and 3 of GOG-218.
- We have binned these cases based upon the specific type of TP53 mutation as in **Figure 1**.
- Most recently we have received the entire list of mutations in TP53 found in the cases from GOG-218 and have begun to study the impact of those mutations on clinical outcomes (**Figure 2**).

Figure 1



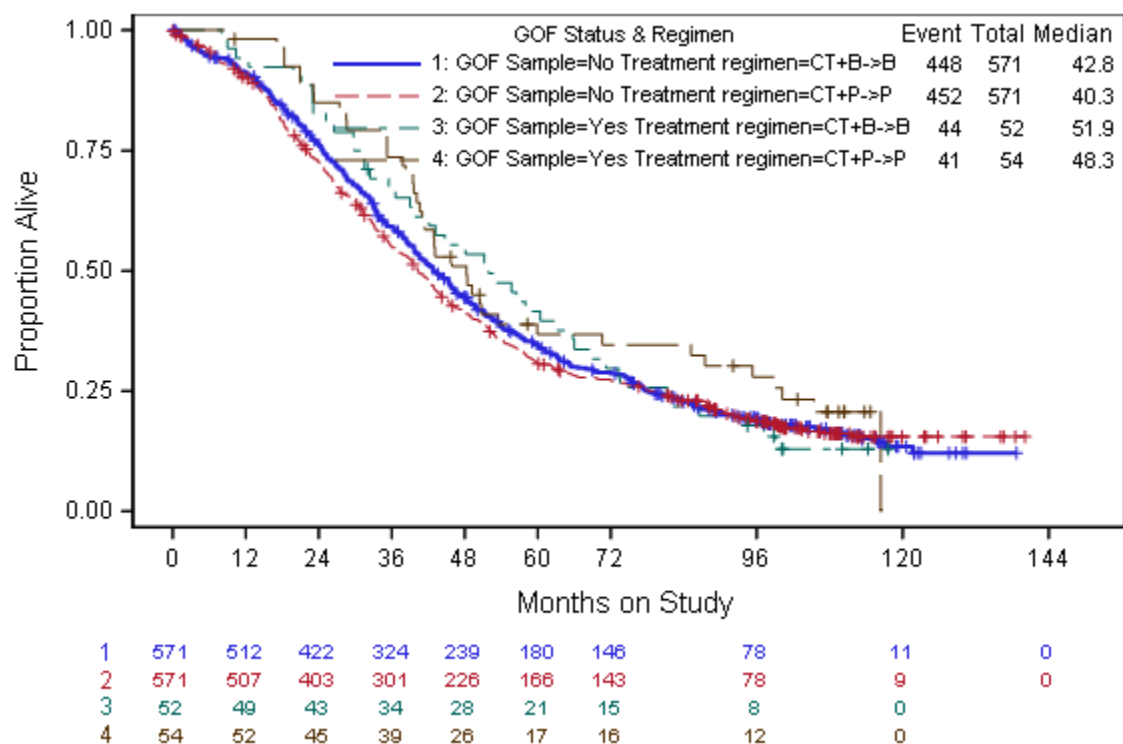


Figure 2. Overall Survival for patients treated with chemotherapy + bevacizumab versus chemotherapy alone from GOG 218. This sample includes only those TP53 mutations that are categorized as “gain of function” or GOF. There is a trend for improvement in overall survival in those high-risk cases with the addition of bevacizumab in this preliminary analysis.

We are now assessing the entire number of cases in GOG study 218 with p53 missense mutations to increase the number of patients and will report these findings in the coming year. **Those data will complete Aim 1.**

Specific Aim 2: Assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations.

Aim 2A: Determine the baseline impact of various GOF mutants on cell cycle checkpoint control and expression of canonical p53 transcriptional targets.

- We have generated both cell line models and patient-derived organoid (PDO) models of ovarian cancer harboring various mutations in *TP53*. These data have been published in two peer-reviewed scientific manuscripts (see **Appendices 2 and 3**). **Table 1** (next page) contains a list of all ovarian PDO models annotated with *TP53* mutational status determined by sequencing and p53 expression level determined by immunohistochemistry.

ID	Histologic subtype	Stage	<i>TP53</i> seq	p53 IHC
ONC-6695	Serous	IA	WT	WT
ONC-6163	Serous	II	WT	WT
ONC-6088	Serous	IIA	WT	OE
ONC-7188	Serous	III	N.D.	N.D.
ONC-6134	Serous	IIIA	R267G	OE
ONC-6045	Serous	IIIB	N.D.	OE
ONC-6069	Serous	IIIB	Del/splice/intron 7 retention	Null
ONC-6072	Serous	IIIB	WT	WT
ONC-7059	Serous	IIIB	N.D.	N.D.
ONC-6693	Serous	IIIC	WT	cytoplasmic
ONC-7253	Serous	IIIC	WT	N.D.
ONC-7485	Serous	IIIC	N.D.	N.D.

ONC-7705	Serous	IIIC	N.D.	N.D.
ONC-7704	Serous	IIIC	N.D.	N.D.
UBC 7391	Serous	IIIC	N.D.	N.D.
ONC-7910	Serous	IIIC	N.D.	N.D.
ONC-6061	Serous	IIIC	WT	WT
ONC-6561	Serous	IIIC	N.D.	Null
ONC-6663	Serous	IIIC	WT	OE
ONC-7052	Serous	IIIC	WT	Null
ONC-6497	Serous	IV	WT	WT
ONC-7077	Serous	IV	G266V	OE
ONC-6140	Serous	IVA	N.D.	N.D.
ONC-6562	Serous	IVA	WT	OE
ONC-7033	Serous	IVA	N.D.	cytoplasmic
ONC-5942	Serous	IVB	C275Y	OE
ONC-6007	Serous	IVB	WT	OE
ONC-6683	Serous	IVB	WT	OE
ONC-7017	Serous	IVB	N.D.	WT
ONC-7791	Serous	IVB	N.D.	N.D.
ONC-7024	Serous		R213-STOP	WT
ONC-7624	Serous		N.D.	N.D.
ONC-7627	Serous		N.D.	N.D.
ONC-7978	Endometrioid	IC	N.D.	N.D.
ONC-7477	Endometrioid	IIIA	N.D.	N.D.
ONC-7962	Adenocarcinoma	IV	N.D.	N.D.
ONC-7063	Clear cell	IA	WT	WT
ONC-5945	Clear cell	IC	WT	WT
ONC-6092	Clear cell	IC	WT	WT
ONC-7395	Mucinous	IA	N.D.	N.D.
ONC-7429	Mucinous	IA	N.D.	N.D.
ONC-7465	Mixed	IV	N.D.	N.D.
UBC 7201	Carcinosarcoma	IIIA	N.D.	N.D.
ONC-7645	Carcinosarcoma	IIIC	N.D.	N.D.
NR1	Carcinosarcoma	IVA	Δ 131 mutation	OE
ONC-7234	Malignant brenner tumor	IIB	WT	N.D.

- We have examined PLAC1 transcriptional control in two ovarian cancer cell lines harboring different p53 mutations (see **Appendix 4**). PLAC1 transcription is a readout for WT p53 activity.
- Finally, we have performed Kinexus phosphoproteomic analysis of key cell cycle controllers in ovarian cancer cells (**Table 2**).

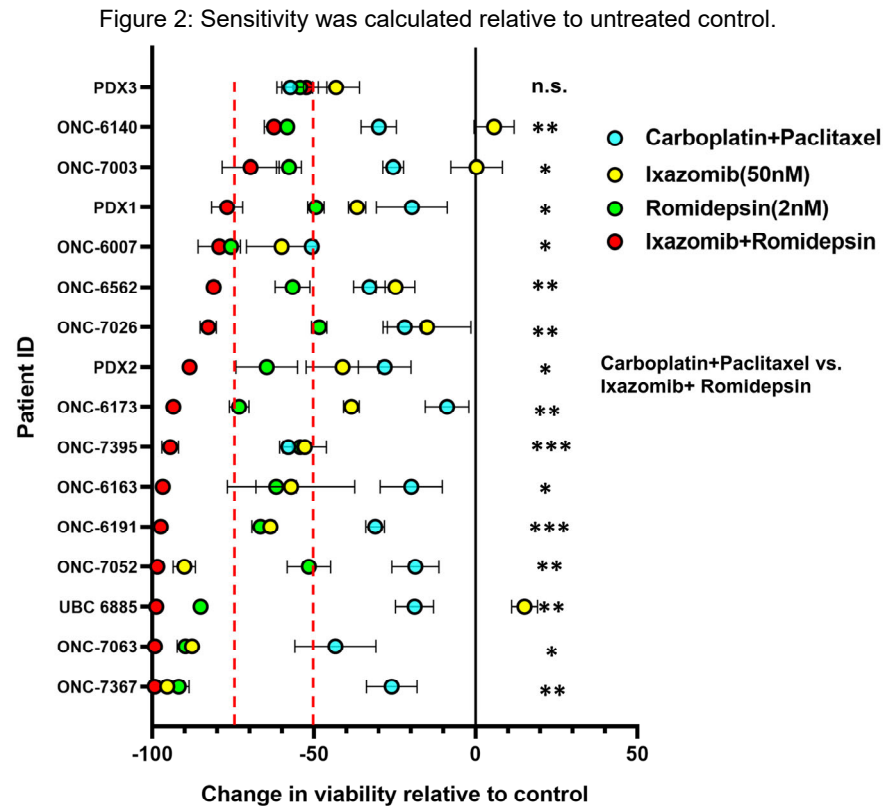
Table 2: Kinexus phosphoproteomic analysis of the impact of bevacizumab or cediranib on protein phosphorylation in CAOV3 ovarian cancer cells. Cells were treated with either bevacizumab or cediranib (1 μ M), followed by assessment of protein phosphorylation using the Kinexus KAM phosphoproteomic array. The signal intensity was normalized to internal controls and calculated relative the untreated control. Lead candidates are identified. %CFC: percent change from control; data are colored from blue to yellow to indicate the significance of the change from control. Pan-specific indicates assessment of total protein expression.

- From these phosphorylation events (**Table 2, below**), we note the consistent enhancement of phosphorylation of two proteins, ABL and EZH2, when treated with anti-angiogenics bevacizumab and cediranib. The phosphorylation of ABL at Y257 inhibits the nuclear localization signal and prevents ABL from shuttling to the cytoplasm from the nucleus, which is required for normal cell proliferation. In addition, the phosphorylation of EZH2 on T487 is an inhibitory phosphorylation event that prevents EZH2 from stimulating cell proliferation. These are two novel signaling events associated with bevacizumab therapy that likely play a role in the improved outcomes noted when bevacizumab is combined with chemotherapy in GOG-218. On the other hand, a number of phosphorylation events are induced that appear to signal potential developing resistance effects, for example, the induction of insulin-like growth factor receptor (IGFR1) phosphorylation at 1161 and 1163 enhance IGFR1 kinase activity, potentially counteracting the therapeutic effects of bevacizumab and cediranib. These data suggest a novel therapeutic strategy to combine anti-angiogenics such as bevacizumab with tyrosine kinase inhibitors of IGFR1. We will test this strategy in cell lines in the coming months.

Target Name	P-Site	Control		Bevacizumab		Cediranib		
		Average Normalized Net Signal	Average Normalized Net Signal	% CFC	Lead	Average Normalized Net Signal	%CFC	Lead
Abl (Abl1)	Y257	3761	7151	90	Priority	7167	91	Priority
Abl2 (Arg)	Pan-specific	6462	8429	30		10193	58	Possible
Abl2 (Arg)	Y439+T440	6574	13841	111	Possible	15058	129	Possible
ACC1 (ACACA)	S78+S80	475	778	64	Possible	717	51	
ACC2 (ACACB)	T1342	7026	14123	101	Possible	16933	141	Priority
ACTB	Y53	2575	3771	46	Possible	1777	-31	
AMPKa2 (PRKAA2)	Pan-specific	9034	7388	-18		13189	46	Possible
AMPKa2 (PRKAA2)	S377	4704	7193	53		9907	111	Priority
Arrestin b	S412	601	940	56	Possible	580	-4	
ATM	Pan-specific	9806	10319	5		15245	55	Possible
CDK9	Pan-specific	547	1015	85	Possible	424	-23	
CLK1	S337	3336	5296	59	Possible	3033	-9	
DLK (ZPK)	S269	8521	8510	0		2626	-69	Possible
ERK5 (MAPK7)	Pan-specific	5747	8469	47	Possible	9386	63	Possible
EZH2	T487	6183	11321	83	Priority	13512	119	Priority
FAK (PTK2)	Y397	3638	8418	131	Possible	5680	56	
FGFR3	Pan-specific	7669	7854	2		12534	63	Possible
FGFR4	Pan-specific	3838	4107	7		5629	47	Possible
Fgr	Pan-specific	11578	12896	11		17239	49	Possible
Flt3 (STK1)	Pan-specific	11462	10718	-6		17339	51	Possible
FOXK1	S441+S445	6302	10532	67	Possible	11558	83	
FOXO1A (FKHR)	S256	3907	4223	8		6029	54	Possible
GFAP	Pan-specific	1925	1849	-4		2829	47	Possible
HDAC5	S498	577	907	57	Possible	437	-24	
HePTP (PTPN7)	S44	5159	8929	73	Possible	4764	-8	
HGK (ZC1)	Pan-specific	1829	9151	400	Priority	7583	315	Priority
HGK (ZC1)	T187	1966	9240	370	Priority	6767	244	Priority
HIPK1	Y352	4323	7794	80		8040	86	Possible
HRAS	Pan-specific	3839	7555	97	Priority	6829	78	
HRAS	Y157	7782	9357	20		11801	52	Possible
IGF1R	Y1161+T1163	2644	5887	123	Priority	4878	84	Priority
ILK1 (ILK)	S343	2518	3820	52	Possible	2236	-11	
Jun (c-Jun)	S243	8758	9146	4		12759	46	Possible
Ksr1	Pan-specific	913	858	-6		381	-58	Possible
MAFG	S124	6485	23087	256	Priority	5204	-20	
MEK2 (MKK2, MAP2K2)	Pan-specific	6378	7041	10		9875	55	Possible
NFKB p65 (Rel A)	Pan-specific	1439	2569	79	Priority	1140	-21	
NuaK1 (ARK5)/Nuak2	T211	7180	8503	18		10713	49	Possible
p38a MAPK (MAPK14)	T180+Y182	9035	14318	58	Possible	11614	29	
p38d MAPK (MAPK13)	T180+Y182	437	1533	250	Priority	616	41	
p53 (TP53)	Pan-specific	4531	7503	66	Possible	5429	20	
p53 (TP53)	S37	1785	2588	45	Possible	2000	12	
p53 (TP53)	S392	6873	14237	107	Priority	5913	-14	
p70S6K (S6Ka, RPS6KB1)	Pan-specific	4504	8433	87	Priority	4950	10	
p70S6K (S6Ka, RPS6KB1)	T252	8986	17413	94	Possible	15747	75	Possible
PCYT1A (CTPCT; CCTA)	T342+S343	497	1571	216	Priority	721	45	
PDK1 (PDPK1)	S241	7643	16441	115		16849	120	Priority
PFKFB3	S461	1592	3034	91	Possible	1783	12	

Aim 2B: Identify the most effective molecular inhibitor to combine with chemotherapy in order to overcome the impact of each GOF mutant.

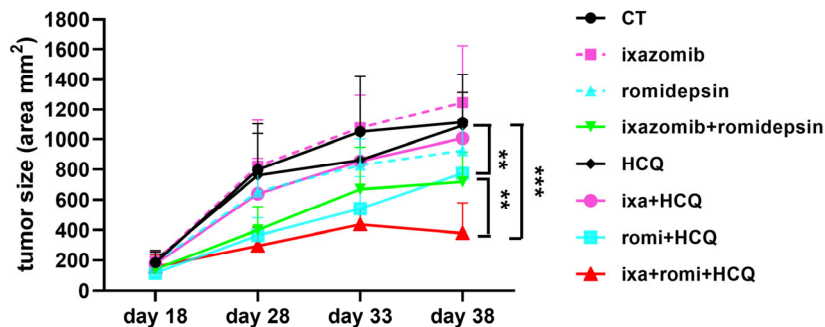
- We have performed a series of studies to interrogate various molecular agents in combination with chemotherapy based upon the specific *TP53* mutational status of the models. First, we found that the antiangiogenic agent cediranib is superior to bevacizumab (see Appendix 3, Bi et al., *Pharmaceuticals*).
- Having identified this alternative angiogenic, our findings set the stage for new clinical trials using cediranib or other tyrosine kinase inhibitor antiangiogenics in place of bevacizumab with chemotherapy.
- We also found that p53 WT activity can be reactivated using a molecular reactivator that converts mutant p53 conformation back to wild type (see **Appendix 4**, Devor et al., *Pharmaceuticals*).
- We also explored agents that induce massive cell death in ovarian cancer models and identified the combination of a histone deacetylase (HDAC) inhibitor with a proteasome inhibitor. We confirmed efficacy in cell lines, PDO models, and a xenograft model of a serous endometrial tumor, a histologic subtype that is very similar to high-grade serous ovarian cancer (example data are provided in **Figure 2** on the previous page; manuscript has been submitted to *Cell Death and Disease* and currently under review).



Aim 2C: Determine the in vivo efficacy of the best combinations from Aim 2B.

- We have determined the in vivo efficacy of the combination of an HDAC inhibitor combined with a proteasome inhibitor in a xenograft model of serous endometrial cancer (**Figure 3**).

A



B

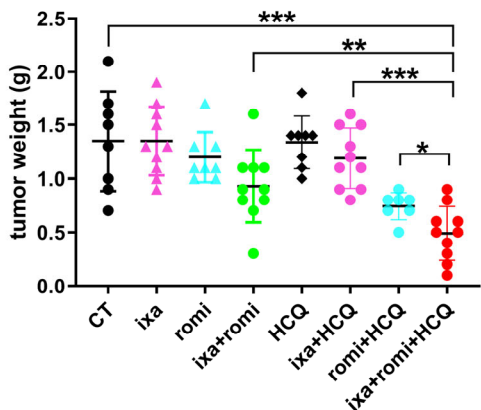


Figure 3: Sensitivity of xenograft models of Hec50 cells to ixazomib+romidespsin. A) Tumor volume; B) tumor weight at the completion of the experiment (Day 38).

Aim 3: Interrogate the function of *TP53* mutations that are somatic variants of unknown function to define the best therapy for patients with these mutations.

- To date, we have binned 210 cases with *TP53* unique mutations in the GOG-218 dataset (**Figure 1**). Of these, nearly all of the 96 missense mutations are sVUFs. In addition, we have identified sVUFs in the PDO models of ovarian cancer that are also available for mechanistic studies.
- We have identified a highly active drug, HO-3867 (analogue of curcumin) and another agent, APR-246** that demonstrate preclinical activity against sVUF and GOF p53 mutants (**Figures 4 and 5**).

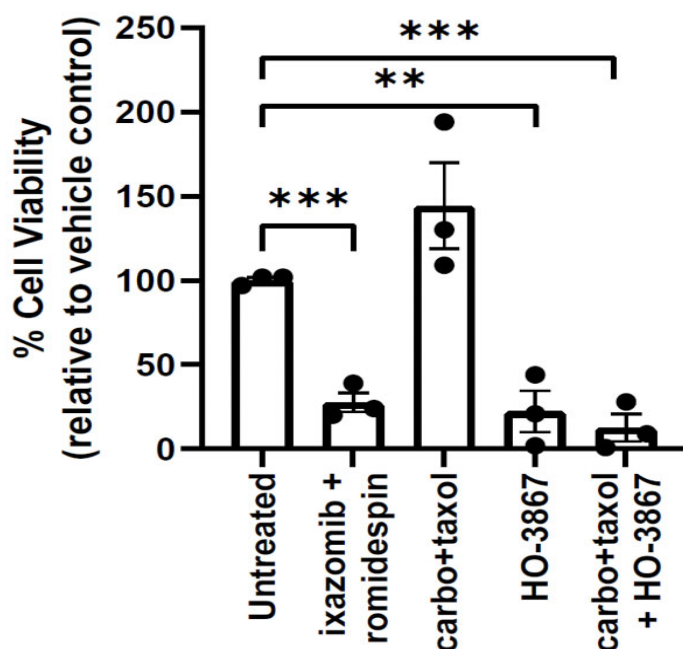
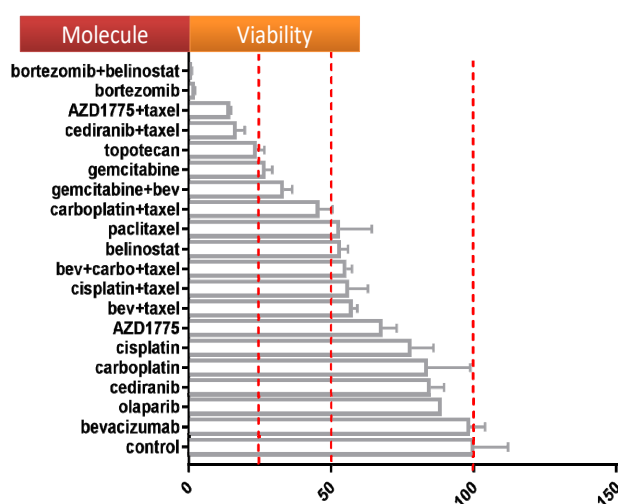
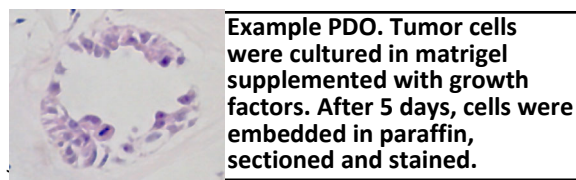


Figure 4. Patient derived organoid expressing a mutation in *TP53* that is a somatic variant of unknown function (sVUF). Note the exquisite sensitivity to the curcumin derivative HO-3867 and its synergy with chemotherapy.

Drug concentration: Cisplatin 1 μ M, Carboplatin 1 μ M, paclitaxel 10nM, bevacizumab 1 μ M, AZD1775 125nM, Bortezomib 10nM, Belinostat 1 μ M, Olaparib 2 μ M, Gemcitabine 100nM, Topotecan 100nM, Cediranib 1 μ M.

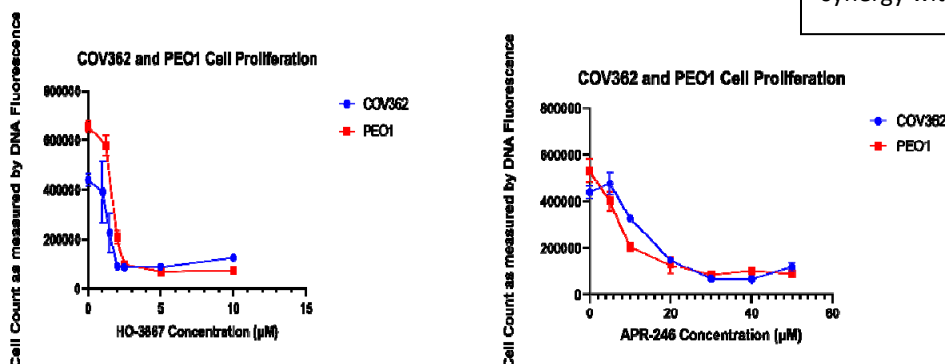


Figure 5. IC₅₀ of HO-3867 and APR-246 in ovarian cancer cells with mutant p53 and BRCA. Concentrations that result in 50% cell death (IC₅₀) are in the low μ M range for both HO-3867 and APR-245.

As a next step building upon the results obtained in Aim 3, we are pursuing the development of agents such as HO-3867 for clinical trials with an industrial partner. This will be an important and clinically meaningful future goal enabled by the current award.

What opportunities for training and professional development has the project provided?

This project was not intended to provide training and professional development opportunities. However, funds from this project have provided training opportunities for a postdoctoral fellow and a post-baccalaureate research intern. In addition, this project has supported in part the research of a clinical gynecologic oncology fellow in training. The purpose of this trainee's project was to create models of chemo-response using patient-derived organoids. A senior scientist on this grant (Co-investigator Dr. Kristina Thiel) has now attained an NCI K22 transition career development award and has accepted an independent faculty position at the University of Iowa. In addition, this grant supported the work of a summer undergraduate student and a high school student who both presented posters at the University of New Mexico Under-represented Minority Poster Session, 2022.

How were the results disseminated to communities of interest?

Results have been disseminated through peer-reviewed publications as described above and in the Appendices. See also Section 6: Products. These results have also been presented in internal works-in-progress meetings at the sponsoring institution.

What do you plan to do during the next reporting period to accomplish the goals?

We will follow the experimental plan as outlined in the funded Project Narrative and the Statement of Work. Specific objectives in Aim 1 are to correlate p53 mutational status with outcomes through a collaboration with the NRG Oncology Statistical Office. For Aims 2 and 3, we will better characterize p53 mutations in PDO models harboring hotspot mutations from the GOG-218 cohort. We will also follow-up on the data from Aim 2 suggesting that the addition of IGFR1 inhibitors to bevacizumab may be a novel therapeutic strategy. We will also test inhibitors of EZH2 and ABL as new therapies in the models. Finally, having identified a potentially active agent in ovarian cancer, HO-3867, we will continue to pursue preclinical studies with this drug in preparation for future clinical trials.

4. Impact**What was the impact on the development of the principal discipline(s) of the project?**

These studies are anticipated to have a significant positive impact on the field by enhancing the design and choice of therapy for ovarian cancer based on specific *TP53* mutations. Moreover, our findings have the potential for immediate clinical application since we are performing retrospective analyses of a positive trial to better define a subgroup of patients that are more likely to respond. We will also prioritize drug choice in Aims 2 and 3 to use agents that have proven safety profiles and efficacy in other cancer types. Hence, our findings can be quickly implemented to provide personalized precision treatment options and improve outcomes for women with ovarian cancer using currently defined and FDA-approved treatments.

What was the impact on other disciplines? These results pertain to other cancers because many other cancer types have identical mutations in *TP53*. For example, our group has published on p53 mutations as predictors of response to bevacizumab-containing chemotherapy in advanced or recurrent endometrial cancer (Leslie et al., Gynecol Oncol 2021 and Thiel, et al., J of Clin Oncol, 2022).

What was the impact on technology transfer? Nothing to Report.

What was the impact on society beyond science and technology? Nothing to Report.

5. Changes/Problems

Dr. Leslie has made a successful transition from the University of Iowa to the University of New Mexico Health Sciences Center. Her laboratory is now well established and continuing these studies.

6. Products**Publications, conference papers, and presentations****Journal publications:**

1. Bi J, Newton AM, Zhang Y, Devor EJ, Samuelson MI, Thiel KW, Leslie KK. Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers (Basel)*. Published on 2021 Jun 10;13(12):2901. doi: 10.3390/cancers13122901. PMID: 34200645; PMCID: PMC8229222. Acknowledgement of federal support: yes
2. Bi J, Dixit G, Zhang Y, Devor EJ, Losh HA, Newton AM, Coleman KL, Santillan DA, Maretzky T, Thiel KW, Leslie KK. Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy. *Pharmaceuticals (Basel)*. Published on 2021 Jul 16;14(7):682. doi: 10.3390/ph14070682. PMID: 34358108; PMCID: PMC8308742. Acknowledgement of federal support: yes.
3. Devor EJ, Schickling BM, Lapierre JR, Bender DP, Gonzalez-Bosquet J, Leslie KK. The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells. *Pharmaceuticals (Basel)*. Published on 2021 Sep 21;14(9):942. doi: 10.3390/ph14090942. PMID: 34577642; PMCID: PMC8465575. Acknowledgement of federal support: yes.
4. Bi J, Zhang Y, Malmrose, PK, Losh HA, Newton AM, Devor EJ, Thiel KW, Leslie KK. Blocking autophagy overcomes resistance to dual histone deacetylase and proteasome inhibition in gynecologic cancer. *Cell Death and Disease* (2022) 13:59. Acknowledgement of federal support: yes.
5. Ghezelayagh TS, Penington KP, Norquist BM, Khasnavis N, Radke MR, Kilgore MR, Garcia RL, Lee M, Katz, R, Leslie KK, Risques RA, Swisher EM. Characterizing TP53 mutations in ovarian carcinomas with and without concurrent BRCA1 or BRCA2 mutations. *Gynecol Oncol* 2021, 160(3): 786-792. Acknowledgement of federal support: yes.
6. Gonzalez Bosquet J, Devor EJ, Newton AM, Smith BJ, Bender DP, Goodheart MJ, McDonald ME, Braun TA, Thiel KW, and Leslie KK. Creation and validation of models to predict response to primary treatment in serous ovarian cancer. *Scientific Reports* (2021) 11:5957. Acknowledgement of federal funding: yes.
7. Thiel KW, Devor EJ, Filiaci VL, Mutch D, Moxley K, Alvarez Secord A, Tewari KS, McDonald ME, Mathews C, Cosgrove C, Dewdney S, Aghajanian C, Samuelson MI, Lankes HA, Soslow RA, and Leslie KK. TP53 sequencing and p53 immunohistochemistry predict outcomes when bevacizumab is added to frontline chemotherapy in endometrial cancer: An NRG Oncology/Gynecologic Oncology Group Study. *J Clin Oncol* 40:3289-3300, 2022. Acknowledgement of federal funding: yes.

Poster Presentations

Star Tahy, Jamie Padilla, Lanie Smith and Kimberly K. Leslie. A novel mechanism of action of anti-angiogenic therapy: inhibition of the epigenetic modulator EZH2. Presented at the Student Poster Session, the University of New Mexico, 2022. Acknowledgement of federal support: yes.

Angelina Licor, Lanie Smith, Jamie Padilla, Vernon S. Pankratz, Kimberly K. Leslie. Efficacy of olaparib and p53 reactivators, separately and combined, to overcome PARP inhibitor resistance in ovarian cancer cells. Presented at the Student Poster Session, the University of New Mexico, 2022. Acknowledgement of federal support: yes. This poster won a "Best Poster" award.

Other Products

Research material: Patient-derived organoid models of ovarian cancer of differing histologies (high-grade serous, carcinosarcoma, clear cell, borderline, see **Table 1**).

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Role	Person months	Institution
Kimberly K. Leslie, MD	PI	No change	University of Iowa

Kristina W. Thiel, PhD	Co-I	No change	University of Iowa
Barbara Norquist, MD	Co-I	No change	University of Washington
Eric Devor, PhD	Co-I	No change	University of Iowa

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Leslie received a new endometrial cancer grant from the CDMRP (CA210610) which does not overlap with this ovarian cancer project.

What other organizations were involved as partners? Nothing to report other than the known collaboration with the University of Washington, as approved with the initial submission.

8. Special Reporting Requirements

Not applicable.

9. Appendices

*Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

Appendix 1: NRG Oncology Ancillary Projects Committee (APC) approval of translational research (APC #20018).

Appendix 2: Bi J, et al, Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers*. 2021.

Appendix 3: Bi J, et al, Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy. *Pharmaceuticals*. 2021

Appendix 4: Devor EJ, et al, The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells. *Pharmaceuticals*. 2021.

Appendix 5: Bi J, et al, Characterization of a TP53 Somatic Variant of Unknown Function From an Ovarian Cancer Patient Using Organoid Culture and Computational Modeling. *Clin Obstet Gynecol*. 2020.

Appendix 6: Bi J, Zhang Y, Malmrose, PK, Losh HA, Newton AM, Devor EJ, Thiel KW, Leslie KK. Blocking autophagy overcomes resistance to dual histone deacetylase and proteasome inhibition in gynecologic cancer. *Cell Death and Disease* (2022) 13:59.

Appendix 7: Ghezelayagh TS, Penington KP, Norquist BM, Khasnavis N, Radke MR, Kilgore MR, Garcia RL, Lee M, Katz, R, Leslie KK, Risques RA, Swisher EM. Characterizing TP53 mutations in ovarian carcinomas with and without concurrent BRCA1 or BRCA2 mutations. *Gynecol Oncol* 2021, 160(3): 786-792.

Appendix 8: Gonzalez Bosquet J, Devor EJ, Newton AM, Smith BJ, Bender DP, Goodheart MJ, McDonald ME, Braun TA, Thiel KW, and Leslie KK. Creation and validation of models to predict response to primary treatment in serous ovarian cancer. *Scientific Reports* (2021) 11:5957.

Appendix 9: Thiel KW, Devor EJ, Filiaci VL, Mutch D, Moxley K, Alvarez Secord A, Tewari KS, McDonald ME, Mathews C, Cosgrove C, Dewdney S, Aghajanian C, Samuelson MI, Lankes HA, Soslow RA, and Leslie KK. TP53 sequencing and p53 immunohistochemistry predict outcomes when bevacizumab is added to frontline chemotherapy in endometrial cancer: An NRG Oncology/Gynecologic Oncology Group Study. *J Clin Oncol* 40:3289-3300, 2022.

Appendix 1



Advancing Research. Improving Lives.™

March 8, 2021

Kimberly Leslie, MD
Department of Obstetrics and Gynecology
University of Iowa
20 Hawkins Drive, 31140 PFP
Iowa City, IA 52242

Dear Dr. Leslie,

We are pleased to report that the NRG Oncology Ancillary Projects Committee (APC) has approved your application, *"Subanalysis of TP53 Mutations as Predictors of Outcome on GOG-0218"* (APC #20018). Austin Miller, PhD, and/or their designee of the NRG Oncology Statistical and Data Management Center (SDMC), will work through project development with you and your team as well as perform the analyses for this project.

Please note, while approval is granted, NRG Oncology cannot immediately provide a timeline for the project's completion due to the resources available and priorities of our organization. The NRG SDMC will work with you and your team to prioritize this project.

Once the analysis is completed and a report provided, you will be expected to develop an abstract for presentation at a national or international scientific session and to prepare a manuscript for publication in a peer-reviewed journal. You are required to work with the NRG Oncology Statistician and the NRG Oncology Publications Department to ensure that all presentations and manuscripts resulting from this project adhere to the NRG Oncology Publications Policy and Guidelines.

Should you have any questions, feel free to contact the APC program, at APC@NRGOncology.org.

Sincerely,

A handwritten signature in black ink that reads "M Machtay".

Mitchell Machtay, MD
Deputy Group Chair for Research Strategy
Ancillary Projects Committee Chair

cc: Austin Miller, PhD
Christopher Purdy, MS
Virginia Filiaci, PhD
Steven Waggoner, MD

Article

Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing

Jianling Bi ^{1,†}, Andreea M. Newton ^{1,†} , Yuping Zhang ¹, Eric J. Devor ^{1,2} , Megan I. Samuelson ³, Kristina W. Thiel ^{1,2}  and Kimberly K. Leslie ^{1,2,*}

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Simple Summary: In the conventional treatment of gynecologic malignancies, most patients receive similar ‘one-size-fits-all’ treatment. However, it is increasingly clear that standard therapies do not work in every patient, and it would be very helpful to have pretreatment predictive assays to provide more personalized regimens. In this study, we describe the routine, successful establishment of patient-derived organoid models (PDOs) of endometrial and ovarian cancer tissues from consenting patients and provide an example of how information from drug screening in PDOs may be a useful predictor of patient response to therapy.



Citation: Bi, J.; Newton, A.M.; Zhang, Y.; Devor, E.J.; Samuelson, M.I.; Thiel, K.W.; Leslie, K.K. Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers* **2021**, *13*, 2901. <https://doi.org/10.3390/cancers13122901>

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Abstract: Developing reliable experimental models that can predict clinical response before treating the patient is a high priority in gynecologic cancer research, especially in advanced or recurrent endometrial and ovarian cancers. Patient-derived organoids (PDOs) represent such an opportunity. Herein, we describe our successful creation of 43 tumor organoid cultures and nine adjacent normal tissue organoid cultures derived from patients with endometrial or ovarian cancer. From an initial set of 45 tumor tissues and seven ascites fluid samples harvested at surgery, 83% grew as organoids. Drug sensitivity testing and organoid cell viability assays were performed in 19 PDOs, a process that was accomplished within seven days of obtaining the initial surgical tumor sample. Sufficient numbers of cells were obtained to facilitate testing of the most commonly used agents for ovarian and endometrial cancer. The models reflected a range of sensitivity to platinum-containing chemotherapy as well as other relevant agents. One PDO from a patient treated prior to surgery with neoadjuvant trastuzumab successfully predicted the patient’s postoperative chemotherapy and trastuzumab resistance. In addition, the PDO drug sensitivity assay identified alternative treatment options that are currently used in the second-line setting. Our findings suggest that PDOs could be used as a preclinical platform for personalized cancer therapy for gynecologic cancer patients.

Keywords: patient-derived organoids (PDOs); endometrial cancer; ovarian cancer; drug sensitivity testing; personalized medicine



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1. Introduction

Ovarian cancer is the fifth leading cause of death of women from cancer worldwide [1]. Due to the lack of symptoms during the early development of ovarian cancer, patients are usually diagnosed at an advanced stage and have the lowest average five-year survival rate (46%) among all patients with gynecologic malignancies [2]. In 2020, over 21,000 women were diagnosed with ovarian cancer and approximately 14,000 deaths occurred from this disease in the United States [3]. Endometrial cancer is the most common gynecologic malignancy. Although many patients present at an early stage and enjoy a good prognosis,

patients with advanced or recurrent endometrial cancer have a very low five-year survival rate (<20%). In 2020, over 65,000 women were diagnosed with endometrial cancer in the United States, and nearly 13,000 women died from this disease [3]. Additionally, endometrial cancer is one of only a few cancers for which the incidence and mortality have been increasing year after year since the 1970s [4].

Current standard treatments for ovarian and endometrial cancer are very similar and use a combination of surgery and chemotherapy [5,6]. Radiation and hormone therapies are two additional methods used to treat endometrial cancer, but for high-grade serous ovarian cancers and high-risk endometrial cancers, chemotherapy with the doublet of a platinum compound and a taxane is typically recommended [7,8]. Although most patients with advanced disease respond well to initial treatment, the majority develop recurrent disease and become resistant to chemotherapy. For recurrent and platinum-resistant disease, additional cytotoxic agents such as gemcitabine, topotecan, doxorubicin and liposomal doxorubicin have been employed as the second-line chemotherapy [9].

Molecular agents which target specific molecules or pathways are also available and used in the adjuvant setting [10]. A class of targeted agents used in ovarian cancer is PARP inhibitors, which are most effective for patients with germline or somatic mutations in *BRCA1/2* [11]. In both preclinical studies and translational studies of completed clinical trials, our group has recently reported that mutations in the tumor suppressor *TP53* predict the benefit of adding the antiangiogenic compound bevacizumab to chemotherapy upfront in advanced endometrial cancer [4,12]. However, for most patients, there are no genetic markers available to predict their response to commonly used agents [13]. Today, even as our knowledge of cancer genetics has increased dramatically, our ability to treat cancers based on the presence of genetic alterations is very limited. Particularly in gynecologic oncology, personalization of medical therapy is an unrealized goal [14].

Understanding patient and tumor genetic diversity and their influence on drug responses is an important step towards personalized medicine. In addition to research models to identify biomarkers of response to novel therapeutics or combinatorial regimens, we propose that patient-derived organoids (PDOs) hold great promise as valuable preclinical models which can provide insights into drug responses that are case-specific. Accumulating evidence indicates that PDOs can predict clinical outcomes in cancer patients [15–17]. Studies in several cancer types have established that PDOs recapitulate both the histologic and genomic features of the lesion from which they were derived [18–20]. Additionally, PDOs can grow with high efficiency in a short period of time which is much faster than generating a patient-derived xenograft (PDX model), enabling a priori prediction of responsiveness to chemotherapy, with the potential to substitute other regimens if primary resistance is demonstrated [21]. PDOs can also be tested for response to novel regimens including combinations of chemotherapy with targeted agents or multiple targeted agents which can be added to the patient's initial round of therapy [22,23].

In this study, we describe progress on the generation of novel endometrial and ovarian cancer PDOs and drug testing. As an example of the power of PDOs to predict clinical outcomes, we present a case in which the PDO results reflected patient resistance to standard therapy, something we argue could be predicted upfront in the future using the PDO method for screening.

2. Materials and Methods

2.1. Clinical Features

All the studies were reviewed and approved by the University of Iowa's IRB, protocol #201809807. The electronic medical record was reviewed to determine oncologic history, diagnosis, neoadjuvant and adjuvant treatment(s) and clinical responses. For patients who received platinum-based chemotherapy, platinum sensitivity was defined as progression-free survival of at least six months after the last platinum-containing therapy. Platinum resistance was defined as recurrence or persistence of disease within six months after the last platinum-containing treatment. Outcome data had not yet matured for some cases

at the time of manuscript preparation; these cases are denoted “TBD” (to be determined). HER immunochemistry (IHC) was performed using clinical standards by the Central Pathology Laboratory at the University of Iowa, a CLIA-certified laboratory.

2.2. Generation of Patient-Derived Organoid Models Using Tumor and Normal Cells

Informed consent was obtained under the University of Iowa’s IRB protocol #201809807. Fresh tumor tissue was collected from each patient’s interval debulking surgery. Each patient’s ascites fluid, tumor and normal tissues were obtained, processed and cultured to create PDO cultures as previously described [20,23]; see additional details in the Supplementary Methods and Supplementary Figure S1. For H&E staining and IHC for HER2, organoids were exposed to 4% paraformaldehyde (PFA) for fixing cells and dissolving Matrigel. After pelleting by centrifugation, histologic gel was added to each sample. The samples were then transferred to a cassette, fixed in 70% ethanol overnight, sectioned and stained with either H&E or the HER2/neu antibody as for the biopsy and primary patient tumor specimens by the Central Pathology Laboratory at the University of Iowa.

2.3. Organoid Viability Assay

Viability of the tumor and normal organoids following drug treatment was performed as previously described [23]; see additional details in the Supplementary Methods. Briefly, organoids were collected with an organoid harvesting solution (Cultrex, R&D Systems, Minneapolis, MN, USA) and digested to single cells with TrypLE Express (Gibco, Waltham, MA, USA). Single cells were suspended in AdDE+++ medium with 10% Matrigel and seeded at a density of 10,000 cells/well in an ultra-low attachment 96-well U-bottom white plate. After 1–4 days, the organoids were exposed to carboplatin (1 μ M), cisplatin (1 μ M), paclitaxel (10 nM), bevacizumab (1 μ M), gemcitabine (100 nM), topotecan (100 nM) or different combinations for 72 h at 37 °C. At the end of the incubation, an equal volume of the CellTiter-Glo 3D reagent (Promega, Madison, WI, USA) was added to each well and incubated for 25 min at room temperature. Luminescence, reflective of cell viability, was measured using a Gen5 Microplate Reader (BioTek, Winooski, VT, USA). All the tests were conducted in triplicate and the data were normalized to untreated controls (set at 100% viability).

2.4. Statistical Analysis

The data were analyzed using the GraphPad Prism software. Statistical significance of differences was determined using two-way ANOVA with the Greenhouse–Geisser correction and Tukey’s multiple comparison test, with individual variances computed for each comparison, or one-way ANOVA with Tukey’s multiple comparison test. All values are expressed as the mean $\pm$ standard deviation (SD) of at least three independent experiments unless otherwise indicated; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Feasibility of Organoid Culture Creation from Freshly Resected Endometrial and Ovarian Cancer

At the time of each patient’s surgical debulking, ascites fluid, tumor and normal tissues were obtained, processed and cultured to create PDO cultures (see the Supplementary Methods and Supplementary Figure S1).

We successfully generated tumor organoid cultures from 43 of 52 tumor samples, an establishment success rate of 83% (Table S1). Successful cultures included 21 ovarian tumors, 22 endometrial tumors and nine normal organoids from normal uterine or fallopian tube samples. While the majority of models were generated using tumor tissues, seven ascites fluid samples were used to create PDOs; among those, six PDOs were generated. Successful growth of PDOs typically occurred within two–three days of surgical resection. In 12 cases with neoadjuvant therapy, six PDOs were generated, for the establishment success rate of 50%. In 40 non-neoadjuvant therapy cases, 37 tumor organoids were generated (success

rate of 93%). We also observed that PDO generation using normal tissues was less successful than using tumor tissues, with the 33% establishment rate. The limited success rate may be due to the small size and the prevalence of stromal cells in the samples.

Next, drug screening was performed on 19 PDOs, a process that can be completed within 7–10 days from the time of surgery. Detailed information on the 19 models tested for drug sensitivity is summarized in Table 1.

3.2. Endometrial and Ovarian Cancer PDO Drug Response

Drug response assays were performed on 19 ovarian and endometrial cancer PDOs (Table 1) using therapeutic agents which are most commonly employed to treat patients with gynecologic cancer: carboplatin, paclitaxel, cisplatin, bevacizumab, gemcitabine and topotecan. PDO models that were created using ascites fluid samples are indicated with an * in Table 1.

Table 1. Patient and tumor characteristics corresponding to the 19 PDOs used for drug screening studies. IV: intravenous; IP: intraperitoneal; NED: no evidence of disease; N/A: not available; TBD: to be determined; * indicates the PDOs that were generated using the ascites fluid samples.

Patient ID	Cancer Type	Stage	Neoadjuvant Treatment	Adjuvant Treatment	Platinum-Sensitive?	Disease Status
ONC-5942	High-grade serous	IVB	Six cycles of carboplatin and paclitaxel followed by two cycles of carboplatin	Three cycles of doxorubicin and bevacizumab followed by bevacizumab	No	NED
ONC-6007 *	High-grade serous	IVB	No	Six cycles of IV/IP cisplatin and paclitaxel	Yes	Alive with disease
ONC-6045 *	High-grade serous	IIIB	No	One cycle of a single agent, carboplatin, followed by six cycles of carboplatin and paclitaxel	Yes	NED
ONC-6069	High-grade serous	Recurrent	Six cycles of carboplatin and paclitaxel followed by olaparib	Olaparib followed by gemcitabine	No	Dead of disease
ONC-6072	High-grade serous	IC	No	None	N/A	Lost to follow-up
ONC-6134	High-grade serous	IIIA	No	Six cycles of carboplatin and paclitaxel	Yes	NED
ONC-6163	High-grade serous	Recurrent	No	Six cycles of carboplatin and doxorubicin followed by olaparib	TBD	NED
ONC-7052	High-grade serous	IIIC	No	Six cycles of IV/IP cisplatin and paclitaxel	TBD	NED
ONC-6061	Low-grade serous	IIIC	No	Two cycles of carboplatin and paclitaxel followed by seven cycles of a single agent, carboplatin, followed by letrozole	No	Alive with disease
ONC-7063	Clear cell	IC1	No	Three cycles of carboplatin and paclitaxel	TBD	NED
ONC-6092	Clear cell	IC	No	Three cycles of carboplatin and paclitaxel	Yes	NED

Table 1. Cont.

	Patient ID	Cancer Type	Stage	Neoadjuvant Treatment	Adjuvant Treatment	Platinum-Sensitive?	Disease Status
Endometrial Cancer	ONC-6096	Endometrioid, grade 1	IA	No	Observation	N/A	NED
	ONC-6191	Endometrioid, grade 1	IB	No	N/A	N/A	NED
	ONC-6051	Endometrioid, grade 2	IA	No	Observation	N/A	NED
	ONC-6173	Endometrioid, grade 2	IA	No	Observation	N/A	NED
	ONC-6071	Endometrioid, grade 3	IA	No	Observation	N/A	NED
	ONC-6057	Serous	IVB	Three cycles of carboplatin, paclitaxel and trastuzumab	Three cycles of carboplatin, paclitaxel and trastuzumab followed by a single agent, bevacizumab	No	Alive with disease
	ONC-6099	Serous	IVB	Six cycles of carboplatin and paclitaxel	Three cycles of paclitaxel and bevacizumab	No	Dead of disease
	ONC-7003	Mixed serous/endometrioid	IA	No	Patient declined adjuvant therapy	N/A	NED

*: PDO models that were created using ascites fluid samples.

Carboplatin, paclitaxel and their combination are the most frequently used in first-line chemotherapeutic regimens for gynecologic malignancies. PDO responses to these agents are shown in Figure 1A. As compared to the untreated controls, nine of the 19 PDOs exhibited a notable decrease in viability when treated with the combination of carboplatin and paclitaxel, with viability ranging from 46.1% to 72.7% after 72 h of treatment. In the combinatorial setting, the effects of the taxane, and not the platinum compound, appear to be the predominant driver of therapeutic effectiveness.

Bevacizumab is an antiangiogenic agent frequently used as an adjuvant therapy in gynecologic cancer [12,24–26]. Therefore, we also screened the PDOs for sensitivity to bevacizumab alone and in combination with the first-line standard chemotherapy. In general, bevacizumab as a single agent had only a modest impact on cell viability and little additional impact on cell killing when combined with chemotherapy (Figure 1B).

As an alternative to the first-line standard chemotherapy via the intravenous (IV) route, cisplatin combined with paclitaxel provides another option for patients to receive chemotherapy by the intraperitoneal (IP) route or via heated intraperitoneal chemotherapy [27,28]. In general, compared to the combination of carboplatin and paclitaxel, cisplatin combined with paclitaxel had similar impacts on cell viability (Figure 1C).

Gemcitabine and topotecan are two commonly used second-line agents for cases of recurrent platinum-resistant gynecologic cancer [29–33]. Herein, we compared the effects of gemcitabine, topotecan and gemcitabine plus bevacizumab on cell viability. For some patients (ONC-6051, ONC-6072, ONC-6092), second-line therapies were more effective with respect to PDO cell killing than the first-line agents, but for most patients, the impact of those agents was not superior (Figure 2).

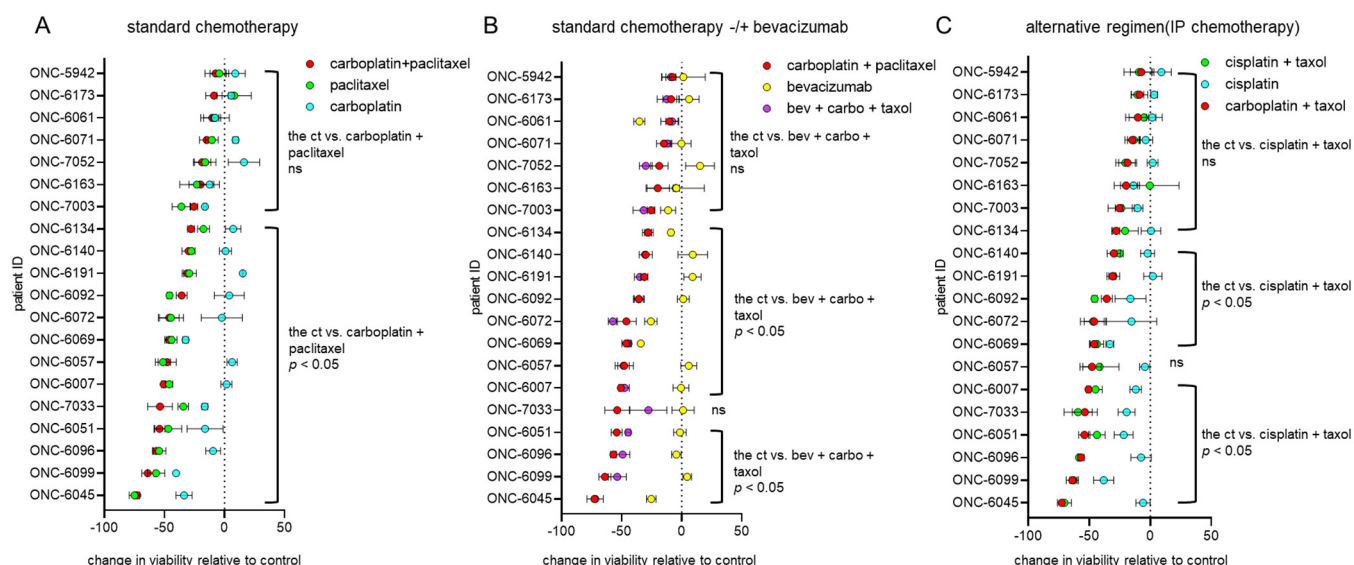


Figure 1. Therapeutic efficacy of the first-line therapies for endometrial and ovarian cancers using PDO models. Endometrial and ovarian cancer PDO response to (A) the standard chemotherapy, (B) the standard chemotherapy $\pm$ bevacizumab and (C) the most common alternative chemotherapy regimen. PDO models were treated with the indicated agents for 72 h, followed by assessment of cell viability. The data were calculated as the change in viability relative to the control which was set at 100% (i.e., no cell death). The samples are ordered based on increasing sensitivity to carboplatin + paclitaxel (red circles). Statistical significance was assessed by two-way ANOVA with the Greenhouse–Geisser correction and Tukey’s multiple comparison test. Significant differences vs. the control are annotated on each panel. All the statistical comparisons are provided in Supplementary Table S2, including specific p -values and comparisons of single agents vs. control or dual treatments.

3.3. Proof-of-Concept: A PDO Model Reflects Treatment Response

3.3.1. Patient Characteristics

Tissues were obtained from a 70-year-old woman who initially presented with shortness of breath, early satiety and postprandial right upper quadrant pain. Imaging was significant for a right pleural effusion, and the patient underwent a thoracentesis with temporary relief in symptoms. Cytology from her thoracentesis revealed PAX8-positive adenocarcinoma indicative of a gynecologic origin. Further imaging revealed multiple pulmonary emboli (PE), carcinomatosis, omental caking, ascites and a thickened endometrial stripe. Her history was negative for postmenopausal bleeding. An endometrial biopsy was obtained, which showed high-grade serous endometrial carcinoma, and HER2 immunostaining was positive (Figure 3A, pretreatment biopsy).

Given her new PE diagnosis and advanced-stage disease, the patient received three cycles of neoadjuvant carboplatin, paclitaxel and trastuzumab, a monoclonal antibody against HER2 (Figure 3B) [34]. Her CA125 decreased from 487 pre-treatment to 141 after the third cycle. Posttreatment imaging showed resolution of ascites, peritoneal carcinomatosis and omental cake. The patient subsequently underwent an exploratory laparotomy, total abdominal hysterectomy with bilateral salpingo-oophorectomy, infragastric and infracolic omentectomy, argon beam coagulation of tumor implants and optimal interval debulking. Final pathology from her debulking revealed her tumor to be of mixed serous and clear cell uterine histologies. Her postoperative course was uncomplicated, and she completed three more cycles of carboplatin, paclitaxel and trastuzumab with plans to continue maintenance trastuzumab until progression or toxicity. The patient received one cycle of maintenance trastuzumab, but before she was able to receive her second cycle (eighth overall), she experienced a rising CA125 and increasing shortness of breath. Imaging showed a recurrent pleural effusion, and a thoracentesis revealed recurrent adenocarcinoma.

Immunohistochemistry (IHC) performed on the cytology from this thoracentesis was estrogen receptor/progesterone receptor (ER/PR)-negative, suggesting that her pleural

effusion was from the clear cell component of her cancer since the clear cell component of the primary tumor specimen was also negative for ER and PR. The patient then received three cycles of a single agent, bevacizumab, during which her CA125 continued to rise, and her dyspnea was stable. Repeat imaging showed no evidence of intraabdominal disease, but she had persistent pleural effusions. Due to poor tolerance of bevacizumab, the patient requested a chemotherapy holiday with as-needed thoracenteses.

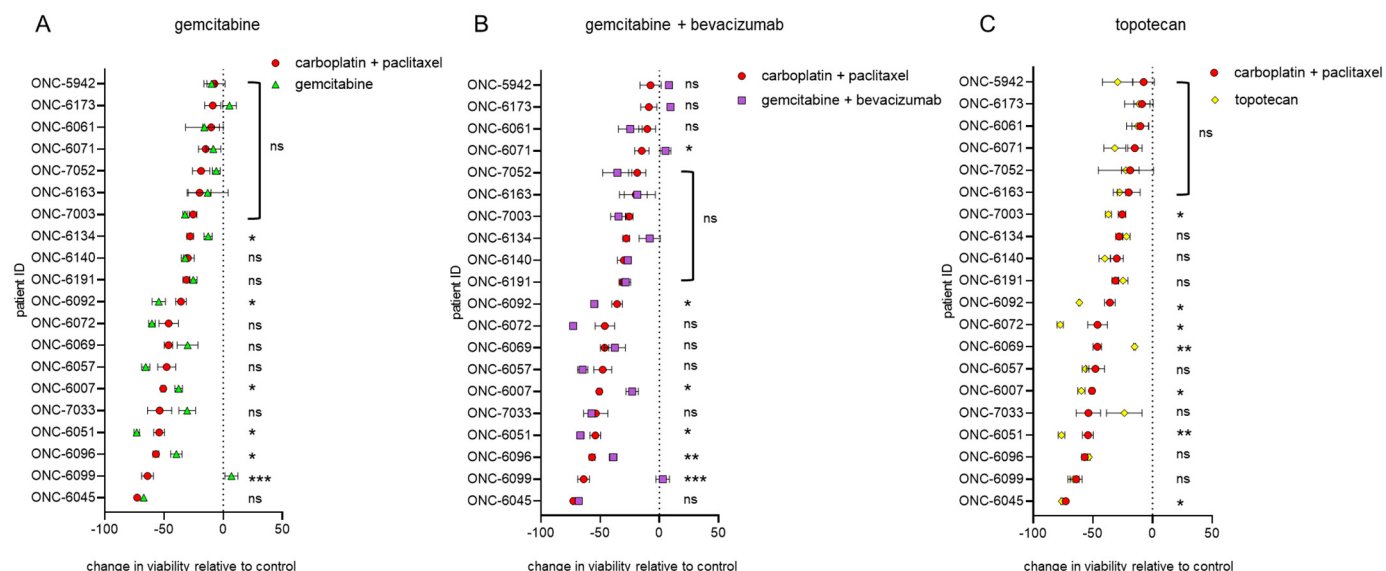


Figure 2. Therapeutic efficacy of common second-line therapies for endometrial and ovarian cancers using PDO models. Endometrial and ovarian cancer PDO response to second-line therapies: (A) gemcitabine, (B) gemcitabine + bevacizumab and (C) topotecan. PDO models were treated with the indicated agents for 72 h, followed by assessment of cell viability as in Figure 1. The data were calculated as the change in viability relative to the control, which was set at 100% (i.e., no cell death). Each panel includes response to the standard chemotherapy (carboplatin + paclitaxel, red circles) for comparison, and the samples are ordered based on increasing sensitivity to carboplatin + paclitaxel. Statistical significance was assessed by two-way ANOVA with the Greenhouse–Geisser correction and Tukey’s multiple comparison test. Significant differences vs. carboplatin + paclitaxel are annotated on each panel; ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All the statistical comparisons are provided in Supplementary Table S2, including specific p -values and comparisons vs. the untreated control. Note that some cases have greater sensitivity to second-line treatments as compared to the standard chemotherapy, whereas others are more resistant. For example, ONC-6099 is relatively sensitive to chemotherapy but had no change in viability when exposed to gemcitabine (panel A).

3.3.2. Trastuzumab Response in the Patient-Derived Tumor Organoid Model

This patient’s tumor was among the PDOs we created. We examined whether the tumor previously exposed to trastuzumab in the neoadjuvant setting before surgical resection would continue to demonstrate sensitivity to this agent or whether neoadjuvant treatment would select for resistant cell clones by the time surgery was performed. Thus, we assessed sensitivity of PDO cells to increasing concentrations of trastuzumab. Our results reflected potential resistance to trastuzumab based upon a lack of reaching an IC₅₀ even at supraphysiologic concentrations (200 μ g/mL) (Figure 3D). The lack of response to trastuzumab was also predicted by the lack of HER2 immunostaining in the postsurgical specimen and the organoid sample (Figure 3A,B). Pathological analysis of the postsurgical specimen indicated some HER2 immunoreactivity in the cystically dilated glandular areas (Figure 3A, right margin of the posttreatment surgical specimen), whereas the stromal region and clear cell component (left side of the posttreatment surgical specimen) were devoid of HER2.

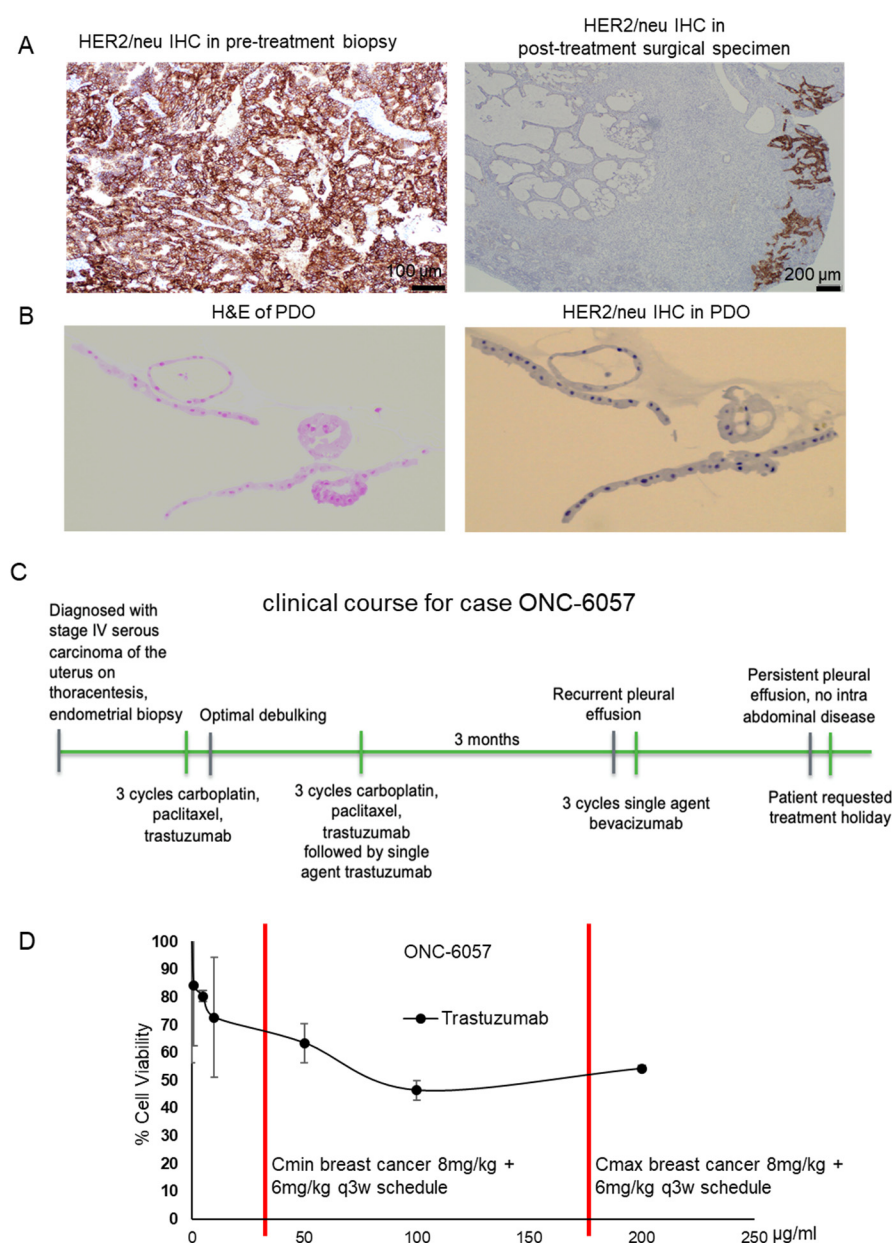


Figure 3. One advanced-stage high-grade serous endometrial carcinoma PDO model (ONC-6057) successfully predicted the patient's clinical platinum and trastuzumab resistance. **(A)** HER2 IHC of the patient's tumor obtained from the pre-treatment biopsy and the post-treatment surgical specimen. Additional images are provided in Supplementary Figure S2. **(B)** H&E staining and HER2 IHC for the corresponding PDO model. Additional images are provided in Supplementary Figure S3. **(C)** Timeline of the patient's clinical course. **(D)** PDO cells were treated with increasing concentrations of trastuzumab for 72 h, followed by assessment of cell viability. The data were calculated as the cell viability (%) relative to the untreated control which was set at 100%. The graph is annotated with the Cmin and Cmax values for trastuzumab used clinically in breast cancer. Note that an IC50 was not achieved with trastuzumab, even at concentrations greater than the Cmax.

Given her new PE diagnosis and advanced-stage disease, the patient received three cycles of neoadjuvant carboplatin, paclitaxel, and trastuzumab, a monoclonal antibody against HER2 (Figure 3B) [34]. Her CA125 decreased from 487 pre-treatment to 141 after the third cycle. Posttreatment imaging showed resolution of ascites, peritoneal carcinomatosis and omental cake. The patient subsequently underwent an exploratory laparotomy, total abdominal hysterectomy with bilateral salpingo-oophorectomy, intra-gastric patient received after failing trastuzumab. Organoid cells were more susceptible to paci-

taxel, with 48.7% cell viability after exposure to this single agent. The patient's PDO was also more sensitive to gemcitabine (65% decrease in viability) and topotecan (56% decrease in viability) as compared to the chemotherapy regimen previously received, carboplatin + paclitaxel.

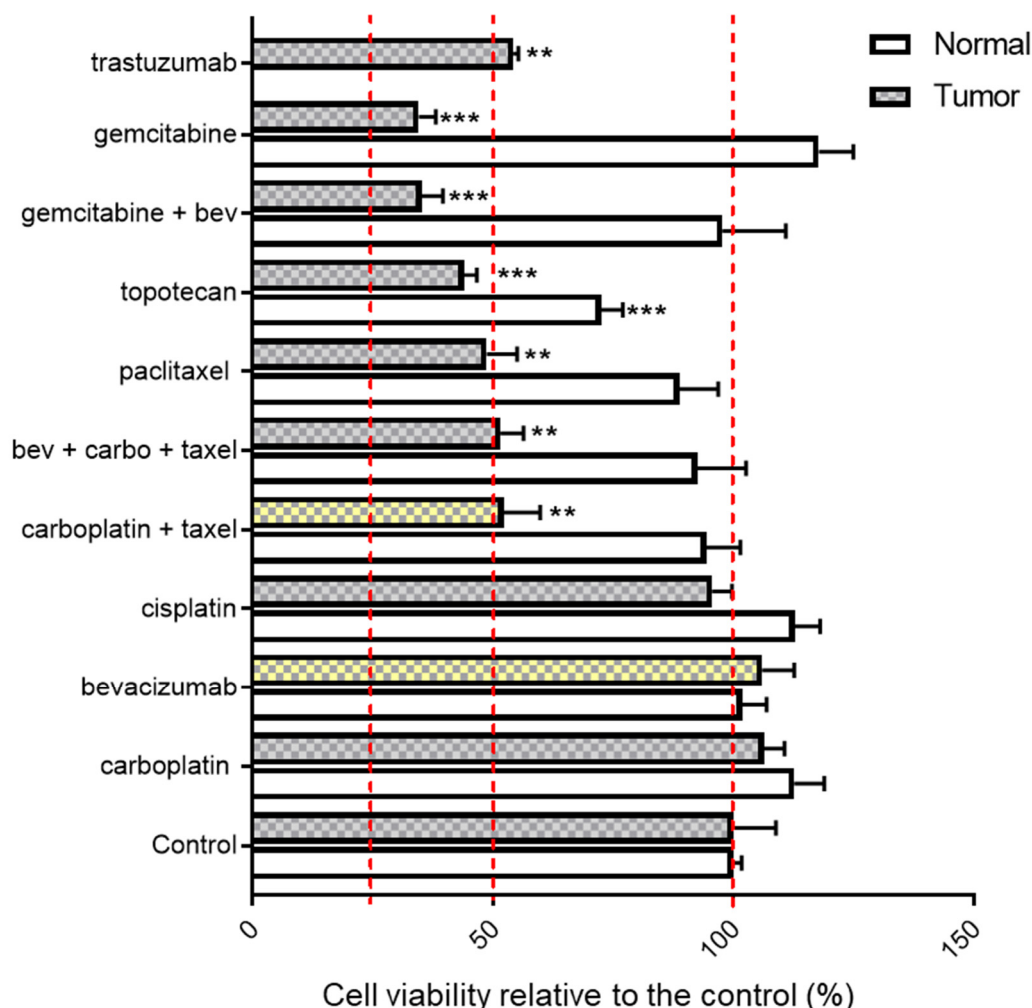


Figure 4. Varying drug sensitivity to the standard and alternative therapeutic options for the ONC-6057 PDO. Waterfall plot of the patient's (ONC-6057) tumor organoid drug sensitivity with the paired normal uterine drug sensitivity. The cytotoxic agents in the chemotherapy regimen (carboplatin + paclitaxel and bevacizumab) the patient received after resistance to trastuzumab was observed are highlighted in yellow. The change in viability was calculated relative to the control which was set at 100% (i.e., no cell death). Statistical significance was assessed by ordinary one-way ANOVA with Tukey's multiple comparison test; ** $p < 0.01$, *** $p < 0.001$ vs. the paired control (normal or tumor specimen).

We also assessed drug sensitivity using a subset of agents in normal tissue organoids from the same patient (Figure 4). Gemcitabine, topotecan and paclitaxel, which induced more than 50% cell death in tumor organoids, all resulted in minimal cell killing of organoids derived from non-malignant tissues.

4. Discussion

We intuitively know that each patient's tumor is different, with potentially unique genetic and phenotypic characteristics. It is becoming increasingly clear that the conventional way to treat patients using a limited panel of first-line agents may miss important opportunities to optimize therapy. In contrast, personalized medicine strives to match the unique patient and tumor characteristics with innovative treatment regimens tailored to each case; however, progress in implementing precision therapies has been hampered by

the lack of sufficient biomarkers that predict response to each therapeutic agent. One way to improve outcomes without the long and laborious process of biomarker identification and validation is to utilize tumor cells themselves as a patient avatar.

PDO culture technology is a novel and powerful tool for generating and maintaining three-dimensional patient tumor and normal tissues in vitro [35]. Since the time from surgical resection to drug testing results takes 7–10 days, use of organoids from primary patient tissues enables the testing of a range of compounds for efficacy even before clinical treatment decisions must be made. In addition, the establishment of organoids from non-malignant tissues from the same patient provides an opportunity to test potential drug toxicities. The methods reported in this study as well as the results reported by others [36] strongly support the concept that establishment of PDOs from patients under treatment for gynecologic cancers is feasible and potentially useful to understand the impact of therapeutic agents on cell viability in a time frame that is congruent with making actionable treatment decisions [37,38]. We propose that testing PDOs for therapeutic response is a methodology worthy of expansion as a means to achieve precision medicine in clinical practice.

Overall, our success rate in generating PDOs was quite high. Among the nine cases which failed to culture, two thirds were from the patients who received neoadjuvant chemotherapy. Neoadjuvant therapy has been shown to negatively affect the success rate of PDO generation [39]. We speculate the reason why tumor tissues from the patients who received neoadjuvant chemotherapy failed to generate organoids is because the tumors may already be undergoing chemotherapy-induced cell death. Accordingly, after we collected the tumor and isolated cells from the tumor, cell viability was too low for organoid culture. In addition, for the vast majority of tissues that were not successfully cultured, we observed that the time from surgical removal to culture was greater than 45 min, though some PDO models were successfully cultured after a delay of several hours. Nonetheless, mitigating the delay from surgical excision to tissue processing will undoubtedly improve the success rate of PDO creation. Other variables include the sample size and the percentage of viable cells within the specimen.

To illustrate how PDO data could be used in the future to predict clinical outcomes, tumor and normal PDOs were established from a patient with advanced high-grade serous endometrial carcinoma. Drug sensitivity testing reflected resistance to all agents given in the neoadjuvant setting. This raises the possibility of tumor evolution after initial exposure to drugs and brings into question the continued use of agents used in the neoadjuvant setting for ongoing adjuvant treatment, i.e., after surgical removal of the tumor. We propose that drug sensitivity testing of PDOs may provide more insight into which agents are most effective at later timepoints. This is based on our proof-of-concept data that the PDO model successfully predicted the patient's clinical platinum and trastuzumab resistance despite her initial response to neoadjuvant chemotherapy.

Although PDOs have proved useful for many applications in basic research and contributed to biomedical advances [40], the road to real-life applications is long, with many technologies to be explored in this area. PDOs have not yet been widely used for translational studies yet due to some limitations [41]. For example, compared with cancer cell lines, organoid culture consumes more time and resources [40]. For most models we created, the amount of tumor tissue we received from the operating room was quite small, and we prioritized drug screening experiments. This precluded extensive molecular characterization for each model, such as comparison of biomarkers between the primary tumor and the PDO model. Given the potential for evolution with prolonged culture, we performed the bulk of experiments on passage 1–2 samples rather than expanding them to get enough cells for additional analyses. While we stored some organoids in liquid nitrogen, we found that the growth rate is significantly slower after cryopreservation. Another limitation is PDO culture may not reflect the tumor environment due to lack of stroma, blood vessels and immune cells [42]. Incomplete representation of intratumoral heterogeneity is another limitation for PDO culture. Many studies have proved that PDOs

can retain the heterogeneous genetic composition of the primary tumor [43,44]. However, each PDO is derived from a small part of the tumor and may not reflect different cell populations of the primary tumor. For example, Roerink et al. have shown the extensive genetic diversification in organoids derived from different regions of the same tumor using comprehensive genetic analysis [45]. Despite these limitations, organoids have emerged as a physiologically relevant ex vivo model to study cancer. In addition, there are several co-clinical trials ongoing in different cancer types that compare drug responses in PDOs to corresponding patient outcomes (e.g., NCT03979170, NCT04859166, NCT04555473).

In conclusion, PDOs provide a valuable preclinical model system to support new opportunities for personalized medicine. Our results demonstrate, first, that PDOs can be established in a high percentage of cases; second, that drug testing on PDOs can be performed in a timely manner and that PDOs have the potential to identify agents with the greatest efficacy in each individual case. This is an important first step in establishing a feasible functional treatment response platform to help more patients with gynecologic cancer receive precision therapeutic care.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cancers13122901/s1>, Supplementary Methods: Protocol for Organoid Generation, Figure S1: Schematic presentation of procedures for the organoid culture of endometrial and ovarian cancer cells derived from patient tumors, Figure S2: Pretreatment biopsy specimen for ONC-6057, Figure S3: Additional H&E and HER2 IHC images of the ONC-6057 PDOs, Table S1: Characteristics of all the specimens attempted to be cultured as PDO models, including the paired normal tissue specimens, Table S2: Statistical analyses for drug screening in Figures 1 and 2.

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Article

Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy

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Abstract: Angiogenesis plays a crucial role in tumor development and metastasis. Both bevacizumab and cediranib have demonstrated activity as single anti-angiogenic agents in endometrial cancer, though subsequent studies of bevacizumab combined with chemotherapy failed to improve outcomes compared to chemotherapy alone. Our objective was to compare the efficacy of cediranib and bevacizumab in endometrial cancer models. The cellular effects of bevacizumab and cediranib were examined in endometrial cancer cell lines using extracellular signal-related kinase (ERK) phosphorylation, ligand shedding, cell viability, and cell cycle progression as readouts. Cellular viability was also tested in eight patient-derived organoid models of endometrial cancer. Finally, we performed a phosphoproteomic array of 875 phosphoproteins to define the signaling changes related to bevacizumab versus cediranib. Cediranib but not bevacizumab blocked ligand-mediated ERK activation in endometrial cancer cells. In both cell lines and patient-derived organoids, neither bevacizumab nor cediranib alone had a notable effect on cell viability. Cediranib but not bevacizumab promoted marked cell death when combined with chemotherapy. Cell cycle analysis demonstrated an accumulation in mitosis after treatment with cediranib + chemotherapy, consistent with the abrogation of the G2/M checkpoint and subsequent mitotic catastrophe. Molecular analysis of key controllers of the G2/M cell cycle checkpoint confirmed its abrogation. Phosphoproteomic analysis revealed that bevacizumab and cediranib had both similar and unique effects on cell signaling that underlie their shared versus individual actions as anti-angiogenic agents. An anti-angiogenic tyrosine kinase inhibitor such as cediranib has the potential to be superior to bevacizumab in combination with chemotherapy.

Keywords: endometrial cancer; bevacizumab; cediranib; patient-derived organoid models; 3D culture; personalized medicine; p53; vascular endothelial growth factor (VEGF)

1. Introduction

Endometrial carcinoma is the most common gynecologic malignancy in the United States. In 2020, over 65,000 women were diagnosed with endometrial cancer and nearly 13,000 women died of this disease [1]. Although the five-year survival rate of patients with early-stage endometrial cancer is relatively high, patients with advanced or recurrent endometrial cancer have a poor prognosis. Additionally, population studies have shown that endometrial adenocarcinoma is one of the only cancers in which prevalence and

mortality are increasing [2]. Recent progress has been made in defining the molecular biology of endometrial carcinoma, which has led to the use of targeted agents to treat this disease. The addition of targeted therapies including anti-angiogenics in conjunction with chemotherapy may be more effective than chemotherapy alone, as we have recently reported [3].

The Gynecologic Oncology Group (GOG, now NRG Oncology) has evaluated a series of anti-angiogenic agents in recurrent endometrial carcinoma. Bevacizumab (Avastin[®], Genentech) is a recombinant humanized antibody against vascular endothelial growth factor-A (VEGF-A), and it has been studied as a single agent in women with recurrent endometrial cancer in study protocol GOG-229E [4]. From this study, bevacizumab significantly improved PFS compared to additional treatment with chemotherapy [4]. GOG-229G, a Phase II trial of combination bevacizumab and temsirolimus (an mTOR inhibitor) was also deemed active in the treatment of recurrent endometrial carcinoma; however, the combination showed significant toxicity [5].

Cediranib is a small molecule multi-tyrosine kinase receptor inhibitor that targets VEGF receptors (VEGFR1/2/3), platelet-derived growth factor receptors (PDGFR α / β and c-Kit), and fibroblast growth factor receptor (FGFR1) [6,7]. GOG-229J was a study of cediranib as monotherapy in advanced endometrial carcinoma that, like bevacizumab, met the criteria for being considered an active agent in advanced endometrial cancer [8]. It was also found to be tolerable when administered as a single agent [8]. Other anti-angiogenic agents have also been investigated, but they have shown limited activity as single agents in unselected patients [9–12]. The GOG-86P trial of bevacizumab with chemotherapy in patients with advanced endometrial carcinoma did not show a statistically significantly increased PFS relative to historical controls when patients were analyzed as an entire cohort [13].

Preclinical studies have clearly demonstrated the activity of single-agent bevacizumab on a variety of tumor models, including endometrial carcinoma. In an orthotopic mouse model of endometrial cancer, bevacizumab significantly reduces tumor volume compared to control [14]. A previous study by our group also showed that bevacizumab inhibits the growth of endometrial cancer in a mouse xenograft model [15]. The anti-tumor activity of cediranib has been demonstrated in tumor xenograft models of various types of cancers including colon, lung, prostate, breast, and ovarian cancer [6,7,16]. In addition, cediranib decreases tumor vessel density and promotes vascular regression [6].

The vast majority of studies of antiangiogenic agents in endometrial cancer have focused on the inhibitory effects on the tumor vasculature. We hypothesized that therapeutic efficacy could also be derived from effects on the tumor cells. To date, cediranib has not been studied in combination with chemotherapy in endometrial cancer despite the documentation from GOG0229J that it has single-agent activity. The studies reported herein were designed to compare the impact of cediranib to bevacizumab to assess whether cediranib may also be effective as an anti-angiogenic and as an adjuvant to chemotherapy. Hence, we compared the efficacy of the two most active anti-angiogenic agents in endometrial cancer, bevacizumab and cediranib, on tumor cell survival and signaling. Whereas previous studies of bevacizumab activity were performed in vivo using xenograft models of endometrial cancer [14,15], herein we used immortalized endometrial cancer cell lines and patient-derived organoid models of endometrial cancer to specifically assess the impact of the agents on the tumor cells. Strengths and limitations of the various models of endometrial cancer have been recently reviewed [17]. Several well-characterized endometrial cancer cell lines are available for in vitro studies, including the poorly differentiated Hec50 and KLE cells. These cell lines are models of more aggressive endometrial cancer and harbor distinct mutations in *TP53* that are frequently detected in patient tumors [18]. This study also includes patient-derived organoid models of endometrial cancer whereby freshly resected tumor specimens are cultured in a three-dimensional model system [19,20]. As compared to patient-derived xenograft models, which also use fresh patient tumor tissue, patient-derived organoids are much more cost-effective with a higher

higher rate of successful culture [17]. Both patient-derived organoid and xenograft models retain intratumoral heterogeneity, which is lacking in immortalized 2D cell lines [17]. A rate of successful culture [17]. Both patient-derived organoid and xenograft models retain intratumoral heterogeneity, which is lacking in immortalized 2D cell lines [17]. A limitation of organoids as compared to xenografts is the absence of tumor vasculature. However, this provided a unique opportunity herein to study the tumor response to anti-angiogenic agents independent of vascular effects. However, this provided a unique opportunity herein to study the tumor response to anti-angiogenic agents independent of vascular effects.

2. Results

2.1. Results: Kinase Receptor Signaling Pathways Are Downregulated by Cediranib but Not Bevacizumab

2.1.1. Kinase Receptor Signaling Pathways Are Downregulated by Cediranib But Not Bevacizumab

We first established the inhibitory potential of bevacizumab and cediranib on ligand-stimulated extracellular signal-related kinase (ERK) activation in human vascular endothelial cells (HUVEC) and endometrial cells (Hec50) (Figure 1A). In contrast to robust inhibition of ERK phosphorylation at Thr202/Tyr204 by both bevacizumab and cediranib in HUVEC cells, the phosphorylation form of ERK displayed no significant induction with VEGF-A treatment and no obvious change with the addition of bevacizumab in Hec50 and KLE cells. These data suggest that the intracellular signaling impact of VEGF-A is not as robust in these tumor cells as compared to the known impact of VEGF-A on the vasculature of the surrounding tumor microenvironment (Figure 1A, middle and right panels) [6].

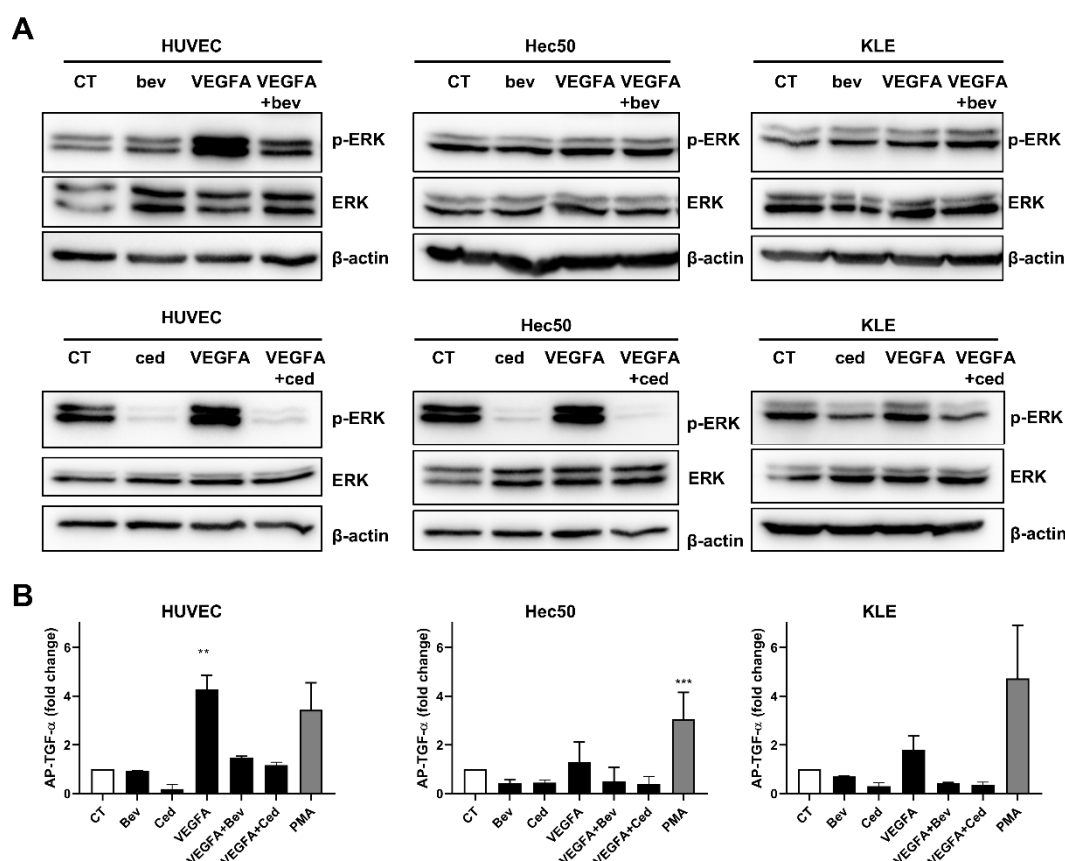


Figure 1. Impact of bevacizumab and cediranib on VEGF/ERK signaling in endothelial cells and endometrial cancer cells. (A) HUVEC, Hec50, and KLE cells were treated with vehicle control (CT), 1 μ M bevacizumab (Bev), 1000 ng/mL VEGF-A, or 1 μ M cediranib (Ced) for 1 hr, followed by assessment of ERK1/2 phosphorylation at Thr202/Tyr204 of total ERK expression by Western blotting. β -actin: loading control. (B) Cells were transfected with the alkaline phosphatase (AP)-tagged ADAM17 substrate TGF- α , and treated as in (A) for 1 hr. The change in soluble AP-TGF- α was assessed and presented as fold change compared to control (CT). PMA (25 ng/mL) served as a positive control for induction of AP-TGF- α shedding. ** $p < 0.01$; *** $p < 0.001$ versus CT by one-way ANOVA with Tukey's multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$ versus CT by one-way ANOVA with Tukey's multiple comparisons test.

2.2. Cediranib but Not Bevacizumab Synergizes with Chemotherapy

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Based on the differential responses of bevacizumab and cediranib in our cell-based assays, we next examined the responses of bevacizumab and cediranib in our cell-based assays. We next examined the impact of the response on cell viability in endometrial cancer models. Studies were first performed in eight patient-derived organoid models of endometrial cancer (Supplemental Table S1) and in eight patient-derived stage IV endometrial cancer cell lines (Supplemental Table S1). The model consists of the previous endometrial cancer cell lines from early-stage/endometrial cancer or primary tumor tissue (serous adenocarcinoma, WRTT virus), which are treated with the combination of tyrosine kinase inhibitors (TKIs) or anti-angiogenic TKIs with the chemotherapy paclitaxel induces massive cell death via mitotic catastrophe [25–27]. We therefore screened the endometrial cancer organoid models for sensitivity to paclitaxel alone or in combination with bevacizumab or cediranib (Figure 2).

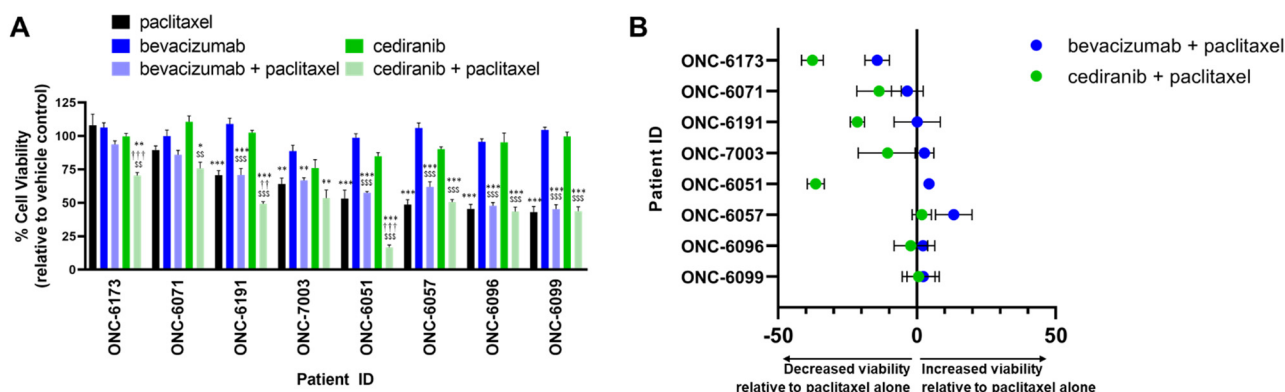


Figure 22. Effect of cediranib and bevacizumab on sensitivity to paclitaxel in patient-derived organoid cultures of primary endometrial tumors. Organoid cultures were treated with 110 nM paclitaxel, 11 μ M bevacizumab, 11 μ M cediranib, or the combination of paclitaxel with bevacizumab or cediranib for 72 hrs, followed by assessment of cell viability. (A) Data were calculated as the percent (%) cell viability as compared to vehicle control and plotted left-to-right by increasing sensitivity to single-agent paclitaxel. (B) The change in viability with the combination of paclitaxel with either bevacizumab or cediranib was calculated relative to paclitaxel alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; ++ $p < 0.01$, +++ $p < 0.001$ versus paclitaxel alone; \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ versus anti-angiogenic agent alone (bevacizumab for bevacizumab + paclitaxel or cediranib for cediranib + paclitaxel treated samples) by ordinary one-way ANOVA with Tukey's multiple comparisons test.

Compared to the untreated control, six of the eight patient-derived organoids exhibited a significant decrease in viability when treated with the paclitaxel, with a maximal decrease in viability of 57% by 72 h (Patient #ONC-6099) (Figure 2A). Both cediranib and bevacizumab as single agents had no impact on cell viability. The decrease in viability of combinatorial regimens was calculated relative to paclitaxel as a single agent.

combinatorial regimens was calculated relative to paclitaxel as a single agent (Figure 2B). In five of the models, cediranib but not bevacizumab further decreased cell viability when combined with paclitaxel as compared to treatment with paclitaxel alone (Figure 2B). In five of the models, cediranib but not bevacizumab further decreased cell viability when combined with paclitaxel as compared to treatment with paclitaxel alone (Figure 2B, ONC-6173–6071, 6191–7003 and 6051). Studies were then extended to well-characterized cell models of advanced endometrial cancer with varying baseline sensitivity to paclitaxel [26,27] (Figure 3). As in the organoid models, bevacizumab did not provide any additional cell killing when combined with paclitaxel (Figure 3A). Dose-response curves using either paclitaxel or cediranib in the presence of a set concentration of the opposing drug indicate synergy when the two agents are combined as compared to a single drug alone (Figure 3B,C). This trend was most pronounced in Hec50 cells, with no synergy observed in KLE cells treated with equivalent doses of the drugs (Figure 3B,C).

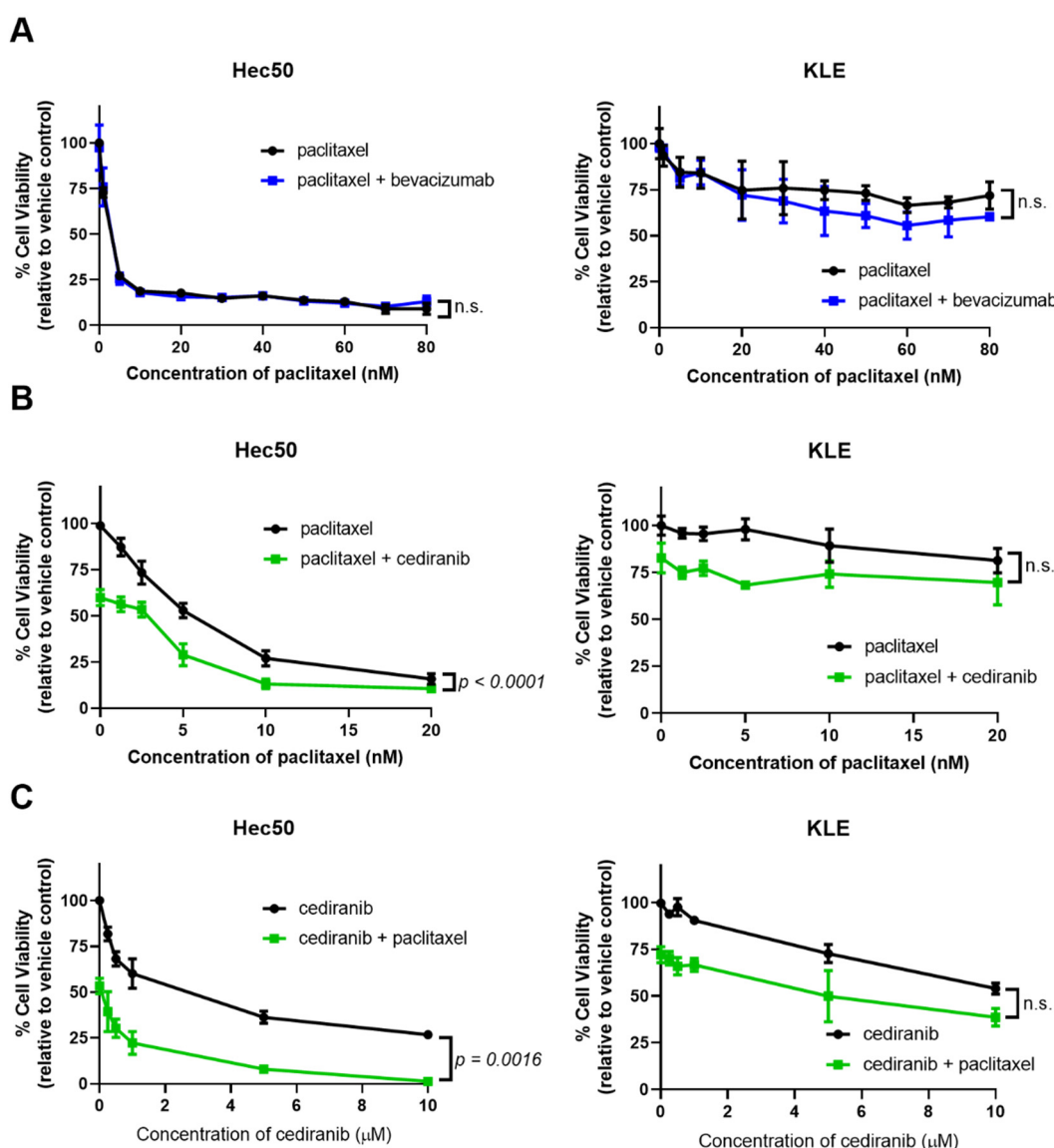


Figure 3. Cediranib but not bevacizumab increases sensitivity to paclitaxel in Hec50 endometrial cancer cells. (A) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of paclitaxel in the absence or presence of bevacizumab for 72 h; cell viability was determined using WST-1 assay. (B) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of paclitaxel in the absence or presence of 1 μ M cediranib for 72 h; cell viability was determined using WST-1 assay. (C) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of cediranib in the absence or presence of 5 nM paclitaxel for 72 h; cell viability was determined using WST-1 assay. For the combinatorial treatments in (B,C) (green lines), the points on the y-axis at 0 reflect treatment with either cediranib alone (B) or paclitaxel alone (C). For example, single-agent cediranib at 1 μ M ((B), Hec50) results in a 40.0% decrease in cell viability as compared to untreated control. Statistical significance was assessed by two-way ANOVA with Sidak's post-hoc test. n.s.: not significant.

2.3. The Combination of Cediranib But Not Bevacizumab with Paclitaxel Increases the Percentage of Cells in Mitosis

Flow cytometric measurements were performed to determine the effects of paclitaxel, cediranib, bevacizumab, and the combination treatment on cell cycle in Hec50 cells, which were more sensitive to the combination of cediranib and paclitaxel in Figure 3. Treatment with cediranib or bevacizumab alone had little effect on cell cycle distribution as compared to control treatment (Figure 4).

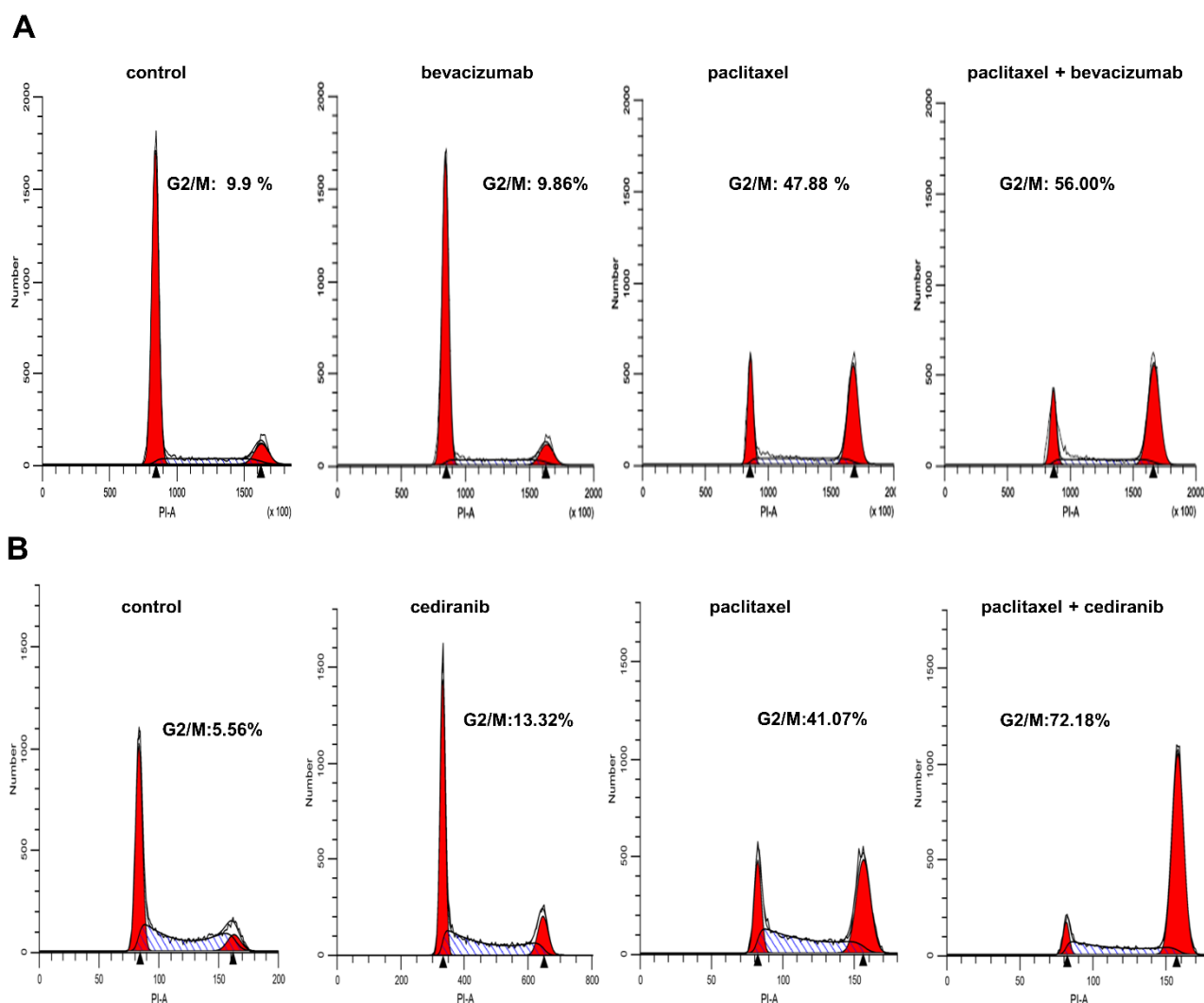


Figure 4. Comparison of the effect of cediranib and bevacizumab on cell cycle distribution in Hec50 cells treated with paclitaxel. **(A)** Cell cycle distribution in Hec50 cells treated with DMSO (Control), 14 nM paclitaxel, 1 μ M bevacizumab, or a combination of paclitaxel and 1 μ M bevacizumab for 24 h. **(B)** Cell cycle distribution for Hec50 cells treated with vehicle (DMSO), 14 nM paclitaxel, 1 μ M cediranib, or a combination of 14 nM paclitaxel and 1 μ M cediranib. Insets denote the percentage of cells in the G2/M phase of the cell cycle.

Paclitaxel is a microtubule stabilizing agent and thus is most effective in mitosis (M phase of the cell cycle). As expected, treatment with paclitaxel alone resulted in a marked shift in cells to the G2/M phase. The combination of paclitaxel and cediranib produced a profound increase in the accumulation of cells in mitosis as assessed by the percentage of cells in G2/M by flow cytometry compared to paclitaxel alone (Figure 4B). Yet there was minimal change in the percentage of cells in G2/M in bevacizumab + paclitaxel-treated cells (Figure 4A). These findings indicate that paclitaxel treatment in the presence of cediranib

results in a significant enhancement of the G2/M population by flow cytometry—the predominance of the cells being in M.

To better understand the mechanism of the mitotic cell death induction by paclitaxel and cediranib, we next examined the expression and post-translational modification of critical regulators of the G2/M checkpoint including cyclin-dependent kinase 1 (cdc2) and cell division cycle 25C (CDC25C). The effect of gefitinib, a tyrosine kinase inhibitor specific for EGFR, in combination with paclitaxel serves as a positive control based upon our previous work that gefitinib and paclitaxel promote premature entry to mitosis, especially in Hec50 cells that are devoid of functional p53 [27].

Treatment of Hec50 cells with cediranib in combination with paclitaxel decreased phosphorylation cdc2. De-phosphorylation of cdc2 is the final signaling step that opens the G2/M checkpoint, thereby allowing cells to enter mitosis. Cells that stop at G2/M have the potential to repair DNA prior to mitosis; however, when the checkpoint is abrogated as reflected by the dephosphorylation of cdc2, cells with damaged DNA enter mitosis and are more vulnerable to paclitaxel (Figure 5A).

The active form of CDC25C, a protein phosphatase responsible for dephosphorylating cdc2 (abrogating the G2/M checkpoint), was significantly increased by this treatment regimen as demonstrated by a loss of phosphorylation at Ser216 and a slower migrating band in the total CDC25C blot. These same signaling events were not observed with bevacizumab was combined with paclitaxel (Figure 5A), indicating a lack of G2/M checkpoint abrogation.

We also examined the effect of combinatorial treatment on KLE endometrial cancer cells, which we previously found to be resistant to paclitaxel + gefitinib [27]. Treatment with anti-angiogenic agents or gefitinib in combination with paclitaxel failed to override the G2/M cell cycle checkpoint, consistent with our previous findings.

To better understand why bevacizumab has no impact on cell survival or cell cycle progression in cancer cells, we performed a phosphoproteomic array using a panel of over 800 phosphorylation sites [28]. We detected similar trends in Akt and ERK/MAPK signaling and G2/M cell cycle checkpoints in response to bevacizumab or cediranib as single agents in Hec50 cells (Figure 5B). Changes in other cell cycle controllers included in the phosphoproteomic array are depicted in Figure 6A. Interestingly, bevacizumab as a single agent promoted more signaling events than cediranib, with only five changes shared between the two treatment groups (Figure 6B,C). Additionally, cediranib largely promoted decreased phosphorylation of signaling molecules, the expected therapeutic effect of a multi-TKI, whereas bevacizumab both increased and decreased phosphorylation events. We hypothesize that increased phosphorylation on pro-growth signaling molecules in response to bevacizumab treatment indicates a potential signal of developing cellular resistance.

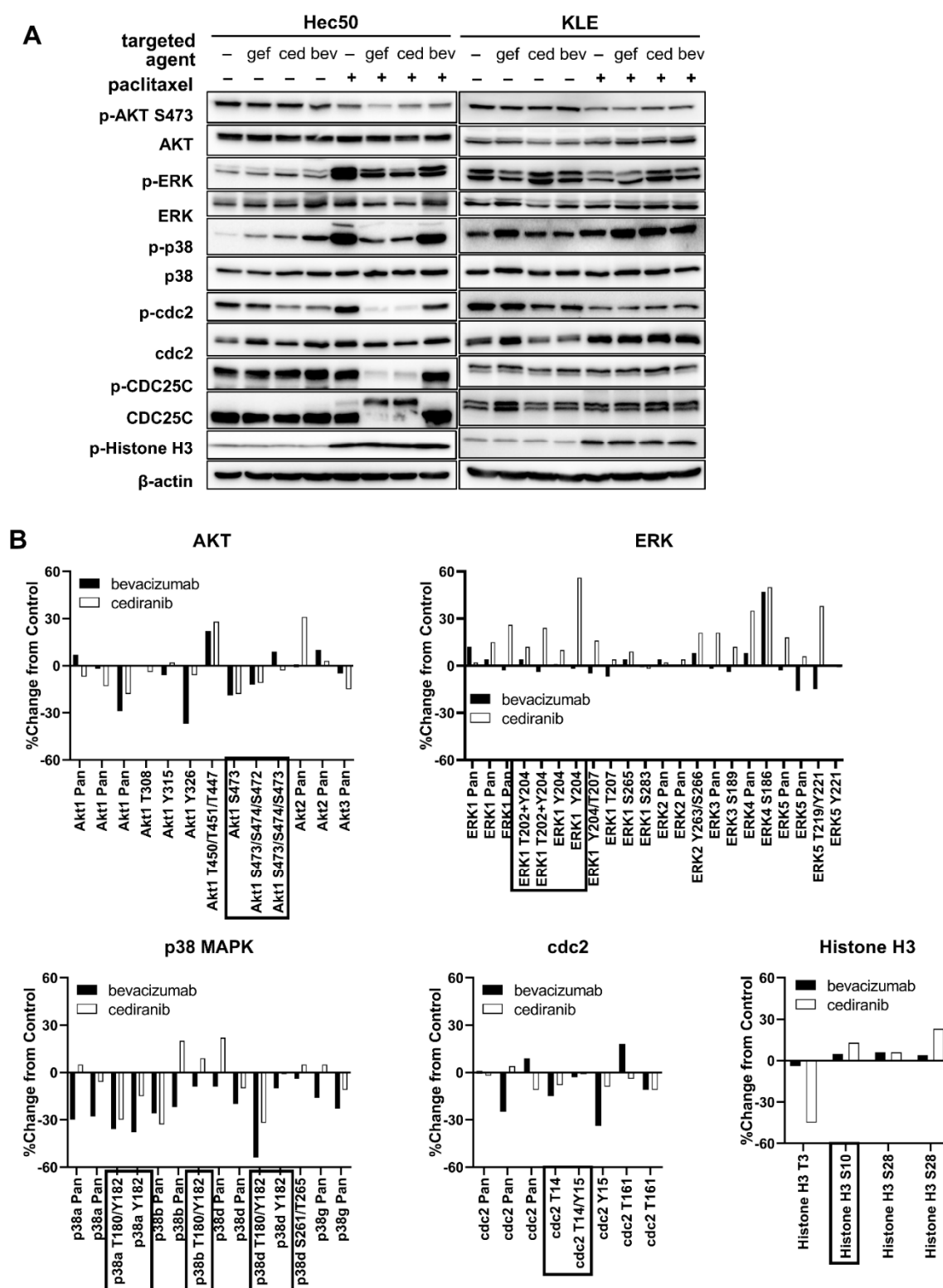


Figure 5. Molecular effects of anti-angiogenic agents on G2/M cell cycle controllers in endometrial cancer cell models. (A) Hec50 (left) or KLE cells (right) were treated with the indicated agents either alone or in combination with paclitaxel for 24 hrs, followed by assessment of cell cycle controllers by Western blotting. Drug concentrations: 10 μ M gefitinib, 1 μ M cediranib, 1 μ M bevacizumab, 14 nM paclitaxel. (B) Expression or phosphorylation of indicated proteins was assessed by Kinex™ KAM-1325 Phosphoproteomic Antibody Microarray in Hec50 cells treated with 1 μ M bevacizumab or 1 μ M cediranib for 24 h. Data were calculated as the percent change from control (%CFC). Phosphosites corresponding to those queried in (A) are indicated with boxes. Pan: antibody total protein expression.

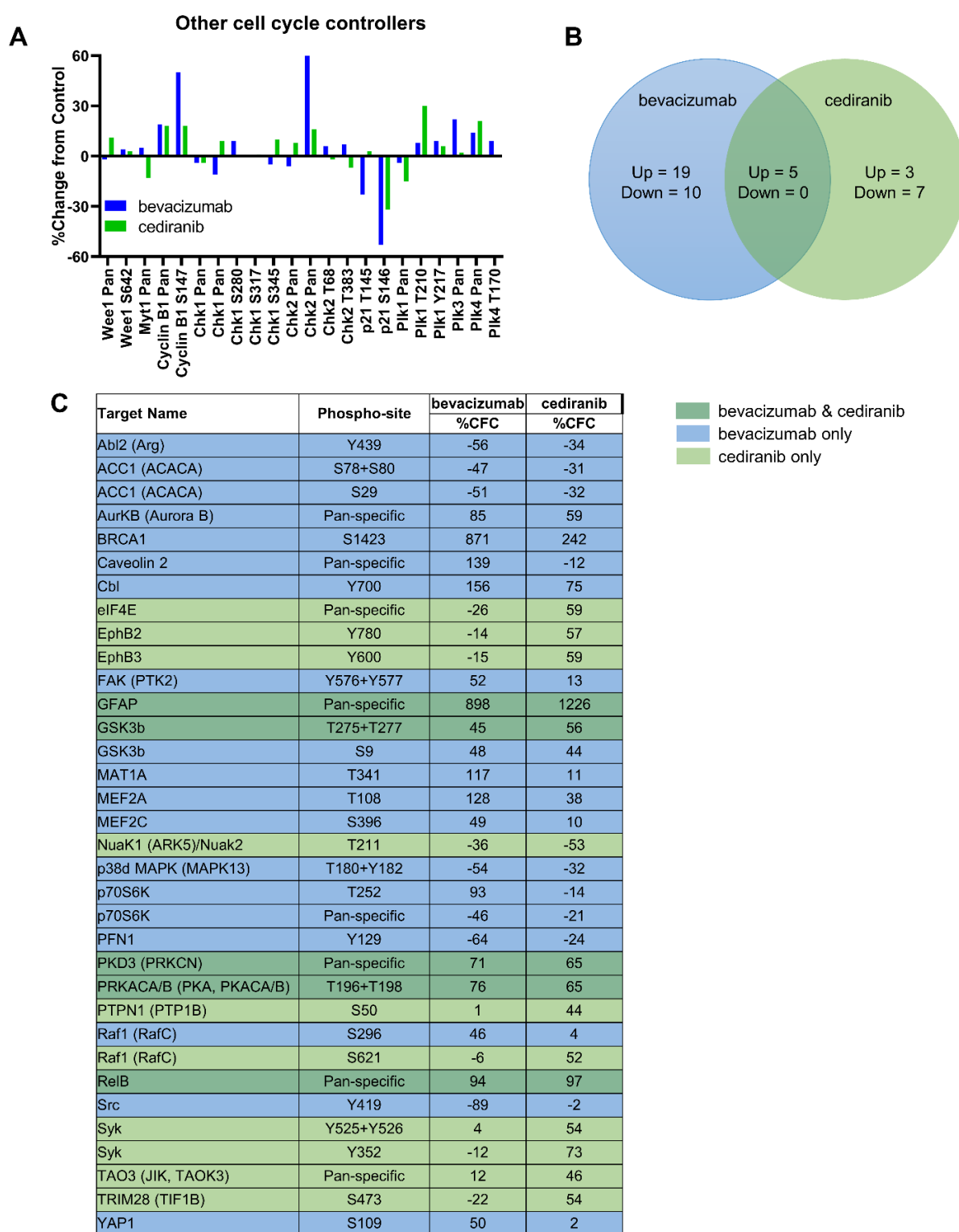


Figure 6. Bevacizumab regulates more signaling events than cediranib in endometrial cancer cells. Signaling events in response to single-agent bevacizumab or cediranib were analyzed by KinexTM KAM-1325 Phosphoproteomic Antibody Microarray after treatment of Hec50 cells with 1 μ M bevacizumab or 1 μ M cediranib for 24 h. (A) Depiction of changes in select cell cycle controllers. Data were calculated as the percent change from control (%CFC). “pan” indicates total protein expression. (B) Venn diagram of lead candidates in response to either bevacizumab or cediranib. Overlap indicates signaling events that are regulated by both agents. (C) Table of all lead up-regulated candidates identified based on phospho-%CFC ≥ 2 . Negative %CFC indicates a decrease as compared to control. Full results are provided as Supplemental Table S2. Negative %CFC indicates a decrease as compared to control. Full results are provided as Supplemental Table S2.

3. Discussion

Angiogenesis plays a crucial role in tumor development and metastasis, and cancer cells frequently upregulate VEGF-A expression to promote angiogenesis. VEGF-A binds to VEGFR1 and VEGFR2, and it is the main stimulator of tumor growth and dissemination. Accordingly, inhibitors of angiogenesis have been tested in clinical trials for a range of cancers, with FDA approval for 11 agents that inhibit either VEGF ligands or receptors [29]. Bevacizumab, which has been FDA-approved for cervical and ovarian cancers, specifically binds to the VEGF-A protein, and thereby inhibits vessel growth in the tumor. Cediranib is a tyrosine kinase inhibitor that targets VEGFR1, 2, 3, PDGFRs, and FGFRs. Cediranib is thought to be effective in the prevention of tumor progression, not only by inhibiting VEGFR2 activity and angiogenesis but also by concomitantly inhibiting VEGFR3 activity and lymphangiogenesis.

While the role of anti-angiogenic agents on tumor vascularization and endothelial cell growth is well-documented [6,7,14,16], few studies have interrogated the impact of anti-angiogenic agents on the properties of tumor cells themselves. In the present study, our objective was to compare the impact of angiogenic inhibitors on endometrial cancer cells. First, we found that cancer cells were not significantly responsive to treatment with exogenous VEGF-A despite the expression of VEGFRs 1 and 3. Bevacizumab, a monoclonal antibody specifically raised against VEGF-A, had little impact on cell viability or cell cycle progression alone as shown in Figures 2–4. In addition, bevacizumab did not synergize with chemotherapy, as we had previously found for other agents that target TKI signaling [25–27]. We provide evidence that synergy was related to the impact on the cell cycle when comparing bevacizumab, with its narrow range of activity, to a multi-targeted anti-angiogenic TKI such as cediranib. Thus, we propose that the ability of cediranib, but not bevacizumab, to inhibit multiple kinases and to dephosphorylate the G2/M gatekeeper cdc2 results in G2/M checkpoint abrogation, premature entry of tumor cells with damaged DNA into mitosis, and cell death through mitotic catastrophe. The significant enhancement of the percent of cells in G2/M when cells are treated with cediranib, and paclitaxel (as shown in Figure 4) is a potential mechanism of synergy. Another explanation for the enhanced response to cediranib versus bevacizumab is the robust expression of cediranib targets VEGFRs, PDGFRs, and FGFRs.

The phosphoproteomic array results reported are valuable as a readout of the numerous signaling events in response to bevacizumab and cediranib treatment at a single time point, 24 h (Figures 5 and 6). The inhibition of phosphorylation of multiple targets related to cediranib activity likely underlies its therapeutic effects as a tyrosine kinase inhibitor [7]. In contrast, the phosphorylation events were more often induced after bevacizumab treatment (Figure 5). Though enhanced phosphorylation is most commonly associated with signaling activation and proliferative signals, in our study, it may suggest that cells treated with bevacizumab instead induce resistance pathways by 24 h. These specific phosphorylation events are worthy of future investigation as they shed light on therapeutic agents that could block the resistance phosphorylation events when added to bevacizumab.

While no direct comparison of efficacy between cediranib and bevacizumab has been reported, we predicted cediranib would have greater benefit due to the additional blockade of all VEGFR isoforms as well as PDGF and FGF receptors. Isoforms of PDGFR and FGFR are expressed in endometrial tumors [30,31], with FGFR2 mutations occurring in ~13% of endometrial cancer cases [32]. We found that cediranib, as a multi-TKI inhibitor, had greater therapeutic effects, including blunting baseline ERK activation, synergizing with paclitaxel in organoid models and cell lines and the anticipated abrogation of the G2/M cell cycle checkpoint.

A limitation of this study, and studies of immortalized cell lines and patient-derived organoid models in general, is the absence of tumor microenvironment features, including immune cells and vasculature. Conversely, a strength of using these models is the ability to determine the impact of bevacizumab and cediranib specifically on the tumor cells without the confounding influence of the tumor microenvironment. We attempted to perform all

studies on organoids within two weeks of development (i.e., obtaining surgical specimens) in order to avoid the effects of tumor evolution in culture. Considering that the size of endometrial tumor specimens is typically very small as compared to other gynecologic cancer types (e.g., ovarian tumors), this approach precluded extensive molecular analyses. Therefore, as a companion, we assessed cell signaling using endometrial cancer cell lines. We did not extend studies to xenograft models of endometrial cancer since both bevacizumab and cediranib have been studied in this system previously by our group and others [6,7,14–16].

Findings from this preliminary study in preclinical models should be expanded to determine the mechanisms of differential sensitivity at the molecular level to cediranib, as evidenced by the lack of synergy when cediranib was combined with paclitaxel in KLE endometrial cancer cells (Figure 3) as well as some organoid models (Figure 2). We originally hypothesized that the mutations in p53, a well-established controller of the G1/S and G2/M cell cycle checkpoints, would predict for enhanced synergy. This hypothesis follows our recently published translational study of GOG-86P in which patients with mutations in *TP53* had significantly improved outcomes when bevacizumab was combined with chemotherapy as compared to other experimental agents [3]. However, the majority of endometrial organoid models used in this study had wild-type (WT) p53 (Figure 2), precluding a definitive analysis of the role of p53 in sensitivity.

4. Materials and Methods

4.1. Chemicals

Gefinitib, cediranib, bevacizumab, and paclitaxel were purchased from Selleck Chemicals, LLC (Houston, TX, USA) and suspended in DMSO.

4.2. Patient-Derived Organoid Models of Endometrial Cancer

All studies using human tissues have been approved by the University of Iowa Institutional Review Board (IRB), protocol #201809807. Patient tumor specimens were obtained within 30 min of surgical resection. Organoids were created per our protocol for ascites fluid [19], with modifications to digest tissue and isolate single cells. Specifically, freshly resected tissue was washed with 10 mL PBS and cut into small pieces. The minced fragments were collected in a 50 mL tube and digested in 5 mL AdDF+++ media (Advanced DMEM-F12 media with 1× Glutamax, 10 mM HEPES and Pen strep) supplemented with 2 U/mL Dispase II, 1 mg/mL collagenase P and 50 µg/mL Dnase I, then incubated at 37 °C for 0.5–1 h. Dissociated cells were filtered through a 40 µm cell strainer, centrifuged at 300× *g* for 10 min, washed twice with PBS, and pelleted. Erythrocytes were removed by incubating the dissociated cells with 2 mL red blood cell lysis buffer for 5 min at room temperature followed by an additional wash with 10 mL AdDF+++ and centrifugation at 300× *g* for 5 min. Finally, the cells were counted and embedded in Matrigel on ice and seeded on pre-warmed 24-well cell culture plates; 500 µL AdDF+++ media was added on the top of the Matrigel to each well.

4.3. Cell Culture

Hec50 cells were kindly provided by Dr. Erlio Gorpide (New York, NY, USA) [18] and grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco Corporation, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. KLE cells were purchased from ATCC and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Human umbilical vascular endothelial cells (HUVEC) were purchased from ATCC and grown in EGMTM -2 MV Microvascular Endothelial Cell Growth Medium (2 BulletKit, Lonza, Alpharetta, GA, USA). To ensure rigor and reproducibility, the identity of all cell lines was confirmed using the CODIS genotyping test (Cat. No. CL1003, Bio-Synthesis, Lewisville, TX, USA).

4.4. Western Blot

Cells were collected and lysed with NP-40 lysis buffer with protease inhibitors. Lysates were analyzed for protein expression/phosphorylation as described previously [25,27]. The following antibodies were used at the indicated dilutions; all antibodies were purchased from Cell Signaling (Danvers, MA, USA): anti-p-AKT-S473 (1:1000, #4060), anti-AKT (1:1000, #4685), anti-p-p44/42 MAPK (Erk1/2)-Thr202/Tyr204 (1:1000, #4370), anti-p44/42 MAPK (Erk1/2) (1:1000, #4695), anti-p-p38 MAPK-Thr180/Tyr182 (1:1000, #9211), anti-p38 MAPK (1:1000, #8690), anti-p-cdc2-Tyr15 (1:1000, #4539), anti-cdc2 (1:1000, #9116), anti-p-CDC25C-Ser216 (1:1000, #4901), anti-CDC25C (1:1000, #4688), anti-p-Histone H3-ser10 (1:1000, #53348).

4.5. Cell Viability Assay

4.5.1. Organoids

Viability of the tumor organoids following drug treatment was performed as previously described [19]. Briefly, patient-derived organoids were collected with organoid harvesting solution (Cultrex, Trevigen, Gaithersburg, USA), and then digested to single cells with TrypLE Express supplemented with 4 μ L 10 mg/mL DNase I stock and 4 μ L 10 mM Y-27632 stock. Single cells were suspended in AdDF+++ medium with 10% Matrigel and seeded at a density of 10,000 cells/well (50 μ L/well) in an ultra-low attachment 96-well U-bottom white plate. After 24 h, cells were exposed to paclitaxel (10 nM), bevacizumab (1 μ M), or cediranib (1 μ M) for 72 h at 37 °C. At the end of incubation, an equal volume of CellTiter-Glo 3D reagent (Promega) was added to each well and incubated for 25 min at room temperature. The luminescence was measured using the Gen5 Microplate Reader (BioTek, Winooski, VT, USA). All the tests were conducted in triplicate and data normalized to untreated control (set at 100% viability).

4.5.2. Cell Lines

Cell viability was determined by WST-1 assay as previously described [26]. Briefly, Hec50 and KLE cells were seeded into 96-well plates (10,000 cells per well) for 24 h and then cultured with increased concentrations of the drugs for an additional 72 h. Cell viability was evaluated using the cell proliferation reagent WST-1 (Roche, Pleasanton, Germany) according to the manufacturer's protocol. The absorbance was measured with a micro-plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the tests were conducted in triplicate and data were normalized to untreated control (set at 100% viability).

4.6. Cell Cycle Analysis

Cell cycle analysis was performed by flow cytometry as described previously [25]. An equal number of cells were plated in 10 cm plates and treated with different drugs for 24 h as for cell viability assays. Cell pellets were harvested and suspended separately in Krishan's solution (3.8 mM sodium citrate, 0.014 mM propidium iodide, 1% NP-40, and 2.0 mg/mL RNase A). Cell suspensions were analyzed using a FacScan Flow Cytometer (Becton, Dickinson and Company, San Jose, CA, USA) and data were analyzed by CellQuest software version 3.3.

4.7. Shedding Assay

HUVEC, Hec50, and KLE cells were grown to 70–80% confluence and serum-starved in Opti-MEM for one hour prior to transfection. Transfection of 1 μ g alkaline phosphatase (AP)-tagged transforming growth factor (TGF)- α were performed according to manufacturer's protocols using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Cells were starved for at least four hours before the stimulation. Recombinant human vascular endothelial growth factor (VEGF-A) was obtained from R&D Systems (Minneapolis, MN, USA) and used at a concentration of 100 ng/mL. The concentration of bevacizumab was 1 μ g/mL. PMA (phorbol-12-myristate-13-acetate) and the AP-tagged TGF- α plasmid have been previously published [33]. Evaluation of AP activity was determined by the colori-

metric assay as described previously [34]. Briefly, the ratio between the total AP activity in the supernatant and the total AP activity in the cell lysate plus the supernatant was computed for normalization. The presented ratios reflect the relative proteolytic activity of a given sheddase toward the AP-tagged ligand TGF- α . No AP activity was detected in the conditioned media of untransfected cells.

4.8. Phosphoproteomic Microarray

Hec50 cells were plated in 15 cm plates and treated with DMSO, 1 μ M bevacizumab, or 1 μ M cediranib for 24 h. Cell pellets were collected and lysed with Kinexus Lysis Buffer with chemical cleavage. The microarray assay and data analysis were performed with Kinex™ KAM-1325 Antibody Microarray Kit by Kinexus Bioinformatics Corporation (Vancouver, BC, Canada). To qualify as a lead, the percent change from control (%CFC) value should be at least 45% higher or lower with fluorescent signals that were at least 1000 counts [28].

4.9. Reverse Transcription and Quantitative Polymerase Chain Reaction

Total RNA was purified from Hec50co and KLE cells using the RNeasy Plus RNA purification kit (QIAGEN, Germantown, MD, USA). Yield and purity were assessed on a Trinean DropSense 16 spectrophotometer and an Agilent Model 2100 Bioanalyzer in the Genomics Division of the Iowa Institute of Human Genetics (IIHG). The mean RNA Integrity Number (RIN) was 9.5 [35]. Next, 500 ng of QC qualified total RNA from each cell line was reverse transcribed using oligo-dT primers in the SuperScript III kit following the manufacturer's recommendations (Invitrogen). The resulting cDNAs were then amplified in the presence of SYBR Green (Thermo Fisher, Waltham, MA, USA) on an Applied Biosystems Model 7900HT platform in the Genomics Division of the Iowa Institute of Human Genetics (IIHG). Locus-specific primers are shown below (Table 1). Raw expression values (Ct) were normalized (Δ Ct) [36] against the endogenous 18S rRNA control.

Table 1. Primer sequences.

Locus	Primer Sequences	Amplicon	Tm (°C) *
VEGFA	For: GGGCAGAATCATCACGAAGT	269 bp	55.0
	Rev: AGGAAGCTCATCTCTCTATGT		54.7
VEGFR1	For: GGACAGTAGAAAGGGCTTCATC	251 bp	55.2
	Rev: CAGGGTAACTCCAGGTCATTG		55.4
VEGFR2	For: GTGGTCTCTCTGGTTGTGTATG	235 bp	55.0
	Rev: CCTCCACACTTCTCCATTCTTC		55.1
VEGFR3	For: CGAAAGTGCATCCACAGAGA	239 bp	54.9
	Rev: AGAGAGAAGATCTCCCAGAGAAG		54.9
FGFR1	For: AGGAACCTTTCAAGCTGC		
	Rev: CATCATGTACAGCTCGTTG		
FGFR2	For: ATGAGGAATACTTGGACCTC		
	Rev: TTAACACTGCCGTTTATGTG		
FGFR3	For: GAAGATGCTGAAAGACGATG		
	Rev: GCAGGTTGATGATGTTTTTG		
FGFR4	For: CTGAGGACAATGTGATGAAG		
	Rev: CCGTTGCTGGTTTTCTTCTTATAG		
PDGFRA	For: GACTTTCGCCAAAGTGGAGGAG	121 bp	58.0
	Rev: AGCCACCGTGAGTTCAGAACGC		62.0

Table 1. Cont.

Locus	Primer Sequences	Amplicon	Tm (°C) *
PDGFRB	For: TGCAGACATCGAGTCCTCCAAC	108 bp	59.0
	Rev: GCTTAGCACTGGAGACTCGTTG		57.8
18S rRNA	For: AACTTTCGATGGTAGTCGCCG	104 bp	57.3
	Rev: CCTTGGATGTGGTAGCCGTTT		57.6

* Tm calculated in the presence of 1.5 mM MgCl₂.

4.10. Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software Version 0.0.0 (121), San Diego, CA, USA). Statistical significance of differences was determined using one-way ANOVA with Sidak's post-hoc test (for comparison of significance between two treatment groups with multiple independent treatment concentrations; Figure 3) or two-way ANOVA with Tukey's post-hoc test (for comparisons of means of multiple groups; Figure 2A). The normal distribution of data was assessed using the likelihood test. All values are expressed as mean $\pm$ standard deviation (SD) of at least three independent experiments unless otherwise indicated.

5. Conclusions

In conclusion, the standard of care for endometrial cancer has not changed beyond chemotherapy, the incidence is on the rise due to the obesity epidemic, and outcomes are worse now than in the 1970s. The data presented in this report are designed to identify agents that should be advanced into clinical trials to improve outcomes for women with endometrial cancer. Based on our findings, we put forth that cediranib should be investigated in future clinical trials in endometrial cancer in the upfront setting in combination with chemotherapy. Specifically, our data suggest that the anti-angiogenic agent cediranib may have additional advantages over bevacizumab as an anti-cancer therapeutic due to its dual effects on tumor cells and vasculature. Our use of endometrial tumor tissue cultured as functional three-dimensional organoids provides highly translational information on the efficacy of anti-angiogenic agents in tumor cells. These data set the stage for future clinical trials to evaluate cediranib in combination with chemotherapy as a treatment for women with endometrial cancer.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ph14070682/s1>: Figure S1: Expression of bevacizumab and cediranib targets, Table S1: Patient characteristics for endometrial cancer organoids, Table S2: Raw data for phosphoproteomic array.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Iowa (protocol code #201809807 approved on 8 March 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Article

The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells

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Abstract: Elevated expression of placenta-specific protein 1 (PLAC1) is associated with the increased proliferation and invasiveness of a variety of human cancers, including ovarian cancer. Recent studies have shown that the tumor suppressor p53 directly suppresses PLAC1 transcription. However, mutations in p53 lead to the loss of PLAC1 transcriptional suppression. Small molecules that structurally convert mutant p53 proteins to wild-type conformations are emerging. Our objective was to determine whether the restoration of the wild-type function of mutated p53 could rescue PLAC1 transcriptional suppression in tumors harboring certain *TP53* mutations. Ovarian cancer cells OVCAR3 and ES-2, both harboring *TP53* missense mutations, were treated with the p53 reactivator HO-3867. Treatment with HO-3867 successfully rescued PLAC1 transcriptional suppression. In addition, cell proliferation was inhibited and cell death through apoptosis was increased in both cell lines. We conclude that the use of HO-3867 as an adjuvant to conventional therapeutics in ovarian cancers harboring *TP53* missense mutations could improve patient outcomes. Validation of this conclusion must, however, come from an appropriately designed clinical trial.

Keywords: ovarian cancer; OVCAR3; ES-2; placenta-specific protein 1 (PLAC1); p53; HO-3867; steric hindrance; reactivator



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1. Introduction

Placenta-specific protein 1 (PLAC1) is a unique 212 amino acid secreted cell surface protein. PLAC1 was initially identified because it is located in a region of the human and mouse X-chromosome that contains other loci suspected of being involved in fetal and placental pathologies [1]. Under normal circumstances, the expression of PLAC1 is almost exclusively limited to the apical surfaces of placental trophoblasts [2]. Placenta-specific expression of PLAC1 has led to its implication in a variety of gestational disorders, including pre-term birth and preeclampsia [3–15]. In the two decades since PLAC1 was initially reported, many papers have demonstrated its co-opted expression in numerous human cancers [16–38]. In all of these studies, increased PLAC1 expression is linked to increased invasiveness, proliferation and/or aggressiveness, as well as poor prognosis, though the precise mechanisms remain poorly defined. Regardless, it is well established that *PLAC1* is an oncogene in addition to being a fetal-placental gene.

Studies of the regulation of PLAC1 expression identified two promoters, termed P1 (distal), located at exon 1, and P2 (proximal), located at exon 4. Transcripts are produced from both promoters simultaneously in cells that express PLAC1 mRNAs [39,40]. However, in placental tissues, *PLAC1* is predominantly transcribed from the P2 promoter, while in tumors and fetal tissues, *PLAC1* is predominantly transcribed from the P1 promoter. Chen et al. [39] identified RXR α and LXR β as promoters of P1 transcription, and later, Chen et al. [41] showed in vitro

that p53 protein suppresses *PLAC1* transcription by blocking the binding of these transcription factors to the *PLAC1* P1 promoter. We subsequently confirmed the p53 suppression of *PLAC1* transcription in a high-grade serous ovarian cancer (HGSOC) tumor panel [32]. However, in that study, we found that *PLAC1* transcriptional suppression was dependent upon the mutation status of the *TP53* gene. Specifically, only wild-type p53 suppressed *PLAC1* transcription, while any *TP53* mutation appears to permit P1 *PLAC1* transcription. We identified a non-canonical p53 binding site just upstream from the P1 RXR α and LXR β binding sites. These results led us to propose that P1 *PLAC1* transcriptional suppression is due to steric hindrance when wild-type p53 is bound. Conversely, P1 *PLAC1* transcription in the presence of mutant p53 is permitted due to the failure of mutated p53 to bind and block the RXR α and LXR β binding sites.

Recognition that mutant p53 permits P1 *PLAC1* transcription in HGSOC tumors and that increased *PLAC1* expression in these cancers leads to poor patient outcomes suggested that altering P1 *PLAC1* transcription can be accomplished by targeting p53 itself. *TP53* harbors both missense mutations, which result in single amino acid changes, as well as frameshift/splice site mutations that result in the loss of protein expression. p53 missense mutants can be targeted by molecules that reactivate their wild-type function. One agent that appears to be well suited to this task is the synthetic curcumin analog HO-3867. In the most extensive study to date, the treatment of various cancer cells harboring non-truncating *TP53* mutations with HO-3867 restored the wild-type transcriptional regulation of a number of key p53 target genes [42]. Herein we report that HO-3867 treatment rescues *PLAC1* transcriptional suppression in *TP53*-mutated ovarian cancer cells. Reestablishment of *PLAC1* downregulation via p53 reactivation could be used in combination with other therapies to improve outcomes for patients presenting with ovarian carcinomas harboring missense *TP53* mutations.

2. Results

2.1. HO-3867 Treatment of Ovarian Cancer Cells Restores Suppression of *PLAC1* Transcription

Our studies were carried out on two well-characterized ovarian cancer cell lines that contain *TP53* missense mutations, OVCAR3 and ES-2. OVCAR3 cells contain the frequent p53 gain-of-function mutant R248Q and were derived from a patient presenting with high-grade serous ovarian cancer, the most common and deadly type. ES-2 ovarian cancer cells are a model of clear-cell ovarian carcinoma and harbor a different p53 binding domain mutation, S241F. While not regarded as a gain-of-function mutant, S241F is classified as pathogenic in COSMIC (<https://cancer.sanger.ac.uk/cosmic> (accessed on 11 October 2019)) and is a DNA contact mutant overrepresented in ovarian cancers [43].

First, the OVCAR3 cells were exposed to 2 μ M and 5 μ M HO-3867 for 24 h and 48 h. *PLAC1* expression relative to untreated OVCAR3 cells is shown in Figure 1. In all cases, HO-3867-treated cells displayed reduced *PLAC1* transcription. Moreover, this suppression was focused at the P1 promoter.

Treatment of the ES-2 cells with 2 μ M and 5 μ M HO-3867 for 24 h and 48 h produced a similar, though not as dramatic, rescue of *PLAC1* transcriptional suppression (Figure 2). Again, transcriptional suppression appeared to be focused on transcripts originating from the *PLAC1* P1 promoter.

2.2. HO-3867 Treatment of Both OVCAR3 and ES-2 Ovarian Cancer Cells Results in a Reduction in Proliferation and an Increase in Apoptosis

Given the importance of p53 in proliferation and apoptosis, we next assessed the functional effect of HO-3867 exposure on both the OVCAR3 and ES-2 cells. Overall, the HO-3867 treatment revealed a dose-dependent decrease in proliferation and a dose-dependent increase in apoptosis in both cell lines (Figure 3). Specifically, treatment of the ES-2 cells with HO-3867 showed decreased proliferation at 1.25 μ M and 2.5 μ M with no change in apoptosis, whereas with higher doses of HO-3867 (5 μ M and 10 μ M), the ES-2 cells had decreased proliferation and increased apoptosis. Interestingly, HO-3867 had a greater effect on the ES-2 cells compared to the OVCAR3 cells for both proliferation and apoptosis, especially at higher concentrations of HO-3867. The results indicate that HO-3867 is capable of arresting cell

growth and inducing cell death through the reactivation of p53 and that the effect of HO-3867 is not only cell-type-specific but also putatively dependent on p53 mutation status.

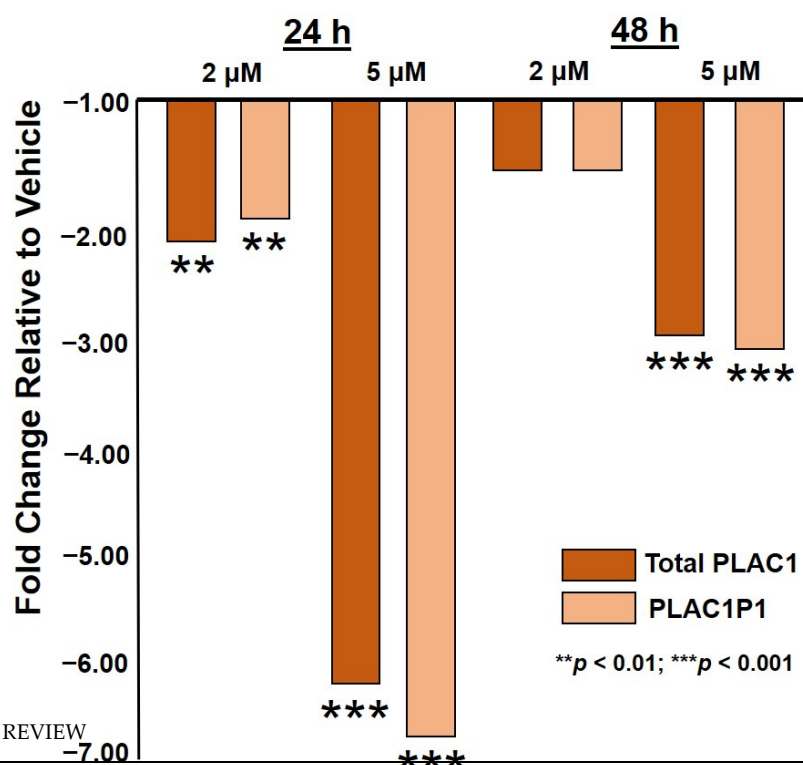


Figure 1. Total PLAC1 and PLAC1P1-specific expression in OVCAR3 ovarian cancer cells treated with 2 μM and 5 μM HO-3867 for 24 h and 48 h.

Figure 1. Total PLAC1 and PLAC1P1-specific expression in OVCAR3 ovarian cancer cells treated

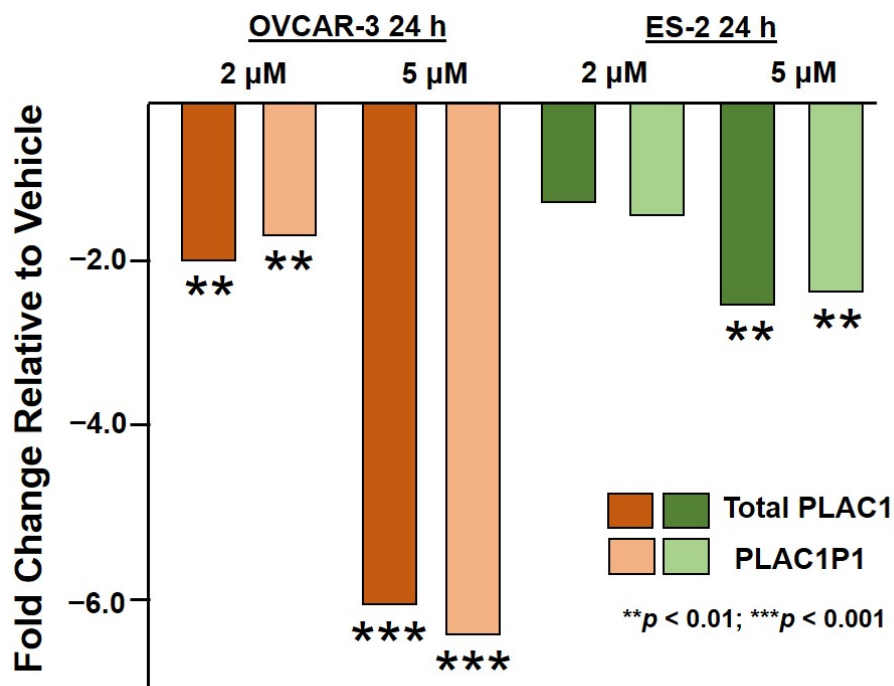


Figure 2. Total PLAC1 and PLAC1P1-specific expression in ES-2 ovarian cancer cells treated with 2 μM and 5 μM HO-3867 for 24 h. ES-2 cells exposed to 2 μM HO-3867 for 48 h did not yield RNA of sufficient quality to perform qPCR and ES-2 cells exposed to 5 μM HO-3867 for 48 h did not survive. The 24 h results for OVCAR3 cells are shown on the left for comparison.

2.2. HO-3867 Treatment of Both OVCAR3 and ES-2 Ovarian Cancer Cells Results in a Reduction in Proliferation and an Increase in Apoptosis

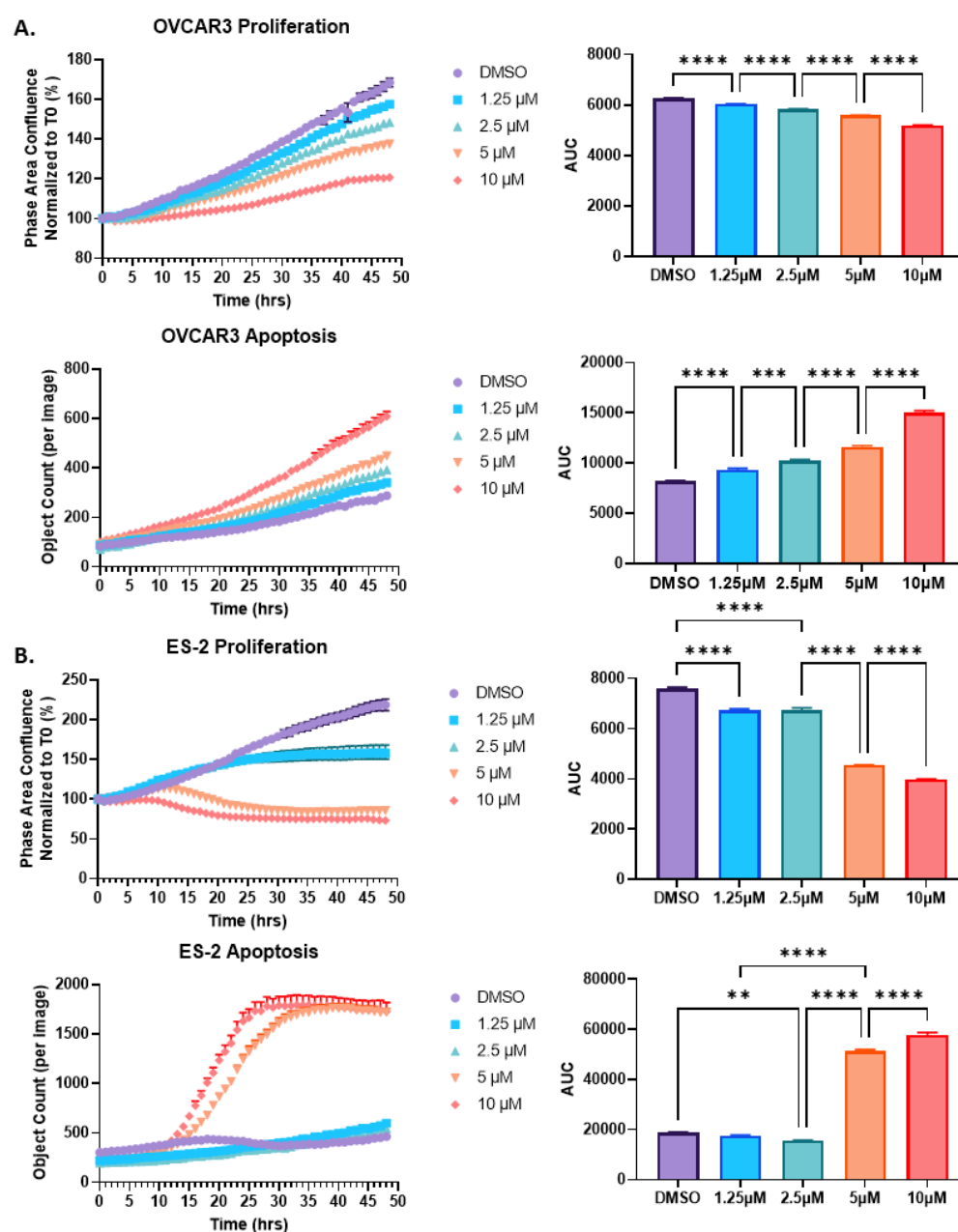


Figure 3. Proliferation and apoptosis in OVCAR3 (A) and ES-2 (B) cells. Cells were treated with increasing amounts of HO-3867, as shown. Proliferation was measured by phase contrast, and apoptosis was assessed by caspase 3/7 activation. Cells were monitored hourly for 48 h using the Incucyte S3 live-cell imaging platform. Area under the curve (AUC) values were calculated automatically by the Incucyte Software and analyzed via ANOVA. Significance values are indicated as ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Discussion

It has long been recognized that a *TP53* mutation is a molecular vulnerability awaiting therapeutic exploitation [44–47]. Wild-type p53 has a wide variety of cellular roles, including being a powerful tumor suppressor. Thus, it follows that the re-introduction of wild-type p53 into tumor cells would result in a positive anti-tumor effect [48]. However, attempts to do this have so far proved unsuccessful [49,50]. For example, gene therapy with p53 failed in clinical trials due to its insufficient accumulation in tumor tissue [49]. A promising alternative strategy would be to introduce a compound that is specific for mutant p53 and would also restore wild-type function. To this end, a number of such

compounds have been developed, including quinuclidines, 2-sulfonylpyrimidines, zinc metallochaperones and thiosemicarbizones [51]. Some of these are well known, such as COTI-2 [52], PRIMA1 and PRIMA1^{met}, now designated as APR-246 [53,54]. Here, we have chosen to assess the less well-known synthetic diarylidenylpiperidone curcumin analog, designated HO-3867, for the purpose of rescuing the suppression of the expression of the oncogene *PLAC1* in ovarian cancer cells harboring a missense *TP53* mutation.

HO-3867 binds p53 at Cys182 and Cys277, creating a “clamp” that holds the β -sandwich in place in a proper DNA-binding configuration while also stabilizing the Zn^{++} binding pocket [42]. The mature p53 protein in this configuration is thus able to function like wild-type p53, including in binding DNA and inducing the transcription of canonical p53 target genes. This was shown by Madan et al. [42] in a panel of 29 human cancer cell lines representing 11 different cancers. These cell lines harbor a total of 26 different missense *TP53* mutations, including six classified as “gain of function” mutants [55]. The effect of exposing these cell lines to HO-3867 was assessed via qPCR expression assays on fourteen known p53 transcriptional target loci [42,56,57]. In the vast majority of these 406 assays (29 cell lines by 14 loci), HO-3867 exposure rescued the appropriate up- or down-regulation of transcription. Indeed, the overall pattern of transcriptional changes was highly significant (5×10^{-211}) [42]. Our study adds to the literature by providing evidence for the appropriate down-regulation of another p53-regulated gene, *PLAC1*, as well as demonstrating the effect in ovarian cancer cells, which were not included in the Madan et al. study [42].

A model describing the effect of HO-3867 exposure on *PLAC1* transcription in cancer cells harboring DNA-binding domain missense mutations is presented in Figure 4. In this model the P1 promoter is sterically blocked when wild-type p53 binds to its nearby binding site. This leads to reduced *PLAC1* transcription. However, mutant p53 is unable to bind to that site, thus opening up *PLAC1* transcription. Exposure to HO-3867 will, in some mutants, restore wild-type configuration, once again sterically blocking the binding site and reducing *PLAC1* transcription.

Where ovarian cancer has been the focus of the effect of HO-3867 is a series of papers originating from The Ohio State University [58–61]. In those papers, it has been demonstrated that HO-3867 is cytotoxic to ovarian cancer cells through the induction of apoptosis and the abrogation of cisplatin resistance. Their primary readout was STAT3 protein expression and STAT3 phosphorylation. It should be noted that STAT3 is also a direct transcriptional target of p53 [56], though it remains unclear if the p53 regulation of STAT3 is the mechanism of action of HO-3867. Thus, it is possible that the changes in proliferation and apoptosis in our study may be due to the restoration of p53 regulation of genes in addition to *PLAC1*.

Finally, while it can be argued that the treatment of ovarian cancer patients with HO-3867—preferably as an adjuvant to conventional therapies—could improve outcomes for these patients, its effect is only possible for those cancers that harbor a *TP53* missense mutation. Obviously, nonsense mutants will often result in the truncation of transcripts that are likely to be eliminated by nonsense-mediated RNA decay [62] or another mechanism that would render the cells devoid of p53 protein expression (i.e., p53 null). Further, the small number of *TP53* missense mutations that occur outside the DNA-binding domain will also not be rescued by HO-3867, as the mechanism of HO-3867 action would not, for example, affect p53 tetramerization. However, taking all of the potential exceptions to the effect of HO-3867 exposure on ovarian cancer into account, there remains around 50% of all ovarian cancers that will qualify. Given the fact that the American Cancer Society estimates that there are roughly 22,000 new cases of ovarian cancer in the United States each year, some 11,000 patients could potentially benefit from adding HO-3867 to their treatment regimens. Naturally, the efficacy of using such an adjuvant therapy can only be assessed through an appropriately designed clinical trial.

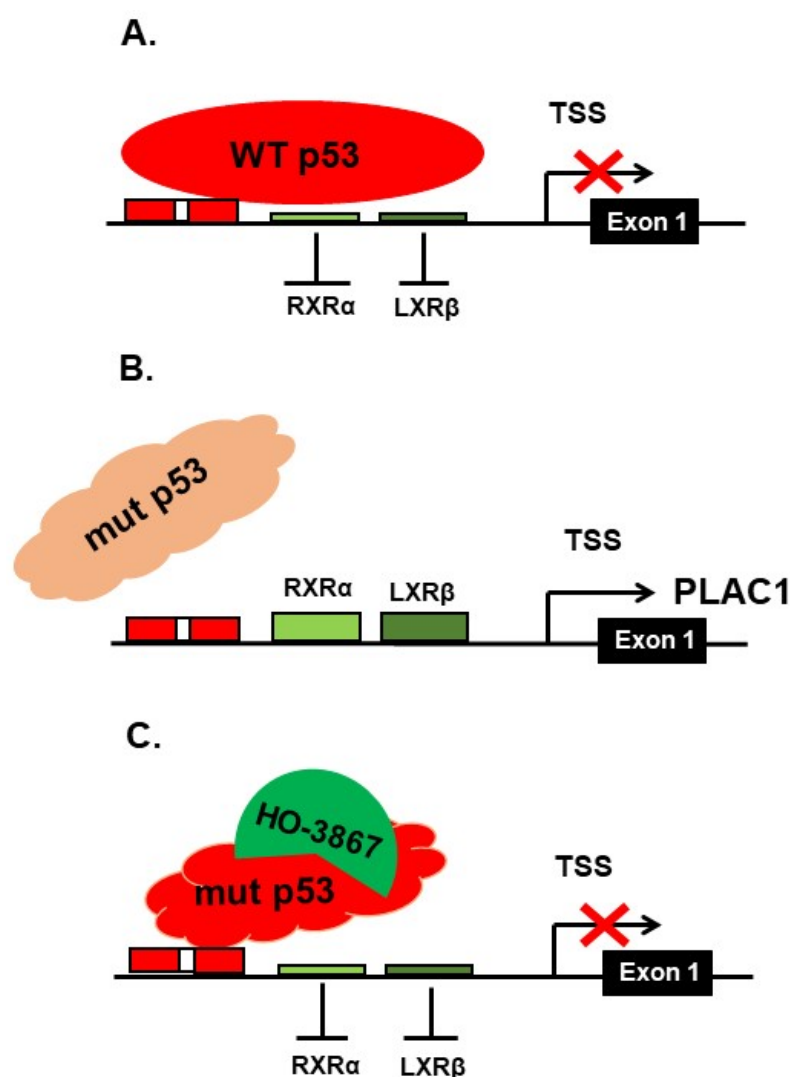


Figure 4. A model of the effect of HO-3867 on p53 binding in the P1 promoter of *PLAC1*. In the top panel (A), wild-type p53 freely binds to its binding site, sterically hindering access of the P1 transcription factors RXRα and LXRβ to their binding sites. This has the effect of suppressing *PLAC1* transcription. However, when p53 is mutated, as in the middle panel (B), p53 is unable to bind to its binding site, thus freeing the P1 transcription factors RXRα and LXRβ to bind to theirs and promoting *PLAC1* transcription. When exposed to HO-3867, as in the lower panel (C), certain p53 mutants are reactivated and will bind to the p53 binding site, once again sterically hindering access of the P1 transcription factors RXRα and LXRβ to their binding sites and hindering *PLAC1* transcription. TSS is the transcription start site.

4. Materials and Methods

4.1. Cell Culture

NIH:OVCAR3 cells were established from ascites from a 60-year-old patient presenting with a poorly differentiated ovarian adenocarcinoma [63]. We obtained these cells from the American Type Culture Collection (HTB-161) and propagated them in RPMI-1640 media supplemented with 20% fetal bovine serum, 0.01 mg/mL bovine insulin and 1% pen/strep antibiotic at 37 °C and 5% CO₂.

ES-2 cells were established from primary tumor tissue from a 47-year-old patient presenting with clear-cell adenocarcinoma [64]. We obtained these cells from AddexBio (C0017006) and propagated them in McCoy's 5A media supplemented with 10% fetal bovine serum and 1% pen/strep antibiotic at 37 °C and 5% CO₂.

Cell line identities were verified via CODIS DNA profiling at BioSynthesis, Inc. (Lewisville, TX, USA) and by direct *TP53* sequencing. Sequencing confirmed that our OVCAR3 cells harbor the R248Q gain-of-function mutant and that our ES-2 cells harbor the unknown function S241F missense mutant as expected.

4.2. HO-3867 Treatment

The HO-3867 reagent was purchased from Cayman Chemical (Ann Arbor, MI, USA, Item No. 21581). Five milligrams of HO-3867 crystals were dissolved in 10.76 mL of DMSO to produce a 1 mM stock solution that was then added to the appropriate cell media to generate the final experimental concentrations.

Depending upon the assay to be carried out, the OVCAR3 and ES-2 cells were seeded in optimum media in 6-well or 96-well culture plates (200,000 cells and 5000 cells per well, respectively) for 24 h prior to the HO-3867 treatment.

4.3. RNA Purification and Quality Control

The total cellular RNA was purified from cells using the RNeasy Plus Kit following the manufacturer's instructions (QIAGEN, Germantown, MD, USA). The RNA yield and purity were assessed in the Genomics Division of the Iowa Institute of Human Genetics (IIHG) using an Agilent Model 2100 DNA Analyzer and a Trinean DropSense 16 spectrophotometer. Samples with an RNA integrity number (RIN) above 7.0 were used for subsequent protocols [65].

4.4. Real-Time PCR

Equal-mass RNA aliquots (500 ng) from treated cells were reverse transcribed in the presence of SuperScript III Reverse Transcriptase and an oligo (dT) primer (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNAs were then amplified in the presence of Power SYBR Green (Thermo Fisher) on an Applied Biosystems Model 7900HT platform in the Genomics Division of the Iowa Institute of Human Genetics (IIHG) using the locus-specific primer pairs shown in Table 1.

Table 1. Primer pairs used for SYBR Green qPCR in this study. Amplification conditions are 95 °C (10:00); 40 (95 °C (0:15), 60 °C (1:00)). A dissociation curve is run at 95 °C (0:15); 60 °C (0:15); 95 °C (0:15) following the amplification.

Locus	Sequence	Amplicon	T _m (°C) *
<i>PLAC1</i>	5'-CACCAGTGAGCACAAAGCCACATT-3'	232 bp	60.3
	5'-CCATGAACCACTCTATGGAG-3'		52.3
<i>PLAC1P1</i>	5'-AAACTTACACGAGGAGTCTGTC-3'	371 bp #	57.2
	5'-CTGTGACCATGAACCACTCTAT-3'	285 bp #	54.2
<i>18S rRNA</i>	5'-AACTTTCGATGGTAGTCGCCG-3'	104 bp	57.2
	5'-CCTTGGATGTGGTAGCCGTTT-3'		54.2

* T_m calculated at 1.5 mM MgCl₂. # Amplicons are from the simultaneously produced P1Long and P1Short transcripts [40].

The raw expression Ct values were normalized against 18S rRNA (Δ Ct). The fold change relative to the untreated cells was calculated via the conventional $\Delta\Delta$ Ct method, where fold change is $2^{-\Delta\Delta C_t}$ [66,67]. The statistical significance of fold changes was assessed via a *t*-test with unequal variances [68]. All experiments were carried out in triplicate.

4.5. Cell Proliferation and Apoptosis

Proliferation: The OVCAR3 and ES-2 cells were plated on 96-well plates. Following a 24 h incubation, the cells were treated with increasing amounts of HO-3867. The cells were imaged using the Incucyte S3 live-cell imaging platform (Sartorius AG, Göttingen, Germany) every hour for up to 48 h. The phase-contrast images were processed using masks and filters to determine cell confluence with the Incucyte Analysis Software (2019B Rev3). Proliferation was plotted as percent confluence normalized to time 0.

Apoptosis: The OVCAR3 and ES-2 cells were plated on 96-well plates. Following a 24 h incubation, the cells were treated with increasing amounts of HO-3867 in combination with 5 μ M Incucyte Caspase-3/7 Green Dye (Sartorius # 4440). The cells were imaged with the Incucyte S3 fluorescence module every hour for up to 48 h. The fluorescent images were processed with the Incucyte Analysis software (2019B Rev3). Caspase 3/7 activation was plotted as the number of fluorescent objects per image. The graphs were created using GraphPad Prism (v9.1.0). All experiments were carried out in triplicate.

5. Conclusions

We have demonstrated that the synthetic curcumin analog HO-3867 successfully rescues the p53- mediated suppression of *PLAC1* transcription in ovarian cancer cells harboring non-truncating *TP53* mutations. As a high expression of *PLAC1* is well known to be associated with poor patient outcomes in a variety of cancers, the use of this compound in conjunction with conventional therapies may serve to improve these outcomes.

Author Contributions: E.J.D. conceived and initiated this project; cell culture, treatments and cell biology assays were carried out by E.J.D., B.M.S. and J.R.L.; RNA purifications and qPCR were performed by E.J.D., B.M.S. and J.R.L.; manuscript writing, reviewing and editing was done by all authors; funding was provided by K.K.L., B.M.S., J.G.-B. and D.P.B. All authors have read and agreed to the published version of the manuscript.

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Characterization of a TP53 Somatic Variant of Unknown Function From an Ovarian Cancer Patient Using Organoid Culture and Computational Modeling

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Abstract

In our proof-of-concept study of 1 patient with stage IIIC carcinosarcoma of the ovary, we discovered a rare mutation in the tumor suppressor, *TP53*, that results in the deletion of N131. Immunofluorescence imaging of the organoid culture revealed hyperstaining of p53 protein. Computational modeling suggests this residue is important for maintaining protein conformation. Drug screening identified the combination of a proteasome inhibitor with a histone deacetylase inhibitor as the most effective treatment. These data provide evidence for the successful culture of a patient tumor and analysis of drug response ex vivo

Keywords

ovarian cancer; p53; organoid culture; drug screen

Introduction

Epithelial ovarian cancer is the most deadly of the gynecologic malignancies¹ and the fifth leading cause of cancer-related death among women.² Treatment normally consists of surgical debulking followed by 6 cycles of platinum and taxane-based chemotherapy. However, the asymptomatic nature of the disease partnered with a high rate of recurrence

results in a long-term survival rate of only 30%.^{2,3} For these women, the evaluation of new agents must be a priority.

The classic model systems used to evaluate new therapeutic agents for ovarian cancer include immortalized cell lines, patient-derived xenograft models, syngeneic and genetically engineered mouse models, and short-term culture of primary tumor cells. Each of these methods has caveats. For example, many immortalized cell lines do not faithfully represent human disease,⁴ with significant genetic drift associated with extended culture. Patient-derived xenograft models retain many of the phenotypic and genetic features of the primary tumor. However, since these models must be created in immunocompromised mice to avoid host versus graft responses, it is not possible to study the impact of the immune system on tumor response. Although syngeneic mice and genetically engineered mouse models do allow for the evaluation of immunomodulatory agents, these models deviate from human tumor features. The short-term culture of primary tumor cells has been attempted for decades, but recent success is attributed to optimized growth media and the use of Matrigel to provide the structural support for 3-dimensional growth.

First developed for colorectal cancer in 2011 by Clevers and colleagues in the Netherlands,⁵ patient-derived organoid culture is an emerging and novel technology to model cancer. The organoid culture technology includes a cocktail of growth factors to recreate the niche of tissue homeostasis ex vivo. The general protocol includes enzymatic digestion of tumor tissue into single cells, followed by embedding the cells into Matrigel and culture with specific medium optimized for the cancer type.

A handful of studies in several cancer types have established that organoids can recapitulate both histological and genomic features of the pertinent lesion from which they were derived. Very recent work by the Soragni laboratory reported a method for high throughput drug screening using organoid cultures.⁶ Importantly, Vlachogiannis et al,⁷ have reported a positive predictive value (predicting that a particular drug works) of 88% and a negative predictive value (predicting that a particular drug does not work) of 100% when comparing organoid sensitivity to the response of a patient with gastrointestinal cancer.

Herein we present proof-of-concept data that we can create an organoid model of ovarian cancer using cancer cells isolated from ascites fluid that was collected at the time of initial debulking.

Materials and Methods

HUMAN SUBJECTS

Human subjects studies were approved by the University of Iowa Institutional Review Board (IRB). Informed consent was obtained under IRB protocol #2018090807, Molecular Genetic Study of Gynecologic Specimens and Data Repository.

SPECIMEN COLLECTION

The patient was taken to the operating room, where a midline vertical incision was made. Two hundred milliliters of ascites was collected in a sterile container and placed in a biohazard bag. This was then immediately handed off to lab personnel for processing.

ISOLATION OF TUMOR CELLS FROM ASCITES FLUID

On arrival, the ascites fluid was transferred from the vacuum container to 50 mL tubes. Next, 5mL of 50% vol/vol Percoll was aliquoted into each of 16 to 20 15 mL conical centrifuge tubes. Five milliliters of ascites was carefully layered onto the 50% vol/vol Percoll cushion in each tube, followed by centrifugation at 1200g for 20 minutes at 4°C using a swinging bucket rotor. Erythrocytes pellet at the bottom of the tubes, while the desired ovarian cancer cells are at the interphase between the ascites fluid and Percoll. Using a 5 mL pipet, cancer cells were carefully withdrawn from the interphase fraction and transferred to a new 15 mL conical tube. To remove residual red blood cells, 2 mL of blood cell lysis buffer was added.

RNA PURIFICATION

Ascites cells were collected and total cellular RNA was purified using the mirVana RNA Purification Kit following manufacturer's (Thermo Fisher) recommendations. RNA yield and purity were determined on an Agilent Model 2100 Bioanalyzer. Sufficient yield was obtained with an RNA Integrity Number (RIN) >9.0 to proceed with RNA sequencing.

TP53 SEQUENCING

Sanger sequencing of *TP53* was carried out using 100 ng of purified cellular RNA. This RNA was reverse transcribed in the presence of SuperScript III reverse transcriptase (Thermo Fisher). The resulting cDNA was then amplified using the *TP53* primers p53mRNAfor: AGCCGCAGT-CAGATCCTA and p53mRNArev: GA AGTCCTGGGTGCTTCTG. The 1302 bp amplicon was prepared for Sanger sequencing using the QIAquick PCR Purification Kit following manufacturer's (Qiagen) recommendations. Using the PCR primers as sequencing primers, the mRNA amplicon was sequenced in both directions on an Applied Biosystems Model 3730XL platform and aligned against the mRNA sequence NM_000546.

COMPUTATIONAL MODELING OF p53

Simulated annealing was used to generate a plausible structure of the S2-S2' region (residues K120-Q136) with the delN131 (Δ N131) mutation. The same protocol was also applied to the WT protein to ensure a fair comparison. The 3KMD crystal structure⁸ was used as our baseline. Zinc-coordinating cysteines 176, 238, and 242 were deprotonated, and the zinc-coordinating H179 was deprotonated at the δ nitrogen. Then, H193 and H214 were deprotonated at the δ and ϵ nitrogens, respectively, as they form a buried π -stack, have a depressed pKa predicted by PROPKA,^{9,10} and were assigned as neutral by Pradhan et al.¹¹ Using Force Field X (FFX),¹² the structure was re-refined with the polarizable atomic multipole AMOEBA 2018 protein force field.¹³⁻¹⁵

Subsequently, chain A was solvated in a 75.951 Å water box with a total of 13,168 water molecules (including crystal waters) with 100 mM NaCl (27 copies) and 8 additional chloride ions to neutralize the system. This was then minimized using the limited memory Broyden-Fletcher-Goldfarb-Shanno algorithm^{16,17} (LBFGS) algorithm to a convergence criterion of 0.1kcal/(mol×Å) using FFX-OpenMM.¹⁸ This was then equilibrated for 2 nsec with 40 kcal/(mol×Å) harmonic heavy atom restraints at 298.15K. Asparagine 131 was then removed from the structure, and the structure re-minimized to the same convergence criterion while constraining all atoms outside the range Y35 to F43 to the equilibrated structure. Both the WT equilibrated structure and the ΔN131 structure were then annealed, initially at 1000K for 500 psec, then 200 psec each of 800K, 600K, 400K, 300K, 250K, 200K, 150K, and 100K, finishing with 500 psec of dynamics at 50K. During this annealing, protein-heavy atoms and the zinc ion outside the range K120 to Q136 were restrained to the equilibrated structure with 100 kcal/(mol×Å) harmonic restraints.

All dynamics were performed in the NVT ensemble using FFX-OpenMM on NVIDIA Titan V accelerators. The NVT ensemble was maintained with an Andersen thermostat¹⁹ with a collision frequency of 91/psec and a RESPA multiple timestep integrator^{20,21} with 0.5 fsec inner timesteps and 2 fsec outer timesteps. The van der Waals interactions were smoothly switched off between 10.8 and 12.0 Å. The Particle Mesh Ewald algorithm^{12,22} was used for long-range electrostatics, with an Ewald coefficient of 0.545, a 7.0 Å real-space cutoff, fifth-order B-splines, and a 96×96×96 grid. Extrapolated perturbation theory²³ was used for mutual polarization.

GENERATION OF ORGANOID MODEL USING CANCER CELLS FROM ASCITES FLUID

Cancer cell pellets were suspended in AdDE+++ medium and filtered with a 40 µm cell strainer. After centrifugation at 2500 rpm for 5 minutes, the cell pellet was suspended with cool Matrigel (1×10⁵ to 5×10⁵ cells/mL) and mixed well with a cold tip. Spheroids (20 µL in Matrigel) were transferred to 1 well of a 96-well plate; the liquid Matrigel was allowed to solidify by placing the plate in a 37°C incubator for 15 minutes. Next, 200 µl AdDE+++ media was added to the top of the Matrigel. Media was changed every 6 to 7 days and organoids were passaged every 2 to 4 weeks.

The AdDE+++ medium recipe (200 mL)

AdDE+++ (advanced DMEM/F12: 1x glutamax, 10 mM HEPES)	191 mL
Noggin (100 µg/mL)	200 µL
Rspo1 (25 µg/mL)	2 mL
B27 (50×)	4 mL
N-Acetylcysteine (500 mM)	0.5 mL
Primocin	0.4 mL
Nicotinamide (1 M)	2 mL
A83-01 (5 mM)	20 µL
FGF 10 (100 µg/mL)	20 µL

Heregulin β -1 (75 μ g/mL)	100 μ L
Y27632 (100 mM)	10 μ L
EGF (500 μ g/mL)	2 μ L
Forskolin (10 mM)	200 μ L
Hydrocortisone (250 μ g/mL)	400 μ L
β -Estradiol (100 μ M)	200 μ L
Total	200 mL

IMMUNOFLUORESCENCE IMAGING

Organoids were grown on glass coverslips and treated with indicated reagents. After fixation with 2% paraformaldehyde and permeabilization with 1% Triton X-100, cells were labeled with a p53 antibody (sc-126; Santa Cruz) and PAX8 antibody (10336-1-AP; Proteintech) followed by incubation with Alexa Fluor-555 (for p53) or Alexa Fluor-488 (for PAX8)-conjugated secondary antibody. Images were visualized by fluorescence microscopy and acquired with an Olympus BX51 camera at $\times 63$ magnification.

HEMATOXYLIN AND EOSIN (H&E) STAINING

Formalin-fixed, paraffin-embedded organoids section were stained using H&E program 8 from Central Microscopy facility, the University of Iowa, dehydrated using 3 washes of ethanol and xylene, and mounted with coverslips.

DRUG SCREENING

The viability of the tumor organoids following drug treatment was determined by the CellTiter-Glo 3D cell viability assay (Promega, Madison, WI). In brief, tumor cells were suspended in AdDE+++ medium with 20% Matrigel and seeded at a density of 3000 cells/well (200 μ L/well) in an ultra-low attachment 96-well U-bottom white plate. After 24 hours, cells were then exposed to cisplatin (5 μ M), carboplatin (5 μ M), paclitaxel (10 nM), bevacizumab (1 μ M), AZD1775 (125 nM), bortezomib (10 nM), belinostat (1 μ M) or olaparib (2 μ M) for 72 hours at 37°C. At the end of incubation, an equal volume of CellTiter-Glo 3D reagent was added to each well and incubated for 25 minutes at room temperature. The luminescence was measured using the Gen5 Microplate Reader (BioTek, VT). All the tests were conducted in triplicate and data normalized to untreated control (set at 100% viability).

Results

PATIENT CHARACTERISTICS

This proof-of-concept study was performed using tumor cells collected from the ascites fluid of a patient undergoing primary cytoreductive surgery. The patient is a 77-year-old woman who presented with abdominal bloating and discomfort. Her imaging was concerning for advanced stage gynecologic malignancy, and she was recommended for primary cytoreductive surgery. During surgery, she was found to have bilateral adnexal masses obscuring the gynecologic organs and obliterating the rectovaginal space, as well as an

omental cake, tumor plaquing of the right hemidiaphragm, and miliary disease along the small bowel mesentery and descending colon. She underwent an optimal tumor cytoreduction consisting of a total abdominal hysterectomy with bilateral salpingoophorectomy with en block resectosigmoid resection with end to end anastomosis, infragastric and infracolic omentectomy, diaphragmatic peritonectomy with diaphragm resection and primary repair, and appendectomy. Her postoperative course was complicated by a bowel leak from her resectosigmoid re-anastomosis, which required a return to the operating room for an abdominal washout and diverting loop colostomy. She recovered in the intensive care unit on broad-spectrum antibiotics and pressors, transferred to the general care unit, and finally discharged on a postoperative day 36 from her initial surgery. The patient went home on enteral nutrition based on her continued poor oral intake. Final pathology was stage IIIC carcinosarcoma of the ovary. She was recommended to complete 6 cycles of carboplatin and paclitaxel but started with single-agent carboplatin until her performance status improves.

GENERATION OF ORGANOID EX VIVO MODEL OF PATIENT TUMOR CELLS

Cancer cells were isolated from the ascites fluid and used to generate an organoid model of the patient's disease as described in *Material and Methods*. We first performed pathologic analysis of the organoids using H&E staining and found that cells form sphere-like structures (Fig. 1A). Immunofluorescent staining with protein biomarkers paired box gene 8 (PAX8) and p53 confirmed that the tumor is ovarian in origin (Figs. 1B, C). Interestingly, p53 was highly expressed in the tumor organoids by both immunofluorescence and immunohistochemistry (Figs. 1C, D).

IDENTIFICATION OF A TP53 VARIANT OF UNKNOWN FUNCTION

We next examined the *TP53* status of the tumor since *TP53* is commonly mutated in carcinosarcomas of the ovary²⁴ and was overexpressed in the tumor organoid (Figs. 1C, D). Mutations in *TP53* can be binned in a variety of ways, but the most commonly used nomenclature is to bin the mutations into 1 of 3 simplistic functional classes: (1) truncating, frameshift or splice site loss-of-function mutations that mainly result in a p53-null state, (2) missense mutations that often result in gain-of-oncogenic function (GOF), and (3) synonymous/silent mutations that are wild-type (WT) equivalent. Of the missense mutations, ~8 mutations are canonically recognized as "GOF" based on a variety of functional studies²⁵: P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L, R282W.

The aligned mRNA sequence from the patient-derived tumor cells revealed a single mutation event in the *TP53* gene. A deletion of 3 bases (AAG) in Exon 5 resulted in an in-frame deletion of asparagine residue 131 (Δ N131).

In a previous analysis of The Cancer Genome Atlas (TCGA) dataset for ovarian cancer, we found that nearly one half of tumors harbor missense mutations in *TP53* that are not classic GOF mutations.²⁶ We term these missense mutations "somatic variants of unknown function" because there is not a standardized nomenclature for them. The often-used terminology "variants of unknown significance" refers to germline variants with unclear risk

of disease, which is not the case when a patient already has cancer. Therefore, we characterize the Δ N131 mutation as a somatic variants of unknown function.

COMPUTATIONAL MODELING TO PREDICT THE FUNCTIONAL IMPACT OF Δ N131 MUTATION

Since the Δ N131 variant is not a traditional loss-of-function or GOF mutation, our next objective was to model the functional consequences of the deletion of N131. p53 is a well-characterized transcription factor with tumor suppressor activities. p53 GOF mutants are typically broadly divided into 2 categories: (1) *contact mutants* that have lost the ability to bind DNA (eg, R273H, R248Q/W); and (2) *structural mutants* that alter the conformation of the protein (eg, R175H, Y220C).²⁷ Our prior TCGA analysis of ovarian tumors demonstrated a high frequency in *TP53* mutations in the DNA-binding domain.²⁶

In the p53 protein structure, asparagine 131 is part of a β -turn between the S2 and S2' β -sheet segments²⁸ (Fig. 2A). The S2-S2' region lies in between sheet S10 and helix H2, and likely holds them together. The H2 helix is notable for directly contacting DNA, while the S10 helix is part of the main β -sandwich structure. As such, it is thought that without the S2 and S2' β -sheet, the H2 helix may become separated from the rest of the DBD and impact binding affinity. Numerous specific interactions hold the S10-S2'-S2-H2 bundle together (Table 1). In particular, L130 forms a hydrophobic contact with helix H2 (Fig. 2B), and it is almost certain that hydrophobic effects are central to protein folding. To confirm this, we used a global structural optimization method called simulated annealing in the FFX program²⁹ to find low-energy conformations of the S2-S2' region (defined as residues K120-Q136). Although the WT structure maintained the L130-H2 interaction throughout the simulation, even at substantially elevated temperatures (Fig. 2B, dark blue), the Δ N131 mutant showed the L130 movement away from H2 within 60 psec and never returned to an H2-interacting conformation (Fig. 2B, red). This suggests that strain introduced in the β -turn by Δ N131 results in loss of key stabilizing interactions in the S10-S2'-S2-H2 region, including a hydrophobic contact made by L130.

DRUG SCREENING DEMONSTRATES SENSITIVITY OF TUMOR ORGANOIDS TO A RANGE OF AGENTS

To demonstrate the ovarian cancer organoids culture platform is suitable for drug screening, tumor organoids were treated with indicated drugs for 72 hours then the cell viability assay was performed with CellTiter-Glo 3D assay (Fig. 3). The results showed that this patient's organoids were most sensitive to the combination of bortezomib and belinostat, and also quite sensitive to bortezomib, paclitaxel, and AZD1775 alone, but not sensitive to bevacizumab or olaparib. It is also important to note that the tumor organoid was relatively sensitive to the combination of carboplatin and paclitaxel, the standard front-line treatment for carcinosarcoma patients.

Discussion

To make meaningful predictions regarding how each patient is likely to respond to therapy, it is critical to have a reasonable understanding of the function of each p53 mutant. Several

algorithms have been developed to predict if mutations are deleterious from the perspective of protein structure,³⁰ but they do not predict how the mutants affect protein function. Rather, these predictions are binary pathogenic versus benign predictions. Missense mutations may be difficult to characterize based upon predictions of structure alone, and overexpression at the protein level is not always an indicator of oncogenic GOF activity.

The ΔN131 mutant results from an in-frame deletion of nucleotides AAC at positions c.391-c.393 of the *TP53* gene (GRCh38 chromosome 17 coordinates 7675175-7675173). This mutation has been reported independently in a Wilms tumor patient,³¹ a serous ovarian cancer patient,³² a patient with neuroendocrine small cell carcinoma of the uterine cervix,³³ and a patient with adenocarcinoma of the stomach.³⁴ In the first 2 studies,^{31,32} the presence of this mutation was marked by elevated production and accumulation of p53 protein. Herein we document that p53 expression is high in the organoid culture that harbors the ΔN131 p53 mutant protein. However, additional studies are necessary to determine the mechanism of hyperexpression, as well as functional consequences. We speculate that this indel results in a GOF-type p53 protein.

The p53 molecule contains a Zn⁺⁺ pocket in the DNA-binding domain formed by the residues C176 and H179 in the L2 loop and C238 and C242 in the L3 loop. There is evidence that disruption of optimal folding of p53 will impact retention of the Zn⁺⁺ and lead to significant loss of DNA-binding specificity.³⁵ Numerous biochemical and cellular studies have confirmed that proper binding and retention of the Zn⁺⁺ divalent cation is crucial to p53 function and that even minor alterations can significantly disrupt that function.³⁶ Our analysis of the structural impact of the N131 deletion suggests that this indel results in loss of Zn⁺⁺ binding and therefore DNA binding.

Our drug screening results may provide insights for doctors in further treatment for this patient in the clinic. GOF p53 is frequently hyper-stabilized by Hsp90, and we and others have found that strategies that promote Hsp90 acetylation (ie, histone deacetylase inhibitor) sensitize cells with GOF p53 to chemotherapy.^{37,38} Our recent study in gynecologic cancer cells with 2 different *TP53* GOF mutations indicates that greater cell toxicity can be achieved by the addition of a histone deacetylase inhibitor to the backbone treatment of a proteasome inhibitor.³⁹ Our long-term vision is to deploy this platform to determine drug sensitivities of primary specimens, which can offer actionable information to help tailor therapy to individual cancer patients.

Acknowledgments

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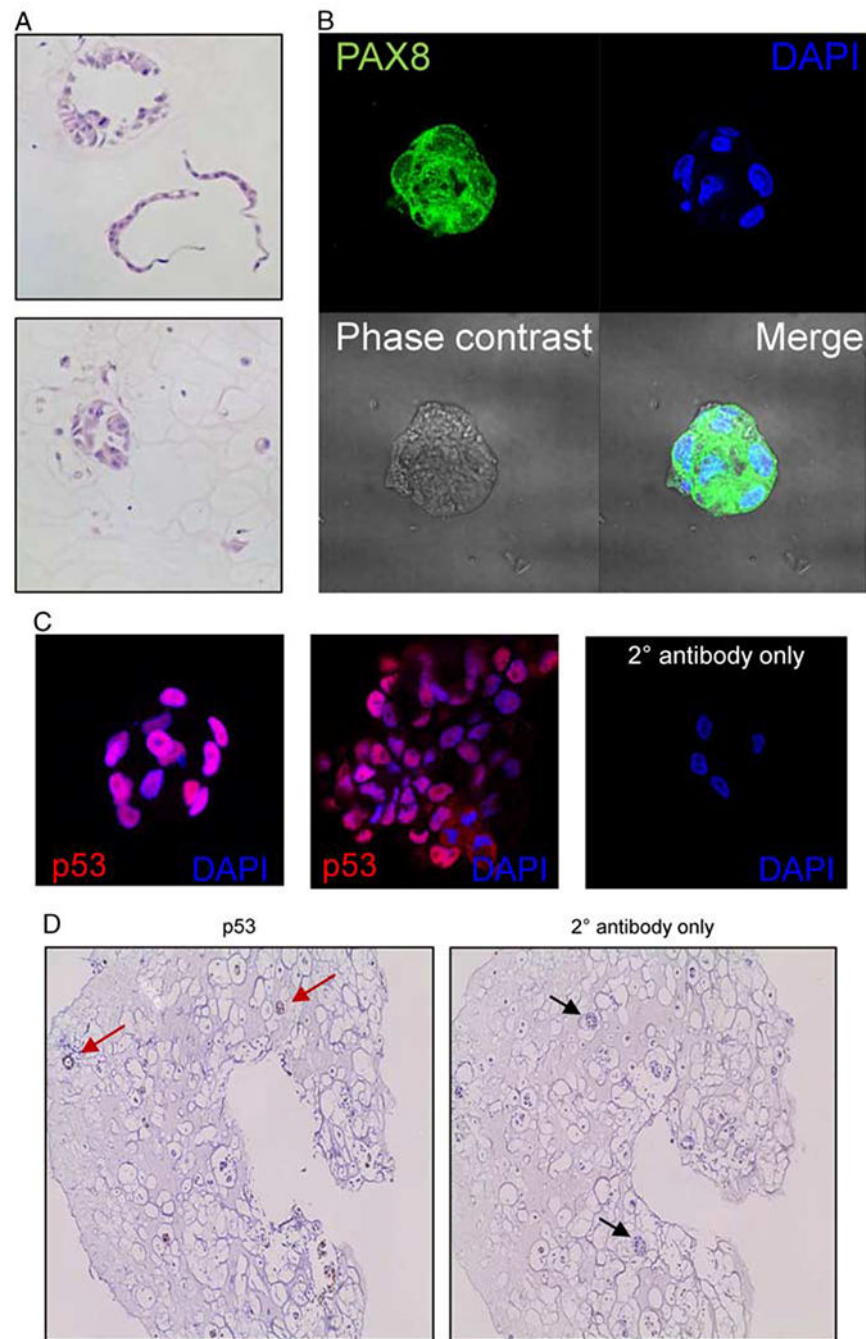


FIGURE 1. Characterization of organoid culture. A, Hematoxylin and eosin staining of paraffin-embedded section. B and C, Immunofluorescent imaging for PAX8 (B) or p53 (C). Nuclei were counterstained with DAPI. D, Immunohistochemistry for p53. Arrows indicate organoids. Other structures are Matrigel.

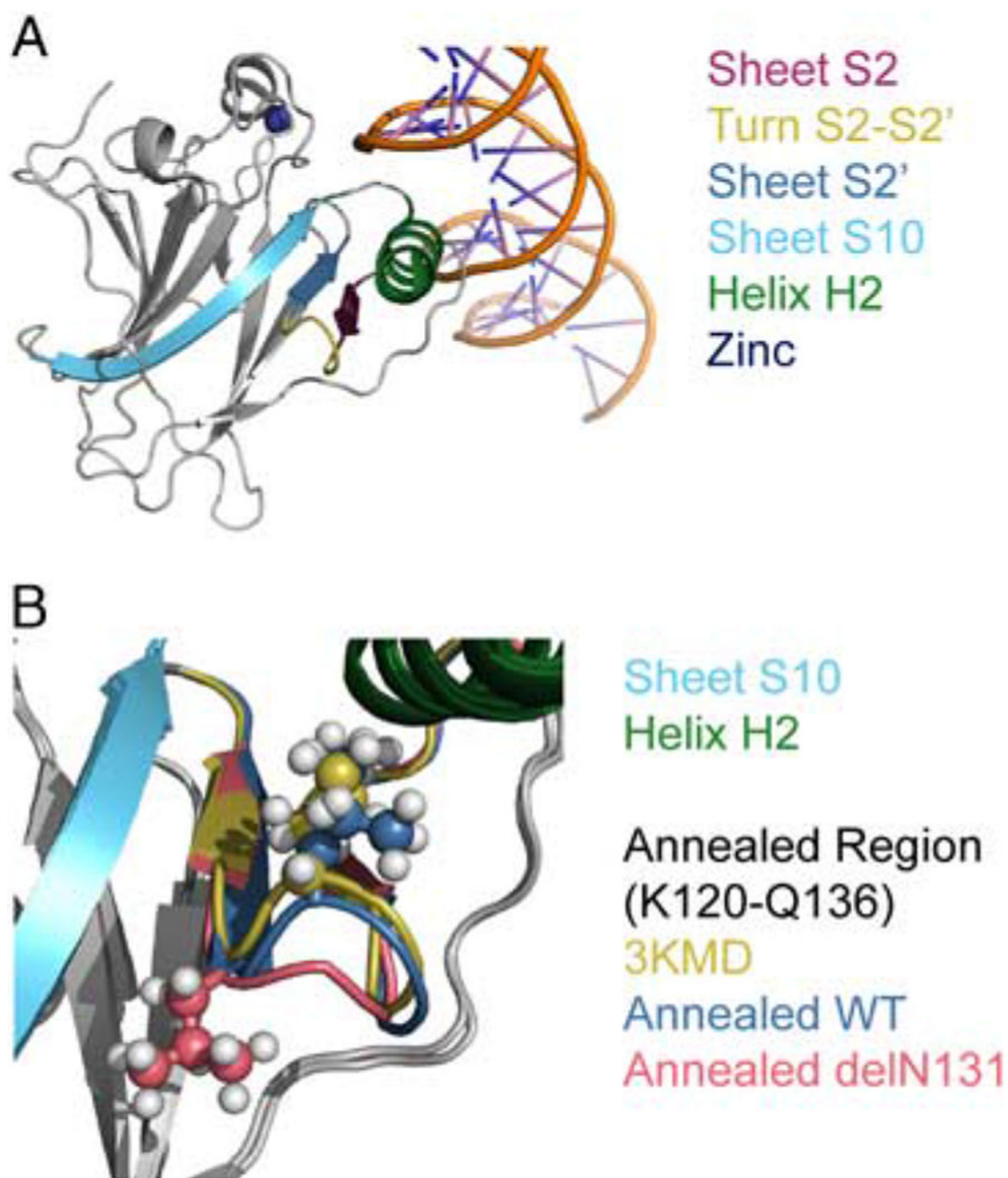


FIGURE 2.

Cartoon representations of p53 (PDB ID: 3KMD). A, Zoomed out view of chain A and the DNA; protein chains B-D are excluded for clarity. B, Overlay of 3KMD chain A with optimized structures from simulated annealing of residues K120 to Q136. The side chain of L130 is shown in ball-and-stick representation. When N131 is deleted, L130 shifts downwards and fails to contact helix H2.

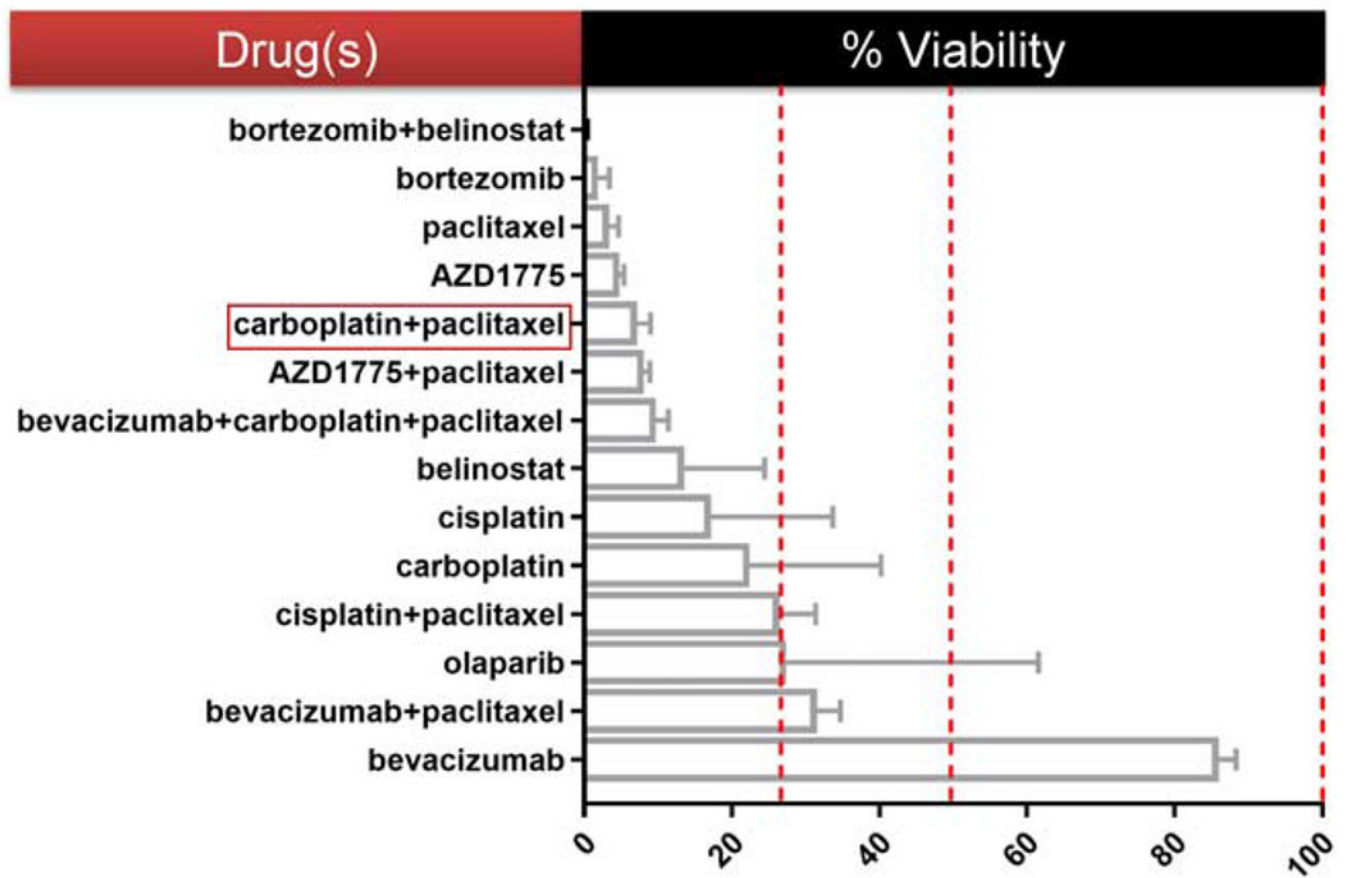


FIGURE 3.

Drug screening using patient organoid. As noted with the red box, carboplatin+ paclitaxel is the standard front-line treatment for ovarian carcinosarcoma.

Key Interactions Formed by Residues in S2, S2', and the Intervening Turn (T2-T2') in p53

TABLE 1.

N-terminal Site	Location	C-terminal Site	Location	Nature of Interaction
C124	S2	C135	S2'	Potential disulfide bond site
T125	S2	F134	S2'	β-sheet H-bond
T125	S2	R282	H2	Side chain H-bond
T125	S2	Y126	S2	Side chain-main chain H-bond
Y126	S2	N131	S2'	Side chain H-bond
S127	S2	L130	T2-2'	Main chain H-bond
S127	S2	L130	T2-2'	Side chain-main chain H-bond
S127	S2	N131	T2-2'	Main chain H-bond
S127	S2	K132	S2'	β-sheet H-bond
S127	S2	K132	S2'	β-sheet H-bond
S127	S2	E286	H2	Side chain H-bond
P128	S2	N131	T2-2'	Main chain H-bond
L130	T2-2'	L289	H2	Hydrophobic contact
N131	T2-2'	S269	S10	Side chain-main chain H-bond
N131	T2-2'	E271	S10	β-sheet H-bond
K132	S2'	E271	S10	Salt bridge
K132	S2'	E271	S10	β-sheet H-bond
M133	S2'	E271	S10	β-sheet H-bond
M133	S2'	R273	S10	β-sheet H-bond
F134	S2'	R282	H2	Hydrophobic contact
C135	S2'	R273	S10	β-sheet H-bond

p53 structures used for this analysis: 3KMD, 2FEJ, 3IGK, 5BUA, 5LGY, 5MCT, and 6FJ5. The major discrepancy between structures in this region is that H2 did not appear to be as well-ordered in many members of the 2FEJ NMR ensemble.

ARTICLE OPEN



Blocking autophagy overcomes resistance to dual histone deacetylase and proteasome inhibition in gynecologic cancer

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Histone deacetylase (HDAC) inhibitors and proteasome inhibitors have been approved by the FDA for the treatment of multiple myeloma and lymphoma, respectively, but have not achieved similar activity as single agents in solid tumors. Preclinical studies have demonstrated the activity of the combination of an HDAC inhibitor and a proteasome inhibitor in a variety of tumor models. However, the mechanisms underlying sensitivity and resistance to this combination are not well-understood. This study explores the role of autophagy in adaptive resistance to dual HDAC and proteasome inhibition. Studies focus on ovarian and endometrial gynecologic cancers, two diseases with high mortality and a need for novel treatment approaches. We found that nanomolar concentrations of the proteasome inhibitor ixazomib and HDAC inhibitor romidepsin synergistically induce cell death in the majority of gynecologic cancer cells and patient-derived organoid (PDO) models created using endometrial and ovarian patient tumor tissue. However, some models were not sensitive to this combination, and mechanistic studies implicated autophagy as the main mediator of cell survival in the context of dual HDAC and proteasome inhibition. Whereas the combination of ixazomib and romidepsin reduces autophagy in sensitive gynecologic cancer models, autophagy is induced following drug treatment of resistant cells. Pharmacologic or genetic inhibition of autophagy in resistant cells reverses drug resistance as evidenced by an enhanced anti-tumor response both in vitro and in vivo. Taken together, our findings demonstrate a role for autophagic-mediated cell survival in proteasome inhibitor and HDAC inhibitor-resistant gynecologic cancer cells. These data reveal a new approach to overcome drug resistance by inhibiting the autophagy pathway.

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INTRODUCTION

Endometrial and ovarian cancer are two of the most common gynecologic malignancies. These diseases combined lead to 25,000 deaths annually in the US [1]. Chemotherapy with the doublet of a platinum compound and a taxane is the first choice for most high-grade serous ovarian and high-risk endometrial cancers [2, 3]. While many patients have a good response to initial treatment, the majority develop recurrent disease and become resistant to chemotherapy. Thus, the development of effective therapies against gynecological cancers is still an unmet medical need.

The ubiquitin-proteasome system is responsible for the degradation of unnecessary or damaged proteins within mammalian cells [4]. Cancer cells rely heavily on hyperactivated proteasomes to evade programmed cell death [5]. Proteasome inhibition thereby prevents clearance of misfolded proteins and leads to cell death, which makes proteasome inhibitors promising therapeutic agents against cancer [6]. Currently, three proteasome inhibitors are approved by the FDA and in clinical use to treat multiple myeloma: bortezomib, carfilzomib, and ixazomib. Ixazomib was the first oral proteasome inhibitor to gain FDA approval [7]. Clinical trials have evaluated the efficacy and safety of bortezomib (NCT00023712) and

carfilzomib (NCT00531284) in ovarian cancer [8]. Bortezomib in combination with conventional chemotherapy has also been tested in ovarian cancer (NCT00059618, NCT00028912, NCT01074411, NCT00620295, and NCT00667641) [8]. While these agents had a good safety and tolerability profile, no therapeutic effect was observed using either bortezomib or carfilzomib as a single agent or in combination with chemotherapy in ovarian cancer [9–11]. To date, analyses have not been performed to explain or overcome resistance to therapy found in these studies.

Histone deacetylases (HDACs) are a class of enzymes that epigenetically regulate many biological processes by deacetylating histones and other regulatory proteins [12]. Cancer cells maintain epigenetic modifications to regulate gene expression patterns that facilitate and sustain tumorigenesis [13]. HDAC inhibitors block the actions of HDACs and affect the expression of genes that regulate many cellular processes, including cell cycle, reactive oxygen species, and angiogenesis [14]. To date, four HDAC inhibitors have been FDA-approved for the treatment of lymphoma: vorinostat (SAHA), romidepsin, belinostat, and panobinostat. HDAC inhibitors employed alone or in combination with chemotherapy have been studied in ovarian and endometrial

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cancer clinical trials (NCT00772798, NCT00976183, NCT00993616, and NCT03018249), including our own group's analysis of entinostat in a surgical window of opportunity trial in endometrial cancer [8, 15, 16]. Similar to proteasome inhibitors, HDAC inhibitors were well tolerated in ovarian cancer. Some studies reported limited efficacy as single agents [15], and a trial of vorinostat with chemotherapy was terminated due to toxicity. However, belinostat in combination with chemotherapy was well-tolerated with an encouraging 43% overall response rate [17].

Instead of using each agent individually or with chemotherapy, where success has been relatively modest, treatment with a proteasome inhibitor in combination with an HDAC inhibitor has achieved remarkable clinical success in hematological malignancies [4]. Numerous preclinical studies have also shown that a proteasome inhibitor and HDAC inhibitor combination can synergistically induce cell death in solid tumors [18–20]. Moreover, multiple clinical trials with proteasome and HDAC inhibitors are ongoing in solid tumors, some of which have shown promising results [21]. For example, the combination of vorinostat with the proteasome inhibitor marizomib was tested in a Phase I clinical trial (NCT00667082) of advanced or recurrent solid tumors. The data showed stable disease in 61% of evaluable patients [22]. Similarly, the combination of vorinostat with bortezomib in patients with advanced solid tumors (NCT00227513) resulted in stable disease in most patients [23]. However, this combination has not been yet tested in clinical trials of women with gynecologic malignancies.

While preclinical studies have demonstrated the activity of HDAC and proteasome inhibitors in solid tumors [24–26], including our own work in gynecologic cancer cell lines [27], the mechanisms underlying sensitivity vs. resistance to this combination are not well understood. Herein we demonstrate that gynecologic cancer cell lines, xenografted animal models, and patient-derived organoids (PDOs) of endometrial and ovarian cancer have differential sensitivity to the combination of the FDA-approved proteasome inhibitor ixazomib and the FDA-approved HDAC inhibitor romidepsin based upon their ability to induce autophagy as a survival mechanism. Thus, our data implicate autophagy as a major mediator of resistance, and inhibiting autophagy significantly enhances sensitivity to therapy. These studies set the stage for novel combinations to treat advanced and recurrent endometrial and ovarian cancer.

MATERIALS AND METHODS

A full description of the Materials and methods are included in Supplemental Methods.

PDO models

All studies using human tissues were approved by the University of Iowa (UI) Institutional Review Board (IRB), protocol #201809807. PDO cultures were created as we previously described [28].

Western blotting

Western blotting was performed in lysates from cells or tumor tissues [29]. Data were normalized to β -actin control and calculated relative to untreated or vehicle control.

Cell viability assays

Analysis of cell viability in PDOs or cell lines was performed as described previously [28, 30, 31]. Data were normalized to untreated control, set at 100% viability. For PDOs, data were calculated as the change in viability relative to control (set at 100%).

Assessment of autophagic flux

The pBabe-puro retroviral expression vector was used to stably express mCherry-EGFP-LC3B. Autophagic flux was calculated as the ratio change in the median fluorescence intensity of mCherry:GFP as determined by flow cytometry [32].

shRNA-mediated Knockdown of ATG5

Hec50 and SKOV3 cells were infected with lentivirus containing either nontargeting shRNA or shRNAs against ATG5 (TRCN0000151963 and TRCN0000151474 obtained from the RNAi Consortium).

Animal studies

Animal studies were performed under animal protocol #0022285-003 approved by UI Institutional Animal Care and Use Committee. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were subcutaneously injected with Hec50 cells and treated starting on day 18 after engraftment.

Immunofluorescence imaging

Immunofluorescence staining was performed using 5 μ m-thick sections of post-treatment tissue samples. Images were visualized by fluorescence microscopy at $\times 63$ magnification.

RESULTS

The majority of endometrial and ovarian cancer PDO models and cell lines are sensitive to dual treatment with a proteasome inhibitor and HDAC inhibitor

To understand the effect of ixazomib and romidepsin on cell viability, drug response assays were performed on 17 PDO models of endometrial and ovarian cancer from patient tumor tissues as well as three endometrial cancer PDX models (Table S1). As compared to untreated controls, PDOs exhibited a notable decrease in viability when treated with ixazomib and romidepsin, with cell-killing ranging from 52.3 to 99.7% (Fig. 1). Similar results were obtained with other HDAC inhibitors and proteasome inhibitors (Fig. S1), indicating this effect is not specific to romidepsin and ixazomib. Models were also exposed to carboplatin plus paclitaxel, the most frequently used first-line

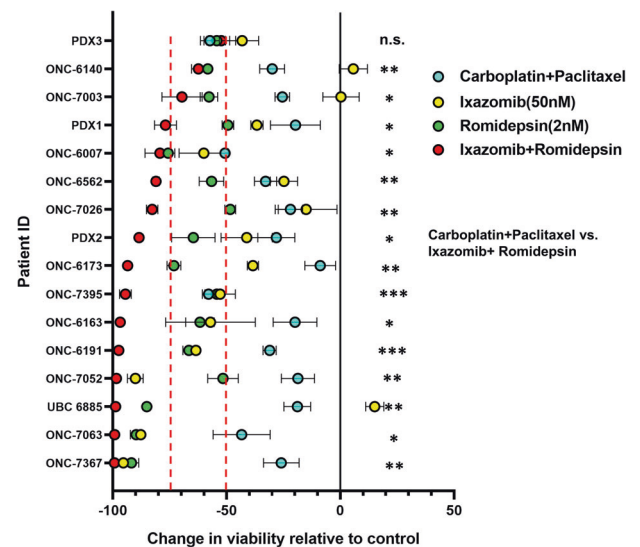


Fig. 1 Most endometrial and ovarian cancer PDO models are highly sensitive to ixazomib and romidepsin as compared to standard chemotherapy. PDOs of fresh patient tumor specimens (indicated by Patient ID) or PDX models cultured in the organoid model system (PDX1-3) were treated with standard chemotherapy (1 μ M carboplatin + 14 nM paclitaxel), ixazomib (50 nM), romidepsin (2 nM) or ixazomib + romidepsin for 72 h, followed by assessment of cell viability. Data were calculated as the change in viability relative to control, which was set at 100% (i.e., no cell death). Statistical significance was assessed by two-way ANOVA with Tukey's post hoc test. Significant differences between carboplatin + paclitaxel vs. ixazomib + romidepsin are annotated for each PDO. n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Patient information is provided in Supplementary Table S1. All statistical comparisons are provided in Supplementary Table S2.

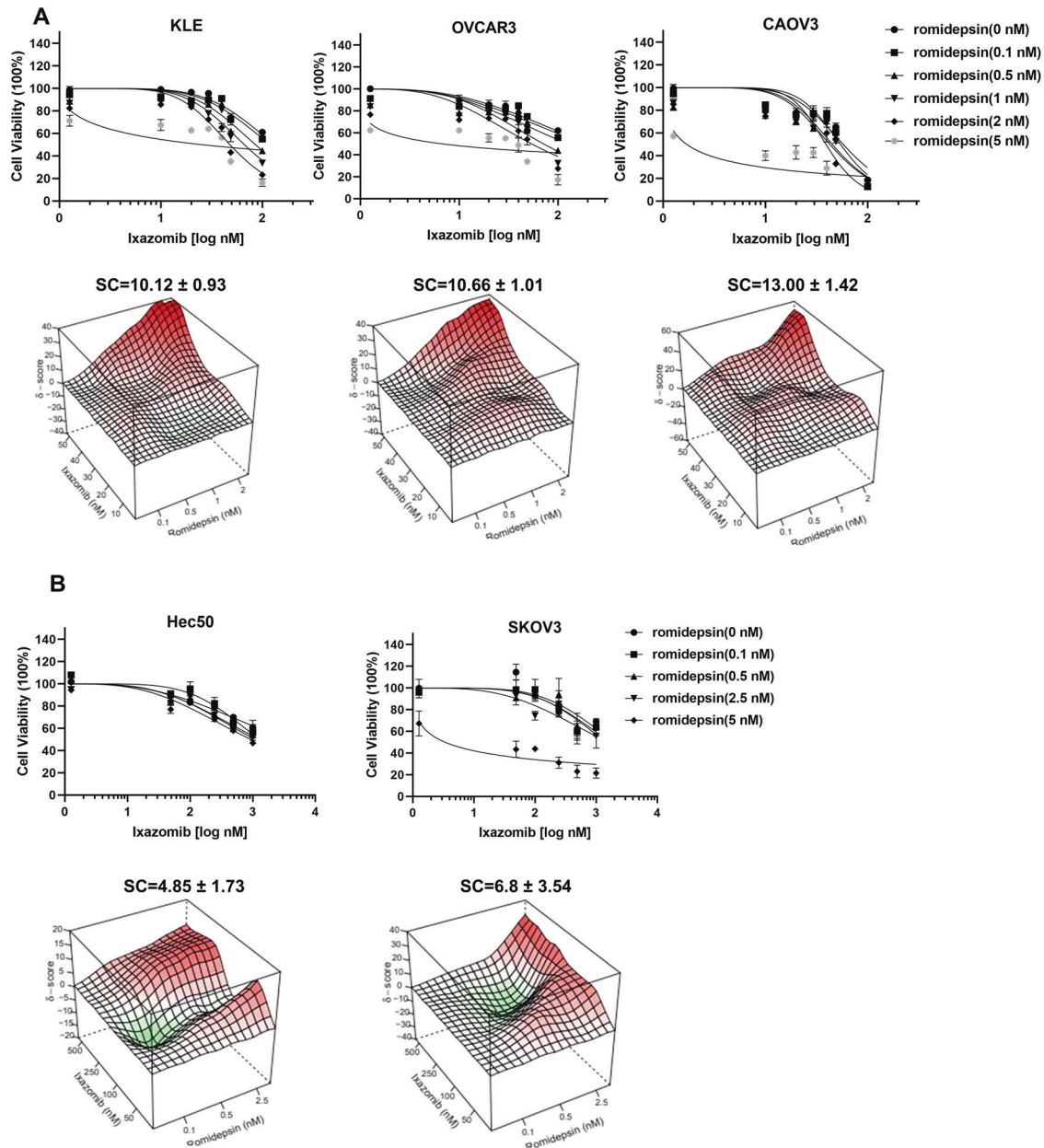


Fig. 2 Gynecologic cancer cell lines display differential sensitivity to the combination of romidepsin and ixazomib. Data are separated into (A) sensitive cells (KLE, OVCAR3, and CAO3) and (B) resistant cells (Hec50 and SKOV3) as determined by viability after treatment with romidepsin ± ixazomib for 72 h. The dose-dependent inhibition curves for drug combinations at varying concentrations are shown on the upper panels. Note that the dose-response curve for ixazomib alone (filled black circles) is denoted as “romidepsin (0 nM).” Lower panels are 3D plots used to determine synergy scores (SC). An SC of >10 indicates synergy.

chemotherapeutic regimen for gynecologic malignancies. Compared to untreated control, PDOs were differentially sensitive to standard chemotherapy, with decrease in viability from 8.8 to 57.9% (Fig. 1). Moreover, 16/17 PDOs were more sensitive to ixazomib and romidepsin as compared to standard chemotherapy (Table S2).

Studies were extended to well-characterized advanced endometrial and ovarian cancer cell models. Synergy between ixazomib and romidepsin was assessed under varying drug concentrations by checkerboard assays followed by analysis using the highest single agent (HSA)-independent method. In KLE, OVCAR3, and CAO3 cells, combining romidepsin with ixazomib produced a significant synergistic effect on cell killing (synergistic score >10; Fig. 2A) at nanomolar concentrations. However, in Hec50 and

SKOV3 cells, combining romidepsin with ixazomib did not result in a synergistic response despite using higher drug concentrations (synergistic score <10; Fig. 2B).

Ixazomib and romidepsin elevate autophagic flux in resistant cells

Recent studies revealed that cancer cells utilize autophagy as a protective cellular survival and defense mechanism to maintain functional mitochondria, reduce DNA damage, and remain viable in response to stress. We hypothesized that resistance to ixazomib and romidepsin in Hec50 and SKOV3 cells is associated with activation of autophagic cell protective mechanisms. We thus assessed the conversion of LC3B-I to LC3B-II by Western blotting. This conversion represents a classic marker of autophagy. To

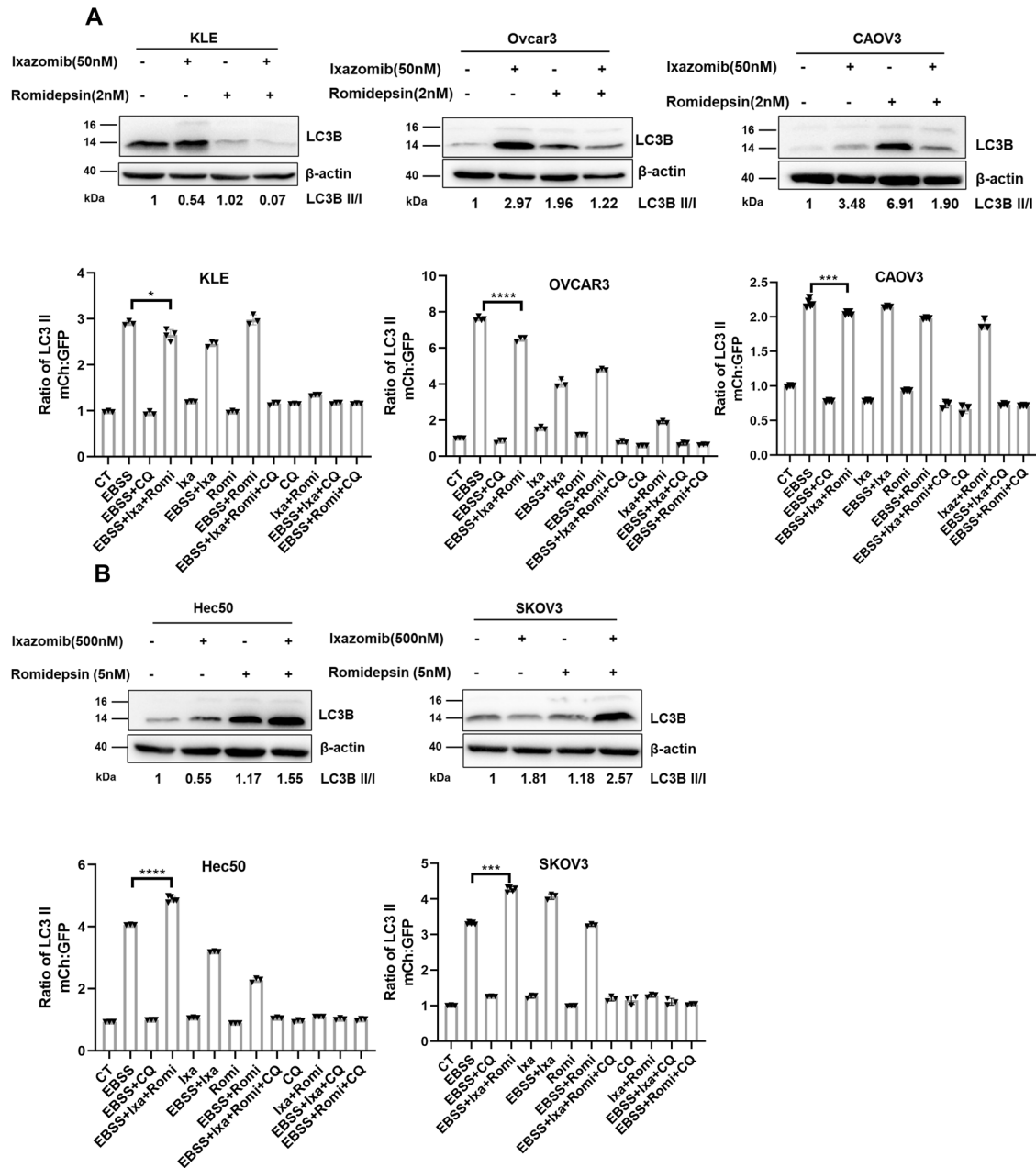


Fig. 3 Ixazomib and romidepsin induce autophagy in resistant Hec50 and SKOV3 cells but not in sensitive cells. **A** Sensitive cells (KLE, OVCAR3, and CAOV3) or **B** resistant cells (Hec50 and SKOV3) were treated with indicated concentrations of ixazomib ± romidepsin for 72 h. BAF-A1 (200 nM) was added in the final 2 h prior to lysis. Upper panels: Western blots were performed to examine autophagic flux as indicated by the conversion of LC3B-I into LC3B-II. The ratio of LC3B-II/I, normalized to control (set at 1), is indicated for each treatment. Lower panels: Sensitive cells (KLE, OVCAR3, and CAOV3) or resistant cells (Hec50 and SKOV3) were transduced to stably express mCherry-EGFP-LC3B, and then exposed to either standard media or EBSS starvation media (to induce autophagy) in the absence or presence of ixazomib, romidepsin and chloroquine (CQ, 50 μ M) for 18 h. Intensity of mCherry and EGF was determined by flow cytometry and autophagic flux calculated as the ratio of mCherry:GFP and normalized to DMSO control (set at 1). * p < 0.05; ** p < 0.01; *** p < 0.001 vs. control by two-tailed unpaired Student's t test.

detect autophagic flux, cells were treated with ixazomib and romidepsin 72 h followed by treatment with the autophagy inhibitor BAF-A1 for 2 h. In contrast to sensitive cells (KLE, OVCAR3 and CAOV3), LC3B-I to LC3B-II conversion increased in resistant cells (Hec50 and SKOV3, Fig. 3A, B, upper panel). These data confirm the induction of autophagy in resistant cells in response to treatment.

Using a pH-sensitive mCherry-GFP-LC3B tandem fluorescent reporter, we examined autophagic flux by flow cytometry. The GFP signal is quenched by the acidic environment of the

autophagosome following fusion with the lysosome, whereas the mCherry signal is more stable [33]. Comparison of the ratio of the median fluorescence intensity of mCherry and GFP provides a quantitative measurement for autophagy [32]. Under nutrient-rich conditions, there was no measurable difference in autophagic flux with ixazomib and romidepsin treatment compared to control and single drug treatment (Fig. 3A, B, lower panels). In comparison, serum starvation with EBSS induced a significant increase in autophagy. The autophagy induced by starvation was decreased by ixazomib and romidepsin in the sensitive KLE, OVCAR3, and

CAOV3 cells but increased in resistant Hec50 and SKOV3 cells (Fig. 3A, B, lower panel). We propose that increased autophagic flux in response to ixazomib and romidepsin treatment in Hec50 and SKOV3 cells indicates a signal of developing cellular resistance.

Inhibition of autophagy sensitizes resistant cells to ixazomib and romidepsin in vitro

To assess whether inhibition of autophagy increases the efficacy of ixazomib and romidepsin, we combined ixazomib and romidepsin with pharmacologic inhibitors of autophagy, chloroquine (CQ), BAF-A1 and LYS05. Autophagy inhibitors increased the sensitivity to ixazomib and romidepsin in previously resistant Hec50, SKOV3, and KLE cells (Fig. 4A–I), but not OVCAR3 and CAOV3 cells that initially demonstrated sensitivity to dual therapy (Fig. S2).

Because CQ, LYS05, and BAF-A1 may have effects on cell survival other than inhibition of autophagy, we also inhibited autophagy by knocking down ATG5, a critical protein required for autophagosome formation [34, 35]. ATG5 silencing is shown in Fig. S3. Hec50 and SKOV3 cells lacking ATG5 had enhanced sensitivity to ixazomib and romidepsin (Fig. 4J–K). These data support the induction of autophagy as a mediator of resistance to the combination of ixazomib and romidepsin.

Autophagy inhibition sensitizes resistant cells to ixazomib and romidepsin by inhibiting HDAC6 activity

Having established that the addition of autophagy inhibitors sensitizes resistant cells to ixazomib and romidepsin, we examined potential mechanism(s).

First, we confirmed induction of autophagy with ixazomib and romidepsin treatment in Hec50 and SKOV3 cells. CQ inhibits autophagy by blocking the fusion of the autophagosome with the lysosome, thereby inhibiting lysosomal-mediated protein degradation. LC3B-II on the surface of autophagosomes is normally degraded after fusion with the lysosome. Hence, after blocking the lysosome with CQ, increased LC3B-II expression following drug treatment indicates induction of autophagy, whereas decreased LC3B-II reflects a reduction [33].

Co-treatment of KLE, OVCAR3, and CAOV3 cells with CQ in combination with ixazomib and romidepsin results in decreased expression of LC3B-II compared to CQ alone (Fig. 5A–C), indicating reduced autophagy. In contrast, CQ, ixazomib and romidepsin treatment of Hec50 and SKOV3 cells increased LC3B-II compared to CQ alone, indicating the induction of autophagy (Fig. 5D, E). These results, taken together with those in Fig. 3, demonstrate that resistance of Hec50 and SKOV3 cells to ixazomib and romidepsin is mediated by increased autophagy.

We next considered the components of the autophagy pathway in order to more fully understand why certain cells may preferentially utilize autophagy as a survival mechanism. HDAC6 is an important regulator in the aggresome and autophagy pathway by chaperoning misfolded proteins to aggresomes for lysosomal degradation [36]. To understand the mechanism of resistance to ixazomib and romidepsin, we examined the acetylation of α -tubulin (ace- α -tubulin) after the inhibition of autophagy. A decrease in HDAC6 activity results in increased ace- α -tubulin. Treatment with ixazomib and romidepsin in combination with CQ did not increase ace- α -tubulin compared to ixazomib and romidepsin treatment in OVCAR3 and CAOV3 cells that do not mount an autophagy survival response and are therefore sensitive to proteasome plus HDAC inhibition (Fig. 5A, B). However, treatment of Hec50, SKOV3, and KLE cells with ixazomib and romidepsin in combination with CQ increased ace- α -tubulin, indicating HDAC6 inhibition (Fig. 5C–E). Since romidepsin is a specific Class I HDAC inhibitor, and HDAC6 belongs to the Class II HDAC family, we surmised that the inhibition of HDAC6 is not due to the direct effects of romidepsin on HDAC6. Supporting this

notion, our data demonstrate a lack of change in ace- α -tubulin in cells treated with romidepsin alone. Increased acetylation of histone H3 confirms the romidepsin effect on Class I but not Class II HDACs [37] (Fig. 5).

We also detected cell-specific variations HDAC6 expression in response to treatment. HDAC6 was markedly increased in sensitive OVCAR3 and CAOV3 cells in response to combination treatment, potentially because these cells countered therapy by producing more HDAC6, but failed to survive regardless. Taken together, our findings indicate that CQ sensitizes Hec50, SKOV3, and KLE cells to ixazomib and romidepsin treatment by inhibiting the activity of HDAC6.

Inhibition of autophagy with hydroxychloroquine sensitizes a resistant cell-derived xenograft model to ixazomib and romidepsin

Given the enhanced cytotoxicity observed in cell cultures with ixazomib and romidepsin in combination with autophagy inhibitors, we asked whether autophagy inhibition augments the response to ixazomib and romidepsin in vivo using a xenograft model of the resistant cell line Hec50. Triple therapy of ixazomib, romidepsin, and hydroxychloroquine (HCQ) resulted in the strongest inhibition of tumor growth and the lowest tumor weight in vivo (Fig. 6A, B). Single drug treatments did not inhibit tumor growth in comparison with vehicle control, but a modest tumor growth inhibition was observed when romidepsin was combined with either ixazomib ($P < 0.01$) or HCQ ($P < 0.05$). The combination of romidepsin + ixazomib + HCQ also induced moderate body weight loss in mice (Fig. S4).

To assess molecular correlates of response, LC3B and ace- α -tubulin were examined in post-treatment tumor tissues by immunofluorescence (Fig. 6C) or Western blotting (Fig. 6D). A marked increase in LC3B-II and ace- α -tubulin was observed in tumors from animals treated with romidepsin + ixazomib in combination with HCQ, consistent with cell line data in Fig. 5. Increased expression of LC3B by immunofluorescence or the conversion of LC3B-I to LC3B-II by Western blotting in tumors treated with the dual or triple-drug combination reflects induction of autophagy. Similarly, we interpret the elevated acetylation of α -tubulin in tumors treated with romidepsin + ixazomib + HCQ as evidence for greater HDAC6 inhibition in response to the triple-drug regimen as compared with romidepsin + ixazomib.

DISCUSSION

In this study, we report for the first time that the combination of ixazomib and romidepsin induces significant cell death in many gynecologic cancer PDOs and cell lines. This was somewhat surprising, as most HDAC and proteasome inhibitors have not been deemed active as single agents in clinical studies of gynecologic cancers [9–11] or in combination with chemotherapy [11, 15]. Supporting this, we saw wide variability in the PDO models to individual HDAC and proteasome inhibitory agents, but the combination was highly effective in majority of models. The PDO culture method provides a novel and powerful platform for studying patient tumors in vitro. Accumulating evidence has emerged that PDOs can predict clinical outcomes in many types of cancers. A previous report from our laboratory substantiated that PDOs have the potential to predict patient responses to chemotherapy [28]. In the current study, the most impressive effects were observed with the combination of HDAC and proteasome inhibitors, especially when compared to standard chemotherapy. Given the increasing recognition that PDOs are reliable surrogates of patient response, our data provide a rationale to pursue the combination of ixazomib and romidepsin clinically despite the perceived failure of single-agent clinical trials.

One of the most important and unexpected findings from this study is that some gynecologic cancer models induce autophagy

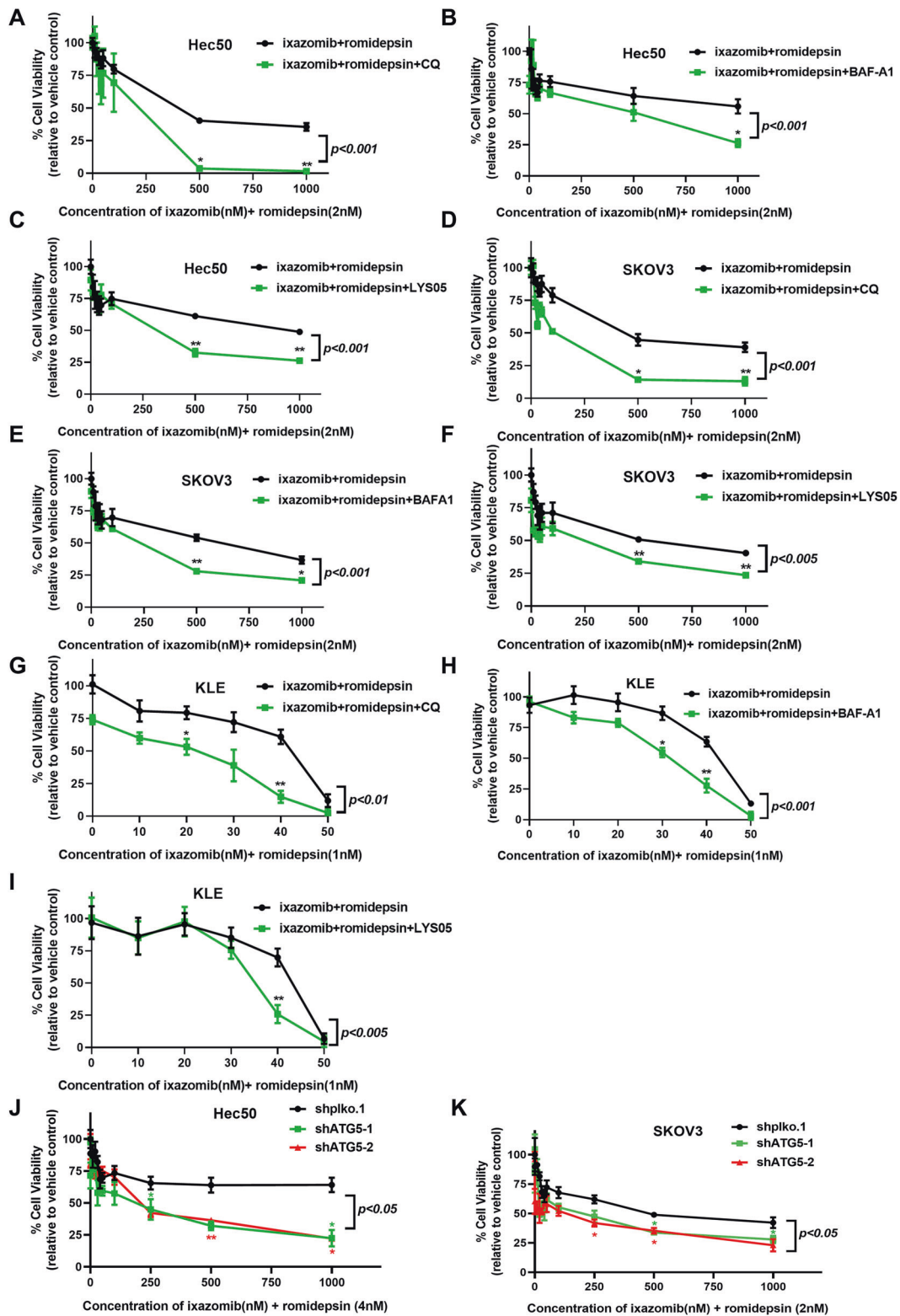


Fig. 4 Inhibition of autophagy increases sensitivity to ixazomib and romidepsin in Hec50, SKOV3, and KLE cells. Hec50 cells were treated with ixazomib and romidepsin with or without autophagy inhibitors 50 μ M CQ (**A**), 1.5 nM BAF-A1 (**B**) or 2 μ M Lys05 (**C**) for 72 h. SKOV3 cells were treated with ixazomib and romidepsin with or without autophagy inhibitors 50 μ M CQ (**D**), 1.5 nM BAF-A1 (**E**) or 2 μ M Lys05 (**F**) for 72 h. KLE cells were treated with ixazomib and romidepsin with or without autophagy inhibitors 50 μ M CQ (**G**), 1.5 nM BAF-A1 (**H**) or 2 μ M Lys05 (**I**) for 72 h. Cell viability was determined using WST-1 assay. For Hec50 and SKOV3 cells, 2 nM romidepsin was set as control (100%); 1 nM romidepsin was set as control (100%) for KLE cells. Cell viability was assessed in Hec50 (**J**) and SKOV3 (**K**) cells with knockdown of ATG5 and treatment with ixazomib and romidepsin for 48 h. Cell viability was determined using WST-1 assay relative to untreated control. Statistical significance was assessed by two-way ANOVA with Sidak's post hoc test. In addition, differences within a concentration were assessed by two-tailed unpaired Student's *t* test. * $p < 0.05$, ** $p < 0.01$ vs. ixazomib+romidepsin (**A–I**); * $p < 0.05$, ** $p < 0.01$ vs. nontargeting shRNA (shplko.1).

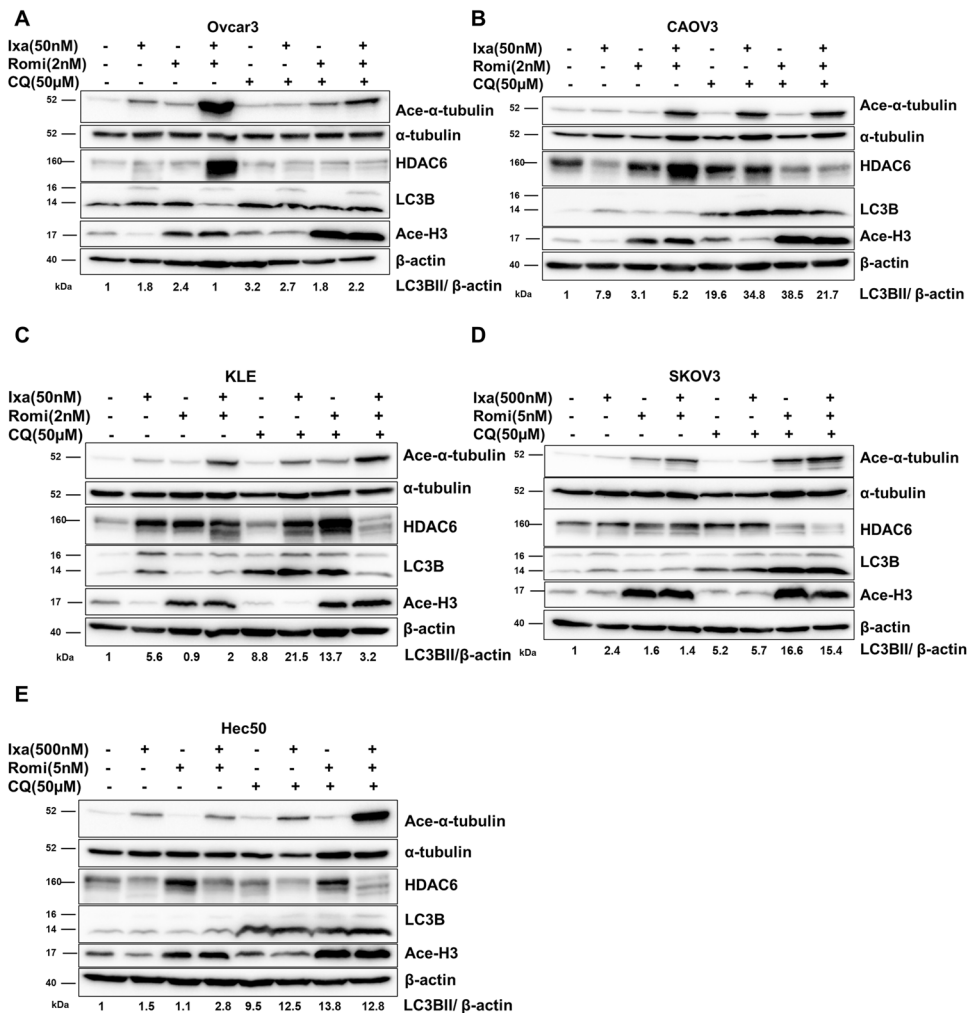


Fig. 5 Autophagy inhibitors increase sensitivity to ixazomib and romidepsin by reducing HDAC6 activity in Hec50, SKOV3, and KLE cells. Ovarc3 (A), CAOV3 (B), KLE (C), SKOV3 (D) and Hec50 (E) cells were treated as indicated for 72 h, and then collected for Western blotting analysis of expression levels of acetylated (ace)- α -tubulin (a marker for HDAC6 inhibition), HDAC6, LC3B, and acetylated-histone H3 (Ace-H3, a marker for class I HDAC inhibition).

as a protective mechanism to sustain viability when exposed to proteasome and HDAC inhibitors. Inhibition of autophagy significantly enhances the synergistic effect of the combination therapy in resistant cell lines as well as a xenograft model in vivo. These preclinical findings for the first time identify autophagy as a biomarker of resistance.

As an evolutionarily conserved degradation process, autophagy eliminates unfolded/misfolded/aggregated proteins and damaged organelles in response to stress or starvation [38]. While the original dogma was that autophagy was a mechanism of cell death, accumulating evidence suggests that autophagy also facilitates survival in response to cellular stresses such as hypoxia, DNA damage, and chemotherapy [39, 40]. Our data add to this body of literature by demonstrating that treatment with an HDAC and proteasome inhibitor also induces autophagy-mediated cell survival. Although preclinical data have supported the use of an HDAC inhibitor in combination with a proteasome inhibitor in some cancers, the role of autophagy in response to this combination has not been fully explored. Moreover, the results of the limited studies that have addressed this question are conflicting. Autophagy is widely thought to contribute to proteasome inhibitor resistance by providing a compensatory mechanism for dysfunctional protein clearance [41]. For example, the combination of bortezomib and romidepsin in gastric

carcinoma induces autophagy, which the authors concluded was a mechanism of cell death [42]. In head and neck squamous cell carcinoma, trichostatin A reduces bortezomib-induced autophagy, and the combination promotes cell death [43]. Treatment with an HDAC inhibitor activates varying molecular mechanisms that can lead to either the activation or suppression of autophagy [44, 45]. To our knowledge, our study is the first to report the role of autophagy in regulating sensitivity to the combination of HDAC and proteasome inhibitors in gynecologic cancers.

In sensitive cell lines, treatment with either an HDAC or proteasome inhibitor alone increased conversion of LC3I to LC3II, whereas the dual treatment did not produce such an effect. We interpret this increase in LC3 conversion as a compensatory mechanism of cellular preservation. In the setting of the dual drug treatment, this is not sufficient to overcome the cytotoxic effects of drugs. Consistent with this interpretation, we observe enhanced cell killing with the double-drug combination vs. single drug for all sensitive cells.

From the perspective of mechanism, we propose that HDAC6 is a critical mediator of resistance through induction of autophagy (Fig. 7). HDAC6 along with the dynein complex recruits and transports misfolded proteins to aggresomes/autophagosomes via the microtubule network for subsequent degradation by lysosomes (Fig. 7). Inhibiting HDAC6 blocks the autophagy pathway in

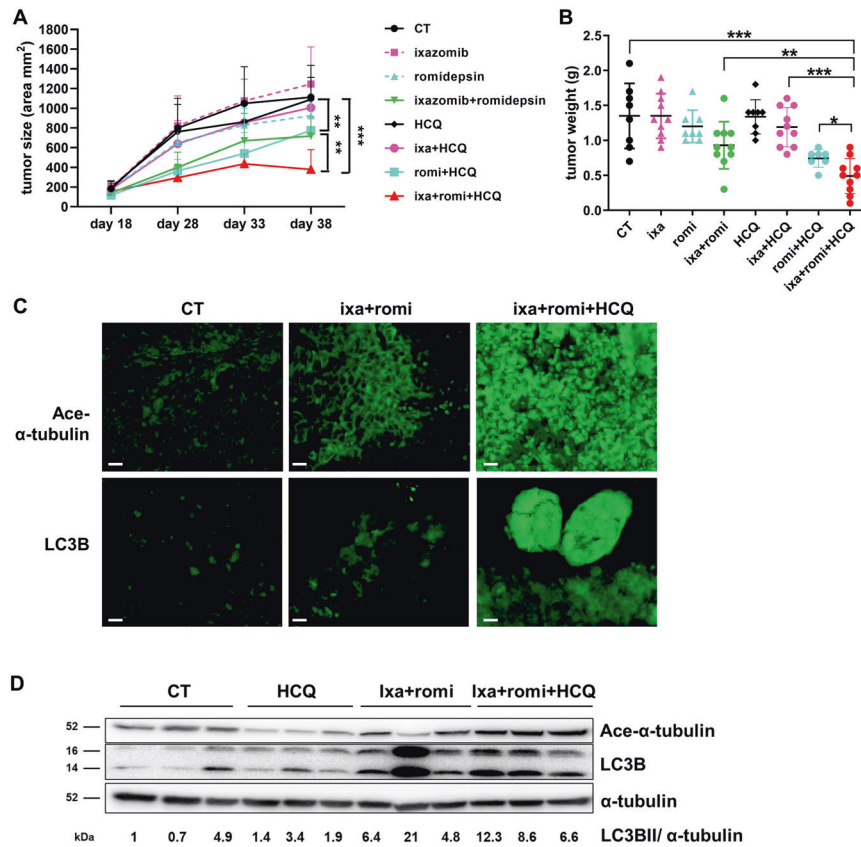


Fig. 6 Hydroxychloroquine increases ixazomib and romidepsin sensitivity in Hec50-derived human endometrial cancer xenografts. **A** Growth curves for tumor volumes. CT control, ixa ixazomib, romi romidepsin, HCQ hydroxychloroquine. Statistical significance was assessed by two-way ANOVA with Sidak's post hoc test. ** $p < 0.01$; *** $p < 0.001$ as indicated. **B** Tumor weight was determined at the completion of treatment. Statistical significance was assessed by student's *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as indicated. **C** Expression of acetylated (ace)-α-tubulin and total LC3B was assessed in post-treatment tumor tissues by immunofluorescence imaging. Representative images are provided. CT control. Scale bar: 10 μm. **D** LC3B-I and -II and ace-α-tubulin were detected by Western blot analysis of post-treatment tumor lysates. Total α-tubulin levels serve as a loading control.

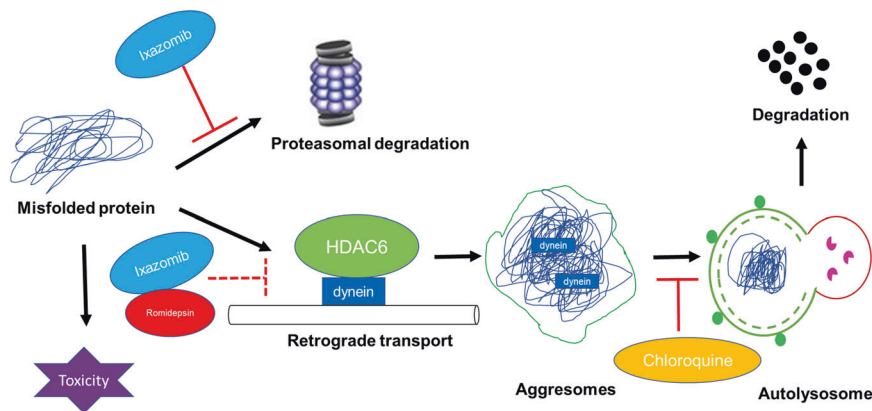


Fig. 7 Schematic depicting the proposed effects of ixazomib, romidepsin, and chloroquine on the proteasome and autophagy signaling pathways.

many cancer types [43, 46, 47]. Thus, HDAC6 has been implicated as a potential target to overcome proteasome inhibitor resistance. Although many HDAC6-selective small-molecule inhibitors have been discovered, not all of them modulate autophagy. No specific inhibitor of HDAC6 has yet been approved for clinical use. Remarkably, in this study, the combination of ixazomib and romidepsin, a Class I HDAC inhibitor that does not directly inhibit HDAC6 (a Class II HDAC [37, 48]), significantly inhibited HDAC6

activity. In sensitive cells, adding an autophagy inhibitor had no additional effect. However, in resistant cells, adding an autophagy inhibitor to the combination of ixazomib and romidepsin further reduced the activity of HDAC6 (as evidenced by increased ace-α-tubulin), leading to cell death. These findings suggest that HDAC6 inhibition can be achieved by treatment with the combination of a proteasome inhibitor and a class I HDAC inhibitor such as romidepsin.

Limitations of this study include the use of autophagy inhibitors that have pleotropic effects. However, the concept that adaptive resistance is mediated by autophagy was confirmed in our studies by demonstrating similar effects using multiple agents and methods, including ATG5 silencing. One potential explanation for the differential sensitivity of the various cell lines is reliance on autophagy for survival in response to cellular stress. For example, the sensitive line OVCAR3 is dependent upon high glucose for survival, whereas the resistant SKOV3 is a low glucose-dependent cell line [49]. Hence, it is possible that SKOV3 cells have an inherent reliance on the autophagy pathway to preserve cellular nutrients. The glucose dependence of the other resistant line, Hec50, has not been investigated.

Another limitation is the lack of a pretreatment biomarker that distinguishes between cells that induce autophagy in response to the combinatorial regimen vs. those that do not. We believe that autophagy is a mechanism that activated in response to drug; thus, that baseline expression of autophagy factors such as LC3B may not be an adequate predictor of sensitivity to therapy. This is the reason we performed the assessment of autophagy biomarkers in the control vs. treated tumor specimens from the xenograft models. This work highlights the need to derive and approve biomarkers for autophagy that can serve as reliable translational endpoints for future trials.

In conclusion, these preclinical studies defined the efficacy of combining a proteasome and HDAC inhibitor in gynecologic cancer cells. Our results suggest that the combination of ixazomib and romidepsin is a possible novel strategy to improve the outcomes of patients with gynecologic cancer, and adding an autophagy inhibitor to this combination in the future may be an option to overcome resistance.

DATA AVAILABILITY

All data are included in this manuscript or the supplemental materials.

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AUTHOR CONTRIBUTIONS

KKL, KWT, and JB designed the study, analyzed data, and wrote the paper. JB performed the majority of the experiments, analyzed data, and wrote the paper. PKM, HAL, EJD, YZ, and AMN contributed specific experiments and data analysis. KKL, KWT, and JB wrote and reviewed the manuscript. All authors read and approved the final version of the manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the University of Iowa Institutional Review Board (IRB, # 201809807). Informed consent was obtained from all subjects involved in the study. All animal studies were performed under animal protocol #0022285-003 approved by the University of Iowa Institutional Animal Care and Use Committee.

COMPETING INTERESTS

KWT is a co-founder of Immortagen, Inc. All other authors declare that they have no competing interests.

ADDITIONAL INFORMATION

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Characterizing *TP53* mutations in ovarian carcinomas with and without concurrent *BRCA1* or *BRCA2* mutations

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Abstract

Objectives: Mutations in the *TP53* tumor suppressor gene are common in ovarian carcinoma (OC) but their impact on outcomes is controversial. We sought to define the relationship of *TP53* mutations to cancer outcomes and their interactions with co-occurrent *BRCA1* or *BRCA2* (*BRCA*) mutations, comparing three different *TP53* mutation classification schemes.

Methods: We performed next generation sequencing on 393 cases of OC prospectively followed for survival. *TP53* mutations were classified according to three schemes termed Structural, Functional, and Hotspot. Mutation distribution was compared between cases with and without *BRCA* mutations. In a subset of 281 cases of high grade serous carcinoma (HGSC), overall survival was compared using Kaplan-Meier curves, logrank testing, and multivariate Cox

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Conflicts of Interest

RAR serves as a scientific consultant to TwinStrand Biosciences Inc. and owns equity at NanoString Technologies Inc. and TwinStrand Biosciences Inc.

regression analysis, both stratified and adjusted for *BRCA* mutation status. Multivariate logistic regression was used to analyze the effects of *TP53* mutation type on platinum resistance.

Results: *TP53* mutations were identified in 76.8% of the total cohort (n=302/393) and 87.9% of HGSC (n=247/281). Cases with *BRCA* mutations demonstrated significantly higher *TP53* mutation frequency overall (n=84/91, 92.3% vs. n=218/302, 72.2%, p<0.001). *TP53* mutations were not associated with overall survival, even when stratified by *BRCA* mutation. *TP53* mutations were associated with platinum sensitivity, even after adjusting for *BRCA* mutation status (OR 0.41, p=0.048). The choice of *TP53* mutation classification scheme was not found to alter any significant outcome.

Conclusions: *BRCA* mutations significantly co-occur with *TP53* mutations. After adjusting for *BRCA* mutations, *TP53* mutations are associated with platinum sensitivity, and this effect is not dependent on *TP53* mutation type.

Keywords

TP53; p53 protein; BRCA1; BRCA2; ovarian cancer; overall survival

Introduction

Mutations in *TP53* are found in greater than 80% of epithelial ovarian cancers; specific rates depend on the histological distribution of the cohort, with the highest frequency in high grade serous carcinoma (HGSC) [1–3]. The corresponding protein p53 functions as a tumor suppressor by mediating the transcription of genes involved in cell cycle arrest, senescence, DNA damage repair, or apoptosis [4]. Although rarely found in the germline (as part of Li Fraumeni syndrome), the vast majority of *TP53* mutations in cancer occur somatically [5]. Prior studies into the frequency and effects of *TP53* mutations in ovarian carcinoma have often used immunohistochemistry studies (IHC) to predict mutational status [6]. While this has recently been optimized to increase specificity of testing, the gold-standard remains tumor sequencing [7].

More research is needed to understand the prognostic value of *TP53* mutations in ovarian cancer. Prior studies have begun to investigate associations between *TP53* mutations and ovarian carcinoma outcomes such as survival and platinum-resistance, however results have been discordant [1, 8–10]. Disparate findings may be due to population or cohort differences but are also likely related to varying methods used to classify and describe *TP53* mutations.

There is no single accepted classification scheme for defining different types of *TP53* mutations, thus limiting cross-literature comparisons. Studies have used classification schemes based on frequency found in cancer (often termed “hotspot” mutations), functional activity, or structural protein changes both to describe the mutations seen in ovarian carcinoma and to investigate effects on outcome such as survival or chemoresistance [2, 10]. There has been increasing interest in mutations that confer oncomorphic or gain-of-function (GOF) activity on p53, rather than just nullification of the tumor suppressor function, but there is not consensus on what defines a GOF mutation [9, 11]. There is also no clear definition of what constitutes a hotspot mutation, with previous studies defining hotspots

based on codon location, individual mutation, specific cancer or all-cancer frequency [10, 12]. Differences in classification schemes may have impacted the conflicting conclusions of whether *TP53* mutations are associated with outcomes in ovarian carcinoma.

Decades of research show that mutations in *BRCA1* and *BRCA2* (*BRCA*), and other genes involved in homologous recombination, are major prognosticators in survival and response to therapy in ovarian carcinoma [13, 14]. It is thus critical to study the interactions between *TP53* and *BRCA* mutations in any analysis of molecular determinants of outcomes, but previous studies have been limited in doing so based on sample size [2, 15]. We aimed to better characterize the landscape of *TP53* mutations and its effects on ovarian cancer outcomes while addressing these limitations by using a large, centrally-reviewed cohort with mutational status of both *TP53* and *BRCA* confirmed through next-generation sequencing.

Methods

This was a prospectively collected cohort of 393 women diagnosed with primary ovarian, peritoneal, and fallopian tube carcinoma, collectively termed ovarian carcinoma (OC), undergoing surgery at the University of Washington Medical Center between 1996 and 2017. The included women provided informed consent for collection of blood and surgical tissue and clinical information, including long term follow-up, through an Institutional Review Board-approved protocol at the University of Washington. Pathology was centrally reviewed. Patients with recurrent OC, cancer diagnosed at time of risk-reducing salpingo-oophorectomy or borderline neoplasm were excluded. Demographic and clinical characteristics including primary histology, stage, grade, and platinum response were collected along with subsequent disease status and survival information on follow-up. The International Federation of Gynecology and Obstetrics (FIGO) ovarian cancer staging classification from 1988 was used to define stage given the timeline of included cases. Overall survival was measured from time of diagnosis. Cancers were classified as platinum sensitive if time to progression after completion of adjuvant platinum therapy was greater than six months or, if that information was unknown, the time between surgical diagnosis and disease progression was greater than fifteen months. All other cases were termed platinum resistant (including those that were platinum refractory). DNA sequencing was performed on the primary neoplastic sample using BROCA, a targeted capture, highly sensitive next generation sequencing platform that was developed at the University of Washington, which identifies all classes of mutations and targets the entire *TP53* gene including intronic sequence [16–18].

TP53 mutation classification

Three different *TP53* mutation classification schemes (Structural, Functional, and Hotspot) were compared in the study analysis in order to determine whether one classification better associated with outcomes (Table 1) [19]. The full list of GOF mutations used is outlined in Supplementary Table S1.

All but two women had germline *BRCA* testing using either BROCA or another testing platform with information extracted from their medical records. The two women without germline sequencing were wildtype for *BRCA* mutations based on tumor sequencing results.

Cases were classified as carrying a homologous recombination deficiency (HRD) mutation if testing revealed deleterious germline or somatic *BRCA1* and *BRCA2* mutations or mutations in other homologous recombination genes (*ATM*, *BARD1*, *BRIP1*, *NBN*, *PALB2*, *RAD51C*, and *RAD51D*) [13, 14, 16]. Deleterious mutations were characterized using the ClinVar database [20].

For confirmatory analysis, distribution of *TP53* class was also investigated using data from The Cancer Genome Atlas (TCGA) and the PanCancer Atlas, accessed through cBioPortal [21–23]. Available mutational information from cases of ovarian serous carcinoma were downloaded and the distributions of *TP53* mutations and *BRCA* mutations were analyzed.

Outcomes and statistical analysis

The distributions of *TP53* mutations were described based on structural, functional, and hotspot classification schemes for the total cohort and within high-grade serous carcinoma (HGSC) only (Table 1). Comparative testing with Fisher's exact test was used to examine *TP53* mutation distribution and associations with clinicopathologic factors.

Kaplan-Meier and logrank testing was performed to compare survival based on *TP53* mutation status. To decrease confounding, outcome analysis was limited to cases of HGSC. *BRCA* mutation status was further used to stratify the analysis to investigate specific effects of *TP53* mutation. A multivariate Cox proportional hazards regression analysis was used incorporating *a priori*-defined clinicopathologic and genetic factors (age at diagnosis, stage, *BRCA* mutation status, primary platinum resistance, optimal cytoreduction, neoadjuvant chemotherapy). To test the proportionality of hazards we used Schoenfeld residuals. Association with primary platinum resistance was investigated as the secondary outcome of interest. Multivariate logistic regression was used to analyze the effects of *TP53* mutation class within high-grade serous carcinoma cases using the same covariates. Stata version 14 [24] was used for all data analyses.

Results

Among the 393 women included in the total cohort, the majority had HGSC (n=281, 71.5%), grade 3 (n=355, 90.3%), and stage 3–4 (n=330, 84.0%) disease (Table 2). *TP53* mutations were found in 76.8% of the total cohort (n=302), 87.9% of HGSC (n=247/281) and 49.1% of other histologies (n=55/112, p<0.001 comparing HGSC with other histologies). Of non-serous cases, the highest rates of *TP53* mutations were found in adenocarcinoma not otherwise specified (94.7%), carcinosarcoma (85.7%) and endometrioid carcinoma (42.9%). Deleterious germline *BRCA* mutations were identified in 65 (16.5%) and somatic *BRCA* mutations were identified in 27 (6.9%). Further somatic or germline deleterious gene mutations conferring HRD (excluding *BRCA* mutations) were identified in 21 cases (5.3%) and included mutations in *BRIP1* (n=5), *RAD51C* (n=4), *RAD51D* (n=4), *ATM* (n=3), *BARD1* (n=2), *PALB2* (n=2) and *NBN* (n=1), most of which have been previously described [16].

Distribution of TP53 mutation types:

Using the structural classification scheme, the majority of cancers carried a missense *TP53* mutation (n=220/393, 56.0%) (Figure 1A). Using the functional classification scheme, loss-of-function (LOF) *TP53* mutations were found in 198 cancers (50.4%) while GOF *TP53* mutations were found in 113 (28.7%). Missense mutations in the nine most frequently-mutated codons in ovarian cancer (termed “hotspot missense”) were found in 93 cancers (23.7%). GOF *TP53* and hotspot missense mutations frequently overlapped; 85 (75.2%) of GOF *TP53* mutations are also located in hotspot codons. HGSC had a significantly higher *TP53* mutation frequency than cases with non-serous histology (n=247/281, 87.9% vs. 55/112, 49.1% respectively, $p<0.001$, Figure 1B).

OC with germline or somatic *BRCA* mutations demonstrated significantly higher *TP53* mutation frequency overall when compared to OC that was *BRCA* wildtype (n=84/91, 92.3% vs. n=218/302, 72.2%, $p<0.001$, Figure 1C). There was no significant difference in distribution of specific *TP53* mutations within each classification scheme (i.e. missense vs. nonsense/frameshift/splice, GOF vs. LOF) between cases with *BRCA* mutations and those without. Cases of HGSC with mutations in *BRCA* also demonstrated higher rates of *TP53* mutations than HGSC without mutations in *BRCA*, though findings were not significant (n=66/72, 91.7% vs. 181/209, 86.6%, $p=0.301$). The full distribution of *BRCA* and *TP53* mutations amongst HGSC and OC of other histologies is displayed in Supplementary Table S2. Even among non-high grade serous histologies, *BRCA* mutations tend to co-occur with *TP53* mutations (Supplementary Table S3). In order to confirm these distribution findings, analyses were repeated using data from TCGA and PanCancer Atlas ovarian HGSC (Figure 1D). Cases with *BRCA* mutations similarly had higher incidence of *TP53* mutations overall (n=32/33, 97.0% vs. n=341/490, 69.6%, $p<0.001$).

Mutations in *TP53* (regardless of class) were more frequently found in carcinomas of patients older at diagnosis (ANOVA $p<0.01$) and in cases of stage III-IV disease (Fisher’s exact $p<0.001$, Table 3). Significant associations between mutation class and clinicopathologic factors are lost, however, when restricting analysis to within the 281 cases of HGSC only (Supplementary Table S4).

Overall survival by TP53 mutation type:

To decrease confounding, survival analyses were restricted to HGSC. Using the structural classification, median survival was 37 months in cases with wildtype *TP53*, 49 months with missense mutations, and 49 months with nonsense/frameshift/splice mutations. Overall survival did not significantly diverge between structural mutation class (logrank $p=0.844$, Figure 2A). Using the functional classification, cases with GOF *TP53* mutations demonstrated a median overall survival of 44 months while LOF *TP53* mutations demonstrated a median survival of 50 months (logrank $p=0.488$, Figure 2B). Cases with hotspot missense *TP53* mutations had a median survival of 41 months (logrank $p=0.305$, Figure 2C).

Having a germline or somatic *BRCA* mutation did confer a survival advantage (logrank $p<0.01$). Cases were stratified by the presence of *BRCA* mutations to further analyze

potential contributions of *TP53* mutations. For cases with germline and somatic *BRCA* mutations, there was no divergence of survival curves between cases with or without *TP53* mutations (logrank $p=0.248$, Supplementary Figure S1A). Cases without *BRCA* mutations also showed no significant difference in survival based on the presence or absence *TP53* mutations (logrank $p=0.994$, Supplementary Figure S1B). Similarly, stratifying by all HRD mutations including *BRCA* did not show a significant effect on survival with *TP53* mutations (logrank $p=0.761$ for cases with HRD mutations, $p=0.780$ for cases without HRD mutations).

On multivariate Cox testing of the associations between *TP53* mutations with overall survival, primary platinum resistance was found to violate the proportional hazards assumption, necessitating stratification on this variable in addition to adjusting for age, presence of a *BRCA* mutation, optimal cytoreduction, and neoadjuvant chemotherapy. The presence of any *TP53* mutation was not independently associated with survival (OR 1.15, $p=0.601$), nor were any specific *TP53* mutations once divided into structural, functional or hotspot classifications (see Supplementary Table S5 for details).

TP53 mutation effects on primary platinum resistance:

There was a similar distribution of structural, functional, and hotspot *TP53* mutations between women with primary platinum sensitive and resistant OC both within the entire cohort (Table 4) and HGSC only (Supplementary Table S4). Cases with concurrent *BRCA* and *TP53* mutations demonstrated similar rates of platinum resistance to *BRCA* mutant cases that were *TP53* wildtype (10/69, 14.5% *TP53* mutation vs. 1/6, 16.7% *TP53* wildtype, $p=1.0$). Cases without *BRCA* mutations also showed similar platinum resistance rates despite *TP53* mutation status (53/165, 32.1% *TP53* mutation vs. 18/61, 29.5% *TP53* wildtype, $p=0.749$). On multivariate logistic regression adjusted for *BRCA* mutation status, age at diagnosis, optimal cytoreduction, and neoadjuvant chemotherapy (with stage omitted due to collinearity) and restricted to cases of HGSC, the presence of any *TP53* mutation significantly increased the odds of platinum sensitivity (OR 0.41, 95% CI 0.17-0.99, $p=0.048$). Individually classified *TP53* mutations each trended toward an association with platinum sensitivity but did not meet statistical significance (see Table 4 for details). In *BRCA*-wildtype cases only, the presence of a *TP53* mutation still trended toward an association with platinum sensitivity though the results were not statistically significant in that smaller population (OR 0.38, 95% CI 0.14-1.01, $p=0.053$).

Discussion

This is the largest single-institution cohort of OC with centralized pathology review and tumor sequencing which allowed for careful analysis of the distribution of *TP53* mutation classes within the context of other clinicopathologic factors. Cases with germline or somatic *BRCA* mutations had significantly higher rates of *TP53* mutations in general (regardless of classification), with few cases (7.7%) being *TP53* wildtype. Our study is one of the largest prospective cohorts specifically examining the distribution of co-occurrent mutations in these two driver pathways and findings were further replicated using TCGA and PanCancer Atlas data. In ovarian and breast carcinoma, p53 IHC analyses have previously demonstrated

more frequent aberrant expression reflective of missense mutations in *BRCA*-mutation carriers [6, 25–27]. Our data suggest that *TP53* mutations may be a pre-requisite in most *BRCA*-associated carcinogenesis. The requirement for p53 protein dysfunction has been previously suggested in *in vitro* models as well as in genetically engineered mouse models [28–30]. These authors hypothesized that losing p53-mediated tumor suppression allows for the cells that exhibit loss of heterozygosity in *BRCA* to escape cell surveillance and apoptosis. The addition of our data further strengthens the argument that there is selection towards the co-occurrence of these mutations in early *BRCA*-associated malignant transformation. *TP53* mutations have been thought to be nearly universal in HGSC, but this does not appear to be the case in *BRCA* wildtype HGSC, with a *TP53* mutation rate of only 86.6% in our study and 69.6% in TCGA.

TP53 mutations were associated with primary platinum sensitivity in HGSC, regardless of type of mutation, after adjusting for covariates including *BRCA* mutation status. Association with platinum sensitivity has also been observed in triple negative breast cancer [31]. Conversely, studies in head and neck and lung cancers suggest that certain *TP53* mutations may associate with platinum chemoresistance [32, 33]. There is scant literature in ovarian cancer regarding the relationship between *TP53* mutations and platinum response, with inconsistent findings from smaller studies neither of which accounted for *BRCA* mutation status [1, 8]. Laboratory studies suggest that chemoresistance and sensitivity depend less on one mutated gene, but a careful interplay between different signaling pathways, transcription factors, and enzymes, and are reliant on the wildtype or mutated function of various genes [34]. While our results show that *TP53* mutations may be used to prognosticate response to platinum, full understanding of chemoresistance likely relies on the analyses of multiple signaling pathways. As *BRCA*-mutated OC are often disproportionately sensitive to platinum-containing chemotherapy, further studies into the effect of *TP53* must take these interactions into account.

Despite the association with platinum sensitivity, the presence of a *TP53* mutation, regardless of mutational classification, was not associated with overall survival in HGSC in our univariate or multivariate analyses. Conclusions from several studies using the TCGA database of high-grade serous ovarian cancers have been mixed, depending on how *TP53* mutations were classified [8, 9]. A study with an expanded panel of GOF mutations based on functional studies, more consistent with our methods, showed a difference between recurrence patterns in OC with wildtype and gain-of-function *TP53* mutations, but no change in overall or progression-free survival [9]. Tuna and colleagues found no difference in survival outcomes between *TP53* missense and nonsense or truncated mutations [10]. They tested hotspot mutations separately based on position, finding that G266, Y163C, and R282 mutations were associated with worsened overall survival compared with other mutations. GOF and hotspot mutations may thus need to be tested individually before being used as prognostic factors in OC, which would require large sample sizes for conclusive analysis. In all such analyses in OC, the confounding influence of *BRCA* mutation status on survival must be considered. In our study, *TP53* mutations were not associated with survival when examined within strata of *BRCA* wildtype or mutated OC. Few prior studies have examined interactions between *TP53* and *BRCA* mutations on survival, although these are the two most common mutations in HGSC. Mandilaras and colleagues [2] similarly

found no association between overall survival and cases with concurrent *TP53* and *BRCA* mutations compared to cases that were *BRCA* wildtype while Li and colleagues [15] found that the presence of *TP53* and *BRCA* mutation conferred longer overall survival when compared with OC without either mutation. These analyses are limited by the rarity of *BRCA*-mutated but *TP53*-wildtype OC, thus making stratification of survival analysis difficult.

This study is the largest unselected cohort study of the effects of *TP53* mutations on survival outcomes in OC. It is also the only study that compared different *TP53* mutational classifications and their interactions with *BRCA* mutations. Our methods were strengthened by centralized pathologic review, rigorous determination of somatic mutations through next generation sequencing, and an enhanced list of defined GOF mutations based on currently available literature. Our cohort was not powered for subset analysis and our general understanding of GOF/LOF classifications is still evolving [35], but this provides valuable preliminary investigation into potential interactions of commonly seen somatic and germline mutations in OC. An understanding of patient-specific mutations and interactions is vital for personalization of cancer therapy. Our data suggest that *BRCA* mutations are a major confounder in associations of *TP53* mutation status and outcome in OC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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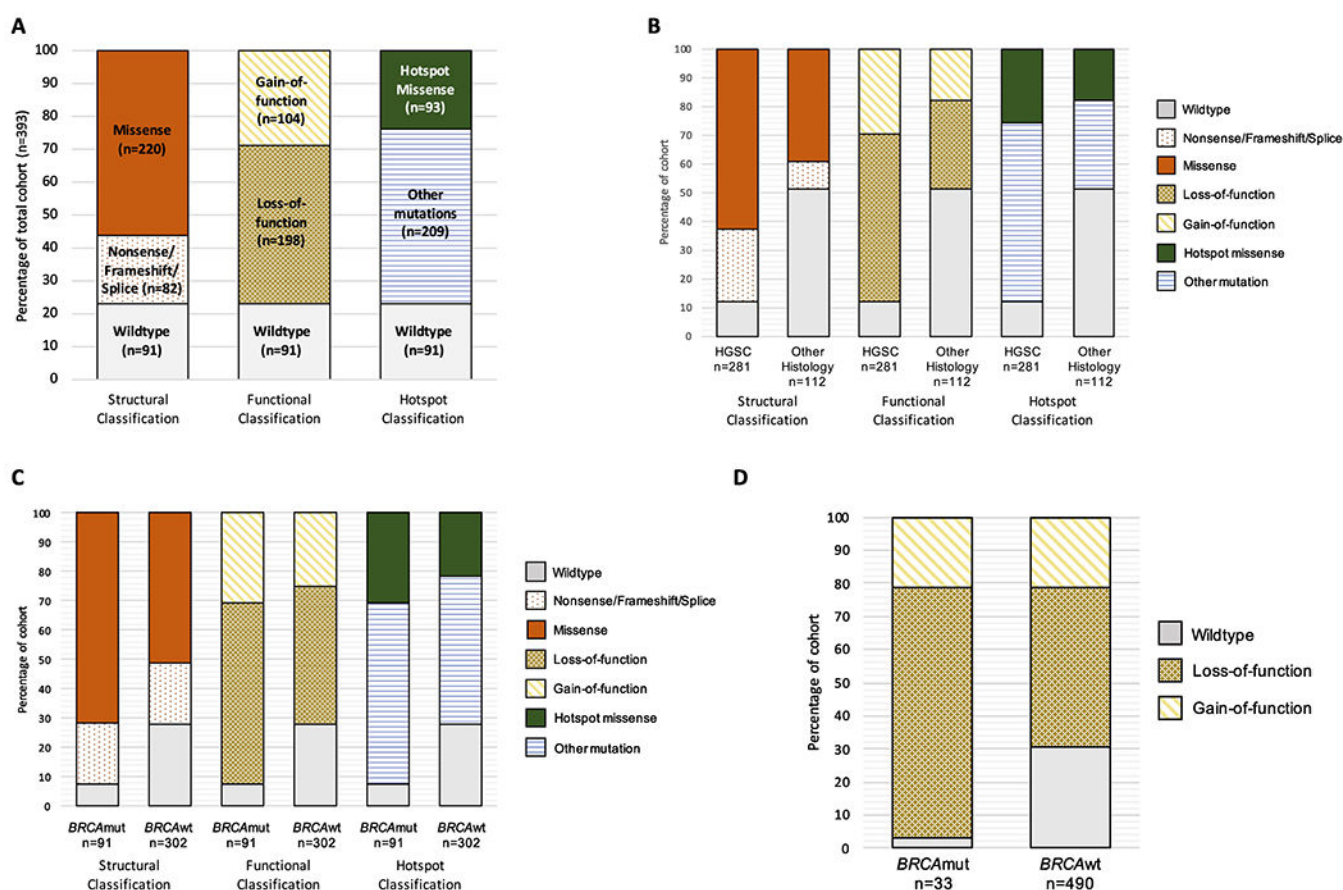
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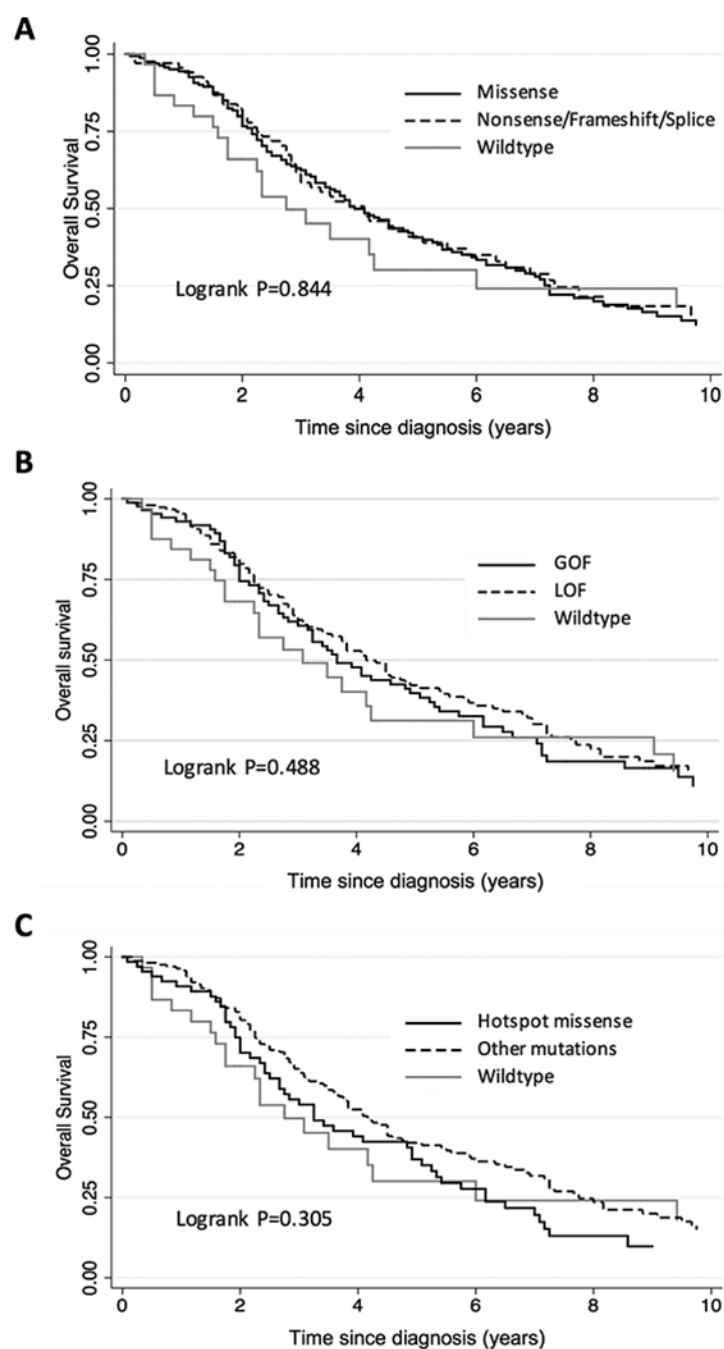
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Highlights:

1. *TP53* mutations were identified in 76.8% of ovarian carcinomas and 87.9% of high grade serous carcinomas in this cohort
2. *BRCA1* or *BRCA2* mutations co-occur with *TP53* mutations in ovarian carcinoma
3. *TP53* mutations are associated with platinum sensitivity, but not overall survival, in high grade serous ovarian carcinoma
4. *TP53* mutations were identified in 76.8% of ovarian carcinomas and 87.9% of high grade serous carcinomas in this cohort
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**Figure 1:**

Distribution of *TP53* mutations using different classification schemes. All cases had somatic tumor sequencing and mutations were classified according to three schemes based on structure, function, and hotspots (Table 1). A) Total cohort (n=393) B) Comparing high grade serous carcinoma to other histologies C) Comparing tumors with *BRCA* mutations (*BRCAmut*) and without *BRCA* mutations (*BRCAwt*) D) Confirmatory analysis using TCGA PanCancer Atlas cases of ovarian serous carcinoma (n=523), showing *TP53* mutations by functional classification and *BRCA* status

**Figure 2:**

Overall survival compared between *TP53* mutation class. Analysis restricted to high grade serous cancer only. A) Structural *TP53* mutation classification (WT = wildtype) B) Functional classification (LOF = loss-of-function, GOF = gain-of-function) C) Hotspot classification

Table 1:

Mutation classification schemes

	Structural Classification:	Functional Classification:	Hotspot Classification:
Definition	Standard definitions of mutation types based on output from Seshat (https://p53.fr/TP53-database/seshat), a web service which performs <i>TP53</i> annotation using data from the UMD <i>TP53</i> database [19]. In-frame mutations were considered missense.	Nonsense/frameshift/splice mutations were considered loss-of-function mutations. Missense mutations were further subdivided into loss-of-function or gain-of-function based on experimental studies demonstrating an oncogenic phenotype, primarily interference with p73 activity, transactivation of genes repressed by wild-type p53 protein, or cooperation with oncogenes for transformation of rat or mouse embryonic fibroblasts (see Supplementary Table 1 for details and sources).	The nine most frequent codons mutated in ovarian cancer in the UMD <i>TP53</i> database (https://p53.fr/tp53-database) [5], each found in greater than 1.5% of ovarian cancer samples, were termed “hotspots”. Missense mutations in these codons (273, 248, 175, 220, 245, 195, 282, 179, 241) were separated from other mutations.
Classes	Wildtype Missense Nonsense/Frameshift/Splice	Wildtype Loss-of-function Gain-of-function	Wildtype Hotspot Missense Other mutations

Table 2:

Cohort demographics

	N (total 393)	%
Mean age at diagnosis (SD)	59.9 (12.4)	
Histology:		
High grade serous	281	71.5%
Low grade serous	21	5.3%
Endometrioid	28	7.1%
Clear cell	21	5.3%
Adenocarcinoma	19	4.8%
Carcinosarcoma	14	3.6%
Other	9	2.3%
Grade:		
1-2	35	8.9%
3	355	90.3%
Unknown	3	0.8%
Stage *:		
I	34	8.7%
II	23	5.9%
III	275	70.0%
IV	55	14.0%
Unknown	6	1.5%
Platinum Response [†] :		
Sensitive	219	68.2%
Resistant/refractory	82	31.8%
Unknown	92	55.7%
Cytoreduction [‡] :		
Optimal	274	69.7%
Suboptimal	106	27.0%
Unknown	13	3.3%
Neoadjuvant chemotherapy:		
Yes	48	12.2%
No	332	84.5%
Unknown	13	3.3%
BRCA1 or BRCA2 [§] :		
Wildtype	302	76.8%
Germline mutation	65	16.5%

	N (total 393)	%
Somatic mutation	27	6.9%
Other HRD genes [/] :		
Wildtype	372	94.7%
Germline or somatic mutation	21	5.3%
<i>TP53</i> :		
Wildtype	91	23.2%
Germline or somatic mutation	302	76.8%

* Staged according to 1988 International Federation of Gynecology and Obstetrics (FIGO) ovarian cancer staging classification.

[†]Platinum sensitive: no recurrence or progression within 6 months of platinum-containing primary adjuvant treatment.

[‡]Optimal: less than 1 cm of residual disease.

[§]One case with both somatic and germline mutation included in both categories.

[/]HRD mutations (excluding *BRCA1* or *BRCA2*) include *ATM*, *BARD1*, *BRIP1*, *NBN*, *PALB2*, *RAD51C*, and *RAD51D*.

Table 3:

Comparison of *TP53* mutation types between clinicopathologic subgroups.

	Wildtype (n=91)	Missense (n=220)	Nonsense/ Frameshift/Splice (n=82)	P*	GOF (n=113)	LOF (n=189)	P*	Hotspot missense (n=93)	Other mutation (n=209)	P*
Mean age at diagnosis (SD)	55.3 (14.8)	60.9 (11.4)	62.2 (11.2)	0.002	61.4 (11.7)	61.2 (11.1)	<0.001	60.5 (11.4)	61.6 (11.3)	<0.001
Histology										
High grade serous	34 (37.4%)	176 (80%)	71 (86.6%)	<0.001	92 (81.4%)	155 (82.0%)	<0.001	73 (78.5%)	174 (83.3%)	<0.001
Other carcinomas	57 (62.6%)	44 (20%)	11 (13.4%)		21 (18.6%)	34 (18.0%)		20 (21.6%)	35 (16.8%)	
Stage [†]										
I-II	31 (35.2%)	17 (7.8%)	9 (11.1%)	<0.001	11 (9.8%)	15 (8.0%)	<0.001	11 (11.8%)	15 (7.3%)	<0.001
III-IV	57 (64.8%)	201 (92.2%)	72 (88.9%)		101 (90.2%)	172 (92.0%)		82 (88.2%)	191 (92.7%)	
Platinum sensitive [‡]	48 (71.6%)	119 (72.1%)	52 (75.4%)	0.881	58 (69.9%)	113 (74.8%)	0.699	48 (69.6%)	123 (74.5%)	0.718
Platinum resistant/refractory	19 (28.4%)	46 (27.9%)	17 (24.6%)		25 (30.1%)	38 (25.2%)		21 (30.4%)	42 (25.5%)	
Optimal cytoreduction ^{‡,§}	41 (75.9%)	127 (64.1%)	48 (68.6%)	0.262	58 (62.4%)	117 (66.9%)	0.240	53 (64.6%)	122 (65.6%)	0.317
Suboptimal cytoreduction	13 (24.1%)	71 (35.9%)	22 (31.4%)		35 (37.6%)	58 (33.1%)		29 (35.4%)	64 (34.4%)	
Germline or somatic <i>BRCA</i> mutation	7 (7.7%)	65 (29.6%)	19 (23.2%)	<0.001	33 (29.2%)	51 (27.0%)	<0.001	28 (30.1%)	56 (26.8%)	<0.001
Cases without <i>BRCA</i> mutation	84 (92.3%)	155 (70.4%)	63 (76.8%)		80 (70.8%)	138 (73.0%)		65 (69.9%)	153 (73.2%)	
Germline or somatic HRD mutation [§]	12 (13.2%)	73 (33.2%)	24 (29.3%)	0.001	37 (32.7%)	60 (31.7%)	0.002	32 (34.4%)	65 (31.1%)	0.001
Cases without HRD mutation	79 (86.8%)	147 (66.8%)	58 (70.7%)		76 (66.3%)	129 (68.3%)		61 (65.6%)	144 (68.9%)	

* ANOVA or Fisher's exact testing. All groups are compared with the Wildtype patient group.

[†] Cases with unknown covariate status excluded from analysis.[‡] Data shown for stage III-IV disease only (total n = 322).[§] Includes mutations any mutations in *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *BRIPI1*, *NBN*, *PALB2*, *RAD51C*, and *RAD51D*.

Table 4:

Multivariate logistic regression examining effects of *TP53* mutation type on platinum resistance in high grade serous carcinoma cases by classification scheme. Models were adjusted by age, presence of a *BRCA1* or *BRCA2* mutation, optimal cytoreduction, and neoadjuvant chemotherapy. Stage was omitted from model due to collinearity.

	Odds Ratio	95% Confidence Interval	Wald <i>P</i>
All <i>TP53</i> mutations	0.41	0.17-0.99	0.048
<i>Structural classification</i>			
Wildtype	1.00 (Ref)		
Missense	0.42	0.17-1.05	0.064
Nonsense/Frameshift/Splice	0.37	0.13-1.06	0.064
<i>Functional classification</i>			
Wildtype	1.00 (Ref)		
Loss-of-function	0.40	0.16-1.00	0.051
Gain-of-function	0.43	0.16-1.15	0.093
<i>Location classification</i>			
Wildtype	1.00 (Ref)		
Hotspot missense	0.44	0.16-1.24	0.121
Other mutation	0.39	0.16-0.98	0.046



OPEN

Creation and validation of models to predict response to primary treatment in serous ovarian cancer

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Nearly a third of patients with high-grade serous ovarian cancer (HGSC) do not respond to initial therapy and have an overall poor prognosis. However, there are no validated tools that accurately predict which patients will not respond. Our objective is to create and validate accurate models of prediction for treatment response in HGSC. This is a retrospective case–control study that integrates comprehensive clinical and genomic data from 88 patients with HGSC from a single institution. Responders were those patients with a progression-free survival of at least 6 months after treatment. Only patients with complete clinical information and frozen specimen at surgery were included. Gene, miRNA, exon, and long non-coding RNA (lncRNA) expression, gene copy number, genomic variation, and fusion-gene determination were extracted from RNA-sequencing data. DNA methylation analysis was performed. Initial selection of informative variables was performed with univariate ANOVA with cross-validation. Significant variables ($p < 0.05$) were included in multivariate lasso regression prediction models. Initial models included only one variable. Variables were then combined to create complex models. Model performance was measured with area under the curve (AUC). Validation of all models was performed using TCGA HGSC database. By integrating clinical and genomic variables, we achieved prediction performances of over 95% in AUC. Most performances in the validation set did not differ from the training set. Models with DNA methylation or lncRNA underperformed in the validation set. Integrating comprehensive clinical and genomic data from patients with HGSC results in accurate and robust prediction models of treatment response.

Abbreviations

HGSC	High-grade serous ovarian cancer
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
lncRNA	Long non-coding RNA
miRNA	Micro RNA
ANOVA	Analysis of variance
AUC	Area under the curve
TCGA	The cancer genome atlas
CI	Confidence interval
PFS	Progression-free survival
OS	Overall survival
IRB	Institutional review board
WHTR	Women's health tissue repository
gDNAs	Genomic DNAs
SNV	Single nucleotide variation
CNV	Copy number variation

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GCE	Gene copy estimation
NCBI	National Center for Biotechnology Information
PCA	Principal component analysis
PC	Principal components
UI	University of Iowa
ROC	Receiver operating characteristic
FIGO	International federation of obstetrics and gynecology
GEO	Gene expression omnibus
FDR	False discovery rate

Despite notable advances in the treatment of ovarian cancer, it continues to be one of the leading causes of cancer death among women in the United States¹. The most common type of ovarian cancer is high-grade serous cancer (HGSC). HGSC typically presents as advanced disease, and standard treatment consists of combined primary cytoreductive surgery and platinum-based chemotherapy². Platinum is considered the most effective drug for HGSC². Patients that respond to initial therapy and have progression-free survival (PFS) of at least 6 months are termed “platinum-sensitive” or “responders” and have a median survival of well over four years³. In patients that have no residual disease after the initial surgery and respond to chemotherapy, median survivals can reach over 10 years³. However, in nearly a third of patients, HGSC progresses during initial chemotherapy (termed “platinum-refractory”) or recurs < 6 months after finishing treatment (termed “platinum-resistant”) ^{2,4–6}. The majority of these patients with suboptimal response to initial treatment (termed “non-responders” herein) will die from their disease within two years^{4,7,8} and are typically treated in the second-line setting with alternative therapies that do not contain platinum².

In recent years, significant efforts have been dedicated to test new targeted drugs in clinical trials to increase PFS of the patients that already respond to primary chemotherapy, with celebrated successes^{9–12}. However, few resources have been dedicated to identify those patients that are at risk of failing initial treatment before its administration, and there is no validated test that can predict robustly and accurately this outcome^{13,14}. By contrast, in breast cancer gene signatures have been identified that can accurately predict recurrence¹⁵ and chemotherapeutic response^{16,17}. These signatures have been validated in independent clinical studies^{17–20}. For example, one of these signatures, OncotypeDx, used 600 cases to create an association model and validated the model in an additional 400 cases^{15,16}. The majority of previous attempts to define predictors of treatment response in HGSC have been limited by a small number of patients, mixture of histological types and stages, and lack of validation in independent datasets^{13,14}. One of the more successful efforts used serum markers, including kallikreins and CA 125^{13,14}. The performance of these prediction models ranges from 75–85% (measured as the area under the receiver operator curve (AUC)). Adding clinical characteristics to serum markers increases the performance of the model to an AUC of 90%¹⁴. Others have integrated the Cancer Genome Atlas (TCGA) genomic data to predict overall survival (OS) and PFS, with performances that ranged from AUCs of 81 to 87%²¹. However, none of these models have been validated in independent datasets, nor have they been validated prospectively.

Using publicly available multi-dimensional datasets with clinical data, like TCGA, we previously built prediction models that distinguish between different outcomes in ovarian and endometrial cancer; we validated these models in independent datasets^{22–27}. However, these models had some limitations due to suboptimal clinical data: many patients were lost to follow-up, and others had little information about clinical variables that influenced treatment response, such as stage of disease or number of cycles of chemotherapy. Also, some datasets did not have complete molecular information because expression analyses were performed on different platforms, with different probes. This last limitation severely impacted the performance of the validation studies²².

Herein we tested the hypothesis that integrating comprehensive clinical and genomic data from patients with HGSC will ultimately result in a more accurate and robust prediction models of response to treatment. The primary objective of our study was to create models of prediction to standard therapy in patients with HGSC. The secondary objective was to validate these models in an independent dataset. Also, we intend to extract maximum genomic information from RNA-sequencing (RNA-seq) so resulting models would be feasible and affordable for any laboratory.

Methods

This is a retrospective case–control study that used clinical and genomic information to create models to predict initial response to standard therapy for HGSC patients. The prediction was made using only data that could be obtained before the administration of the initial chemotherapy. Also, as we mentioned, we intend to extract maximum genomic information from RNA-sequencing (RNA-seq) experiments.

Outcomes definition. HGSC patients were classified as responders or non-responders. Responders were those with a progression-free survival of at least 6 months after the first platinum-based treatment. Non-responders were those who did not respond (platinum-resistant) or progressed during treatment (platinum-refractory).

Patient inclusion criteria. Ovarian cancer patients with high grade serous histology and complete clinical and pathological data were included. Patients that had less than 6 months of follow-up after completing initial chemotherapy, unknown disease status after 6 months of completion of initial chemotherapy, or incomplete data about the chemotherapy delivered were excluded. Also, patients without DNA and RNA of sufficient quality (see below) for RNA-seq or DNA methylation analysis were excluded from the study. Based on the definition of response to treatment, there were a total of 50 patients classified as responders and 38 as non-responders included in the study (Fig. 1). All patients received combination platinum-based chemotherapy initially. How-

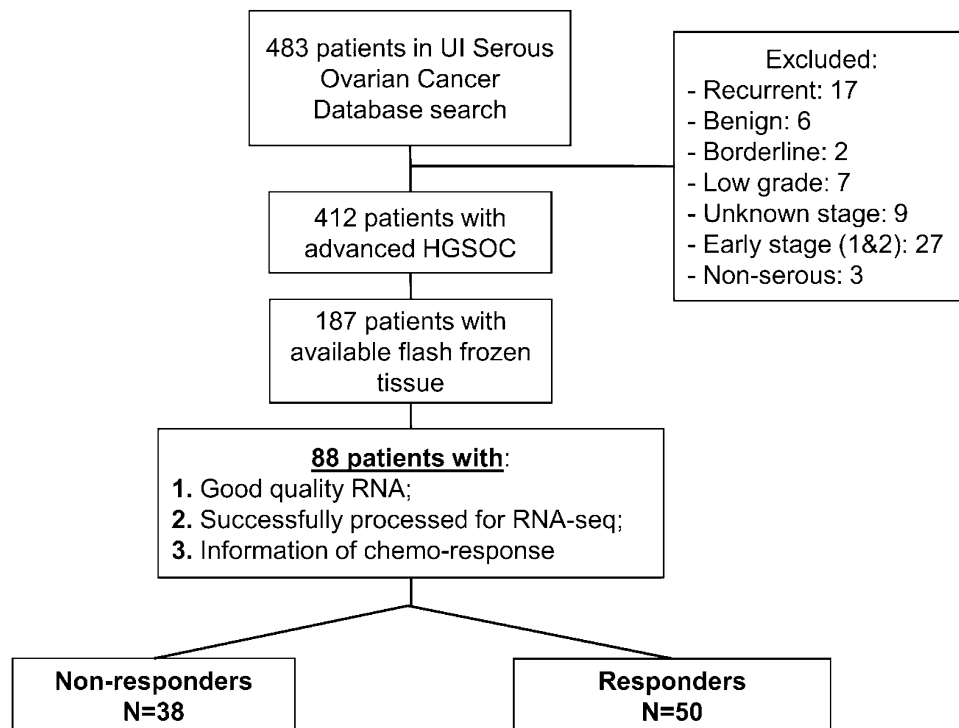


Figure 1. Selection criteria for patients in clinical prediction analysis.

ever, in 2 patients the regimen was changed before finishing because of disease progression in one case, and stable disease in the other. 66% of patient in our analysis received Taxol as initial treatment.

The institutional review board (IRB) of the University of Iowa (UI) approved the current study including human subjects/materials on April 25, 2018 (IRB Number 201804817: ‘*Prediction Models in Ovarian Cancer*’). The UI Department of Obstetrics and Gynecology maintains a Women’s Health Tissue Repository (WHTR) containing more than 60,000 biological samples, including more than 2500 primary gynecologic tumors²⁸. All tissues in the WHTR are collected under informed consent of patients in accordance with University of Iowa IRB guidelines (IRB Number 200910784 and IRB Number 200209010). Tumor samples were collected, reviewed by a board-certified pathologist and flash frozen. HGSC diagnosis was confirmed in paraffin. Specimens had less than 30% of necrosis.

Clinical data. Clinical and pathological data were collected from the electronic medical record. Clinical variables previously observed to be associated with treatment response were included in the data collection²⁷. Only baseline clinical and pathological characteristics that can be obtained before starting initial chemotherapy were collected. Table 1 shows the main clinical variables collected for the study. Differences between clinical variables between responders and non-responders were assessed with logistic regression. P-values < 0.05 were considered statistically significant. All clinical variables initially used in the analysis are described in Supplementary Methods. Statistical analysis and graphics were performed with R statistical package and computer environment²⁹.

Biological data. *RNA purification and sequencing.* Of the 187 patients identified in the original HGSC panel, 88 primary tumor tissues with sufficient RNA yield and quality were available for analysis; 50 were responders and 38 non-responders (Fig. 1). Most tumors were collected from the ovaries, 63%; 30% were extracted from the omentum, 3% from a pelvic mass and the rest, 4%, from an abdominal mass. There were no differences between both groups ($p=0.2$), responders and non-responders, in this distribution. At the time of diagnosis, these HGSC were considered of ovarian origin. Now we assume they would be tubal. Only 3 of them had no ovaries (previously removed) and were considered as primary peritoneal.

Total cellular RNA was purified from primary tumor tissue using the mirVana (Thermo Fisher, Waltham, USA) RNA purification kit following the manufacturers’ instructions. Yield and quality of purified cellular RNA was assessed using a Trinean DropSense 16 spectrophotometer and an Agilent Model 2100 bioanalyzer. Samples with an RNA integrity number (RIN)³⁰ greater than or equal to 7.0 were selected for RNA sequencing.

RNA processing and sequencing has been described elsewhere³¹. Briefly, equal mass total RNA (500 ng) was quantified by Qubit measurement (Thermo Fisher, Waltham, USA). Each qualifying tumor was fragmented, converted to cDNA and ligated to bar-coded sequencing adaptors using Illumina TriSeq stranded total RNA library preparation (Illumina, San Diego, CA, USA). Molar concentrations of the indexed libraries were confirmed on the Agilent Model 2100 bioanalyzer and libraries were then combined into equimolar pools for sequencing. The concentration of the pools was confirmed using the Illumina Library Quantification Kit (KAPA Biosystems,

			Responders	Non-responders	p-value
			N = 50	N = 38	
Age (median, range)			56 (25–81)	64 (33–83)	0.009
Charlson Comorbidity Index*	1–3		9	6	0.039
	4–6		35	21	
	> 6		1	6	
	Unknown		5	5	
FIGO stage	3		39	25	0.069
	4		7	12	
	Unknown		4	1	
Disease in upper abdomen (other than omentum) by imaging	Yes	Large bowel (N = 4)	28	29	0.051
		Porta—hepatis (N = 4)			
		Mesenteric mets (N = 4)			
		Other (N = 22)			
	No		22	9	
Disease in the chest by imaging	Yes	Chest (N = 4)	6	0	0.991
		Pleural effusion (N = 5)			
	No		44	38	
Grade	2		8	11	0.146
	3		35	23	
	Unknown		7	4	
Residual disease after surgery	Microscopic		12	3	0.053
	Macroscopic		37	35	
	Unknown		1	0	
	Optimal (< 1 cm)		37	20	0.039
	Suboptimal (≥ 1 cm)		13	18	
Removal of pelvic LN	Yes		9	4	0.333
	No		41	34	
Removal of para-aortic LN	Yes		5	3	0.734
	No		45	35	
Surgical complexity score [‡]	Low		22	23	0.990
	Intermediate		28	12	
	High		0	3	
Neoadjuvant chemotherapy	Yes		2	10	0.008
	No		47	28	
	Unknown		1	0	
Number of cycles delivered	< 6		2	8	0.344
	≥ 6		48	30	
Dose dense chemotherapy ⁺	Yes		1	1	0.844
	No		49	37	

Table 1. Patient characteristics and association with treatment response. *Charlson Comorbidity Index is a measure of the prognostic burden of all associated morbidities to predict mortality, and is the most validated measure of the prognostic impact of multiple chronic illnesses (www.charlsoncomorbidity.com). [‡]Surgical complexity score: score to predict surgical morbidity and 90-day mortality after primary debulking surgery for HGSC⁶⁷. ⁺Dose dense chemotherapy: increases the dose intensity of the regimen. In serous ovarian cancer, dose dense therapy consists in increasing IV administration of paclitaxel from every 3 weeks to weekly.

Wilmington, MA, USA). Sequencing was then carried out on the Illumina HiSeq 4000 genome sequencing platform using 150 bp paired-end SBS chemistry. All library preparation and sequencing were performed in the Genome Facility of the University of Iowa Institute of Human Genetics (IIHG). Quality control (QC) of both DNA methylation arrays and RNA-seq experiments were performed to minimize technical biases (see details in Supplementary Methods).

DNA methylation assay. Genomic DNAs (gDNAs) were purified from frozen tumor tissues using the DNeasy Blood and Tissue Kit according to manufacturer's (QIAGEN) recommendations. Yield and purity were assessed on a NanoDrop Model 2000 spectrophotometer and used a 260 nm/280 nm absorbance ratio of ~1.8 with minimal to no degradation as shown through horizontal agarose gel electrophoresis. For more details please see the original publication of DNA methylation assessment in HGSC³². Bisulfite converted gDNAs from HGSC

tumors were submitted to the Genomics Core Facility of the IIHG for processing on methylationEPIC arrays. The Illumina Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, CA, USA) allows quantification of more than 850,000 methylation sites across the human genome. Bisulfite-converted samples were denatured and neutralized before they were isothermally amplified overnight. The amplified product was fragmented enzymatically. After isopropanol precipitation, fragmented DNA was resuspended and placed onto Illumina methylationEPIC BeadChip and hybridized. There are two different bead types for each CpG locus, representing methylated or unmethylated DNA. The BeadChip was washed to remove unhybridized DNA, followed by extension and staining. The arrays were scanned with the Illumina iScan and methylation intensity measured. Analysis was performed using the Minfi R statistical package³³.

Pre-processing of biological data. RNA-seq reads were mapped and aligned to the human reference genome (version hg38) using STAR, a paired-end enabled algorithm³⁴. BAM files were produced after alignment. We used featureCount to measure gene expression from BAM files³⁵. After the gene counts were generated, we used DESeq2 package to import, normalize and prepare data for analysis³⁶. We independently used gene expression and micro RNA (miRNA) expression for the association analysis. Exon specific expression needed different mapping references for alignment, therefore ENSEMBL was used to annotate single exons during the mapping process. Then, single exon features were extracted from these newly created BAM files with the DEXSeq package³⁷. BAM files for each sample were also used for genomic or single nucleotide variation (SNV) discovery and base-calling against the human genome reference utilizing SAMtools and BCFtools for sorting and indexing³⁸. After filtering for duplicates, known non-synonymous single-nucleotide variants, and synonymous variants, results were annotated with ANNOVAR and formatted to display the number of variants per gene and sample³⁹. We included only non-synonymous variants. To estimate gene copy we used SAMtools and CopywriteR using BAM files as input⁴⁰. CopywriteR is a suite of tools that uses off-target sequenced data to detect CNV and, initially, was conceived to be used with DNA sequencing products. However, due to the particularities of the method, that uses off-target (not exonic) reads uniformly distributed along the genome, which also are available even in low-coverage sequencing, we used this method to create variables that would be proxies for gene copy in the prediction model (gene copy estimation, or GCE). CopywriteR software uses the segmentation algorithm CBS (circular binary segmentation) to create segmentation files that contain log₂-transformed, normalized ratios of read counts, that can be used to do further prediction analyses. Long non-coding RNA (lncRNA) were determined using BAM files as input⁴¹. Fusion-genes were determined from *fastq* files processed with the STAR-Fusion suite⁴². Supplementary Fig. S3 depicts the pipeline and analytics used for file pre-processing before modelling.

Statistical analysis. *Variable selection for prediction modeling.* In the prediction model, we only used those variables that could be assessed at baseline, prior to initiation of treatment. RNA features were used only to create predictions models of response to treatment, not for other comparisons. Most RNA features were used as continuous variables. Only presence and absence of SNV and fusion-genes were used as dichotomous variables: present or not. Our approach was to (1) reduce the number of variables using a univariate selection of prediction variables with cross-validation; (2) utilize those significant variables from the univariate selection process in a multivariate model to predict response risk. Rather than introducing all variables directly in the prediction model, this approach was chosen because it would likely lead to a model with less complexity (i.e., fewer variables) and can be more easily validated retrospectively and prospectively. To reduce the number of variables, initially, we introduced only features that were different for both groups in a univariate analysis with ANOVA (p -value < 0.05). Then, cross-validation with 10 replicates for each fold (tenfold) was applied to select those variables that were more informative for prediction of response, as implemented by the *caret* R package⁴³. Features that were selected by this univariate analysis were then used for multivariate *lasso* regression modeling. Unless resampling is included in this initial feature selection step, cross-validation of the subsequent models could be biased⁴⁴. Thus, variable selection for all classes of clinical and biological data (gene, miRNA, exon and lncRNA expressions, GCE, fusion-gene, genomic variation, and DNA methylation analysis) was performed with cross-validation to decrease the possibility of overfitting the final model⁴³. As result of this selection process, poorly annotated features, present in one or few samples, were eliminated early in the analysis.

Prediction model construction. Selected clinical and types of molecular variables from the k-fold cross-validation process were analyzed individually and in combination to determine their prediction potential for treatment response. The lasso method, as implemented in the glmnet R package⁴⁵, was used to develop a regression model to predict responders versus non-responders. We selected lasso because it is a multivariate regression method that allows simultaneous selection and estimation of the effects of variables, while accounting and adjusting for confounding factors. In our experience, lasso consistently lowers number of samples and computes AUC without reporting any errors, as compared to other prediction methods²². We evaluated the performance of our model using the AUC and its 95% confidence interval (CI). AUC was estimated with 1,000 replicates of tenfold cross-validation to avoid over-fitting of the model (internal validation)⁴⁶. Bias-corrected and accelerated bootstrap CIs were computed for resulting AUCs. A value of 0.5 indicates a lack of model predictive performance, and 1.0 indicates perfect predictive performance, or the best model.

Model validation. For external validation of response prediction models created with UI data, we used a publicly available TCGA HGSC dataset⁴⁷. We included only patients with follow-up of at least 6 months after completing initial chemotherapy, with known disease status after 6 months of completion of initial chemotherapy, and data about type of chemotherapy delivered²⁵. As before, only baseline (pre-treatment) *clinical data* were used for validation (see Supplementary Methods). Not all clinical data used in the UI cohort was available in TCGA.

This is a limitation of using TCGA data for model building²⁷. Based on these inclusion criteria, there were a total of 189 patients classified as responders and 149 as non-responders in TCGA for validation of prediction models (Supplementary Table S2).

To make validation possible, only TCGA patients with RNA-seq HGSC tumors were included in the analysis. BAM files, resulting from RNA-seq alignment to hg38 human genome version were downloaded (DbGaP Access #16,003—NCBI) and pre-processed as for the UI dataset (see *Pre-processing of biological data*). Fusion-genes need to be determined from *fastq/fq* files, so we converted BAM files into *fq* files with BCFtools³⁸. After sorting and indexing, *fq* files were processed with the STAR-Fusion suite to obtain fusion-genes⁴².

The *validation analysis* applies the UI-built model to the TCGA data to predict or discriminate between responder or non-responder classes. For validation of clinical data, we constructed new UI models with clinical variables available in TCGA. The same was done for other types of data with missing variables in TCGA dataset, DNA methylation and fusion-genes. For validation, we used the best UI-built models of treatment response. Next, we used the R package *pROC* to determine thresholds, or cut-offs, for the UI-built model applied to the TCGA data (see details in Supplementary Methods)⁴⁸. Threshold values that yielded sensitivities of > 90% were ranked from highest to lowest sensitivity, negative predictive value and AUC. Among the ranked results, the top-ranked set of tuning parameters was used to fit a final score of the model to the entire set of patients and define the classification rule. A sensitivity threshold of over 90% will identify most of the patients at risk of failing treatment. Our goal is to capture the highest proportion of non-responders for clinical use of the model, while also aiming for acceptable specificity. Similar thresholds have been used to assess tests for malignancy, recurrence or failure of ovarian cancer treatment^{49–53}. We coupled high sensitivity with high accuracy measured by AUC: 0.8–0.9 is considered ‘a very good’ diagnostic accuracy, 0.9–1 is considered ‘excellent’.

In previous studies we observed that TCGA patient population has different genetic admixture than UI patient population⁵⁴. That difference may influence the performance of validation analyses. To account and adjust for those genetic differences we extracted genotypes from VCF files obtained after RNA-seq. Then we employed two different strategies for the adjustment: 1) we performed a principal component analysis (PCA) to differentiate genotypes from UI and TCGA datasets and used the first 3 principal components (PC) for adjustment; 2) we performed a lasso regression analysis (PCA) to obtain the genotypes that differentiated UI from TCGA, and used them for adjustment (for details see Supplementary Methods).

Survival analysis. To assess the association of survival with response, survival analysis was performed using Cox proportional hazard ratios.**

Ethical approval and consent to participate. Tumor samples were obtained under informed consent after approval by the University of Iowa Institutional Review Board: IRB# 201,804,817 (approved 5/9/2018) and 200,209,010 (approved 9/19/2005). The institutional review board (IRB) of the University of Iowa (UI) approved the current study including human subjects/materials on April 25, 2018 (IRB Number 201804817: ‘*Prediction Models in Ovarian Cancer*’). All data collection and processing, including the consenting process, were performed after approval by the University of Iowa IRB.

Consent for publication. All authors have reviewed and approved the manuscript for submission.

Results

In the UI database, 43% of patients were non-responders, and in TCGA HGSC dataset 44% of patients were non-responders, chi-square p-value = 0.88 (Table 1 and Supplementary Table S1, respectively). Non-responder UI patients had higher Charlson comorbidity index score, more residual disease after surgery, and received more frequently neoadjuvant chemotherapy before surgery (Table 1). Non-responder TCGA patients had more residual disease after surgery (Supplementary Table S2). Median survival was 39.3 months (95% CI: 31, 58.2) for UI responders and 57.7 months (95% CI: 44.3, 82) for TCGA responders. Median survival was 12.5 months (95% CI: 8, 19.1) for UI non-responders and 22.7 months (95% CI: 15.9, 26.3) for TCGA non-responders. Based on these 95% CIs, there were no differences in survival for responders and non-responders in the UI and TCGA datasets.

Variable selection for prediction modeling. After the univariate analysis of all clinical and genomic data with ANOVA as described in *Methods*, we identified those variables that were more informative for the outcome of interest: treatment response (Fig. 2). The number clinical, gene, miRNA, exon and lncRNA expressions, GCE, fusion-gene presence, SNV, and DNA methylation variables selected after the univariate and multivariate analysis, and included in the prediction analyses are detailed in Table 2. Notably, in the genomic variation analysis, we found BRCA2 variants in 24% (12 out of 50) of responders and in 26% (10 out of 38) of non-responders (p = 0.52); and BRCA1 variants in 36% (18 out of 50) of responders and 50% (19 out of 38) in non-responders (p = 0.06). These differences were non-significant; therefore BRCA1&2 were not selected for the prediction analysis.

Prediction model construction. Prediction models of response were built initially with one type of selected data: clinical, gene, exon, miRNA, and lncRNA expression, SNV, GCE, fusion-gene presence, or DNA methylation (Table 2). See Supplementary Table S3 for more details about the variables after lasso prediction. Next, we built models integrating 2 and 3 types of data. The performance of all models was evaluated using the AUC and its 95% CI. By integrating clinical and genomic variables, we achieved prediction performances of over 95%. Figure 3 represents all prediction models with 1, 2, or 3 variables with AUC over 90% (N = 59). Adding 4 or

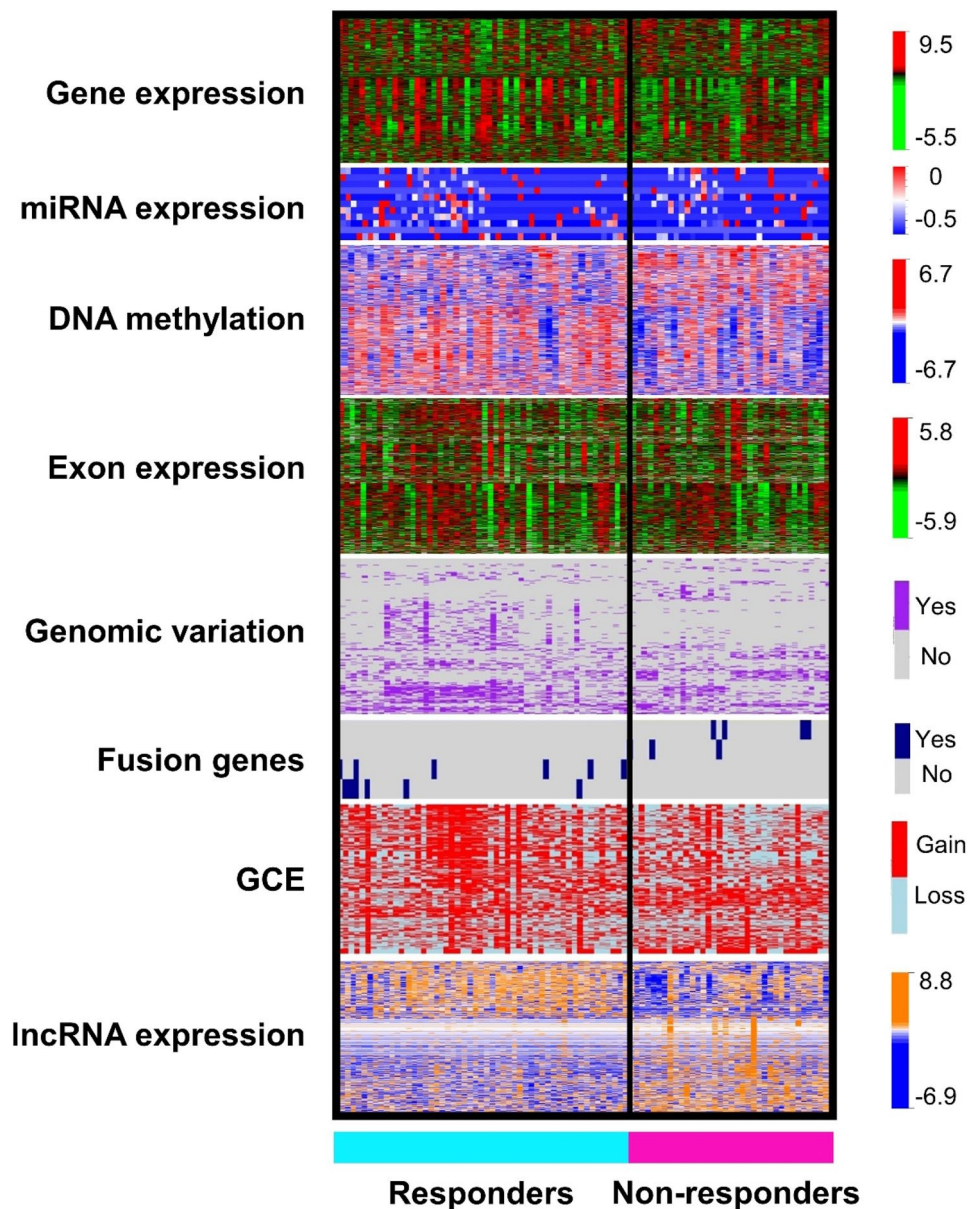


Figure 2. Heatmap of selected variables after univariate ANOVA analysis. Representation of the significant variables after univariate analysis ($p < 0.05$) for different types of genomic data: gene, miRNA, exon, and long non-coding RNA (lncRNA) expression, DNA methylation, genomic variation, fusion-gene presence, and gene copy estimation (GCE). At the right side of each heatmap there are color-coded range of values for all genomic variables. Heatmaps were generated with R package *Heatplus*⁶⁸ (R version 3.6.3. <http://www.r-project.org>).

more types of data increased model complexity without a significant improvement in performance. For details about all prediction models review Supplementary Figure S7.

Model validation. One of the limitations of using TCGA for validation of prediction models was that not all clinical data used in the UI cohort were available in TCGA dataset. For validation analysis, we took all models built using 1, 2, or 3 variables in the UI dataset and inserted TCGA data to assess how well the UI-built models discriminate between responders and non-responders in the TCGA dataset (107 different models)⁴⁷. We selected a sensitivity threshold over 90% in order to identify most of the patients at risk of failing treatment (see the rationale in methods). Notably, validation of models containing DNA methylation and lncRNA data underperformed (Fig. 4A). If we eliminated models with DNA methylation and lncRNA, 80% (51 out of 64) of UI-built models had an AUC 95% CI in the TCGA validation set that overlapped with the UI training set interval (Fig. 4B).

Type of data	Initial number of variables	Variables after selection: univariable ANOVA analysis with k-fold cross-validation	Variables after multivariable prediction model with lasso
Clinical	45	–	7
Gene expression: mRNA	23,528	2214	62
miRNA expression	1914	12	11
Gene copy estimation: GCE	28,917	1098	83
Genomic variation	13,840	327	54
DNA methylation	66,042	4961	35
Long non-coding RNA	16,325	773	69
Fusion genes	597	147	104
Individual exon expression	63,677	4387	61

Table 2. Variable selection and variables after prediction model construction with type of data. To reduce the number of variables, we used univariate analysis of all data with ANOVA to select the variables that were more informative for prediction of response, with a p-value < 0.05 (3rd column). Features that were statistically significant in this univariate analysis were then used for multivariate *lasso* regression modeling. In the last column are the number of variables resulting after performing that prediction model with only one variable. Variables in this last column were used to build prediction models integrating 2 or 3 types of data.

To adjust for different population genetic backgrounds between UI and TCGA cohorts, we used PCA and lasso regression analysis with genotypes that differentiated UI from TCGA. The preliminary studies with PCA did not increase validation performances (see Supplementary Methods), so we next carried out the adjustment with genotypes that differentiated UI and TCGA datasets. When adjusting for genetic variation, validation of the UI-built models had an AUC 95% CI in the TCGA validation that overlapped 97% (62 out of 64 models) with the UI training set interval (Fig. 4C). We did not observe any improvement after adjusting for genotypes in models containing DNA methylation and lncRNA data (Fig. 4D). These results indicate that differences in performance between UI-built and TCGA validation models that do not contain DNA methylation and/or lncRNA data may be related to different genetic background of both datasets.

Discussion

Prediction of treatment response in HGSC patients before treatment initiation is a difficult task. Not many groups have attempted these types of predictions and, of the few reported models, none have been introduced successfully into clinical practice. The reasons are varied but can be classified into two groups: (1) prediction model performances did not warrant their introduction in routine practice^{2,13}; or (2) models could not be validated in independent datasets and thus could not be generally applied^{14,21}. We present a comprehensive study of treatment response prediction in patients with advanced stage HGSC. Most importantly, these prediction models were validated in an independent dataset with similar in-depth genomic assessment, TCGA.

Prediction models were built by integrating different types of clinical and molecular data. Simpler models were built after selecting those variables more informative for the outcome, treatment response, and using cross-validation to minimize over-fitting. As we increased model complexity (with 2–3 types of data), performances on the training set reached levels over 95% in terms of AUC. These performances are promising and could provide robust clinical decision support to discriminate responders versus non-responders. The most predictive models included diverse types of data, but notably, those around 100% AUC included epigenetic regulators of gene expression, either by DNA methylation or miRNA expression. Also, other gene modulators like lncRNA expression and individual exon expression, a readout for splice variant expression⁵⁵, were involved in these high performing models. Finally, clinical information was also an important component of the best prediction models. Other components of high performing models, like fusion genes, have not been characterized yet in HGSC outcome prediction or prognosis, but have been associated with acquired resistance⁵⁶.

Epigenetic gene regulation is one of the mechanisms that regulate treatment response. For example, whole genome DNA methylation analysis found that epigenetic regulation of potentially clinically relevant genes predicts response to platinum^{57–59}. Also, lncRNAs have been associated with epigenetic regulation of HGSC⁶⁰, and specific lncRNAs have been associated with chemo-resistance⁶¹. Therefore, it is not surprising that some of the best prediction models are composed of diverse lncRNAs. The role of miRNAs in response to treatment in HGSC in vitro is well documented⁶², and some miRNAs also have been associated with chemo-response modulation⁶³. The presence of epigenetic regulators and modifiers of treatment response in high performance prediction models of response to therapy seems to support these regulatory mechanisms. We must be cautious about extrapolating functional conclusions from prediction models. Prediction analysis is a form of statistical learning that uses data obtained in the past to predict outcomes, or behavior, of other individuals in the future. Prediction analysis is based on association and does not infer causation⁶⁴.

Prediction models of response created with UI data were validated in TCGA HGSC data. Notably, models containing DNA methylation and/or lncRNA data did not perform well in validation analyses. Moreover, when we adjusted for different genetic backgrounds between UI and TCGA samples, validation performances of models *not* containing DNA methylation or lncRNA improved, with 97% of overlap in the 95% CI of AUCs. Conversely, validation models containing DNA methylation and/or lncRNA did not improve despite adjusting for genetic background. We speculate that genetic background differences between the UI-sampled population and TCGA

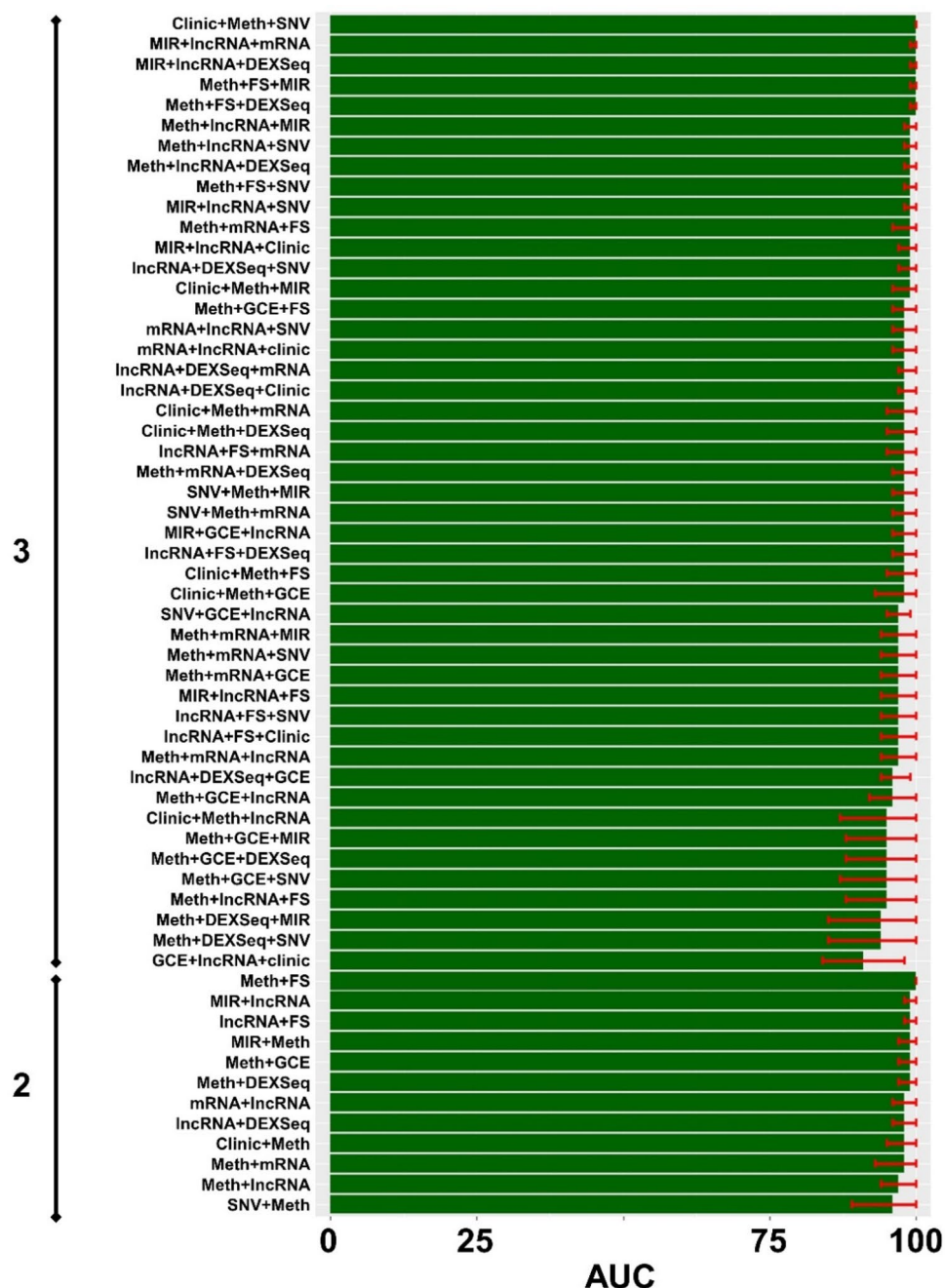


Figure 3. High performing prediction models of response. On the left is the number of types of data: 2: combination of 2 types of data; 3: combination of 3 types of data. Different performances are displayed in ascending order. The x axis is AUC as a percentage (0–100%). Although we tested 107 models with 1, 2 and 3 variables, we only represented those models with performances over 90% measured in AUC, $N=59$ (to see all of them see Supplementary Figure S7). FS: Fusion genes; Meth: DNA methylation; SNV: single nucleotide variation; GCE: gene copy estimation; DEXSeq: exon expression; IncRNA: long non-coding RNA; MIR: micro RNA; mRNA: gene expression. Graphics were generated with R package *ggplot* (R version 3.6.3. <http://www.r-project.org>)⁶⁹.

account for some variation in the validation of these prediction models of treatment response, except for those models including DNA methylation and/or IncRNA. The poor performance of validation TCGA prediction models including DNA methylation and/or IncRNA data was likely due to other reasons. DNA methylation analysis for TCGA HGSC data was performed using Illumina Infinium HumanMethylation27K BeadChip arrays⁴⁷. Methylation in UI was performed with an EPIC BeadChip 850 k arrays (both arrays from Illumina Inc.). The 27 K methylation array interrogates mainly CpG islands in gene promoter regions, while the 850 K array also explores DNA methylation outside the promoter areas with whole genome coverage³². To determine IncRNA

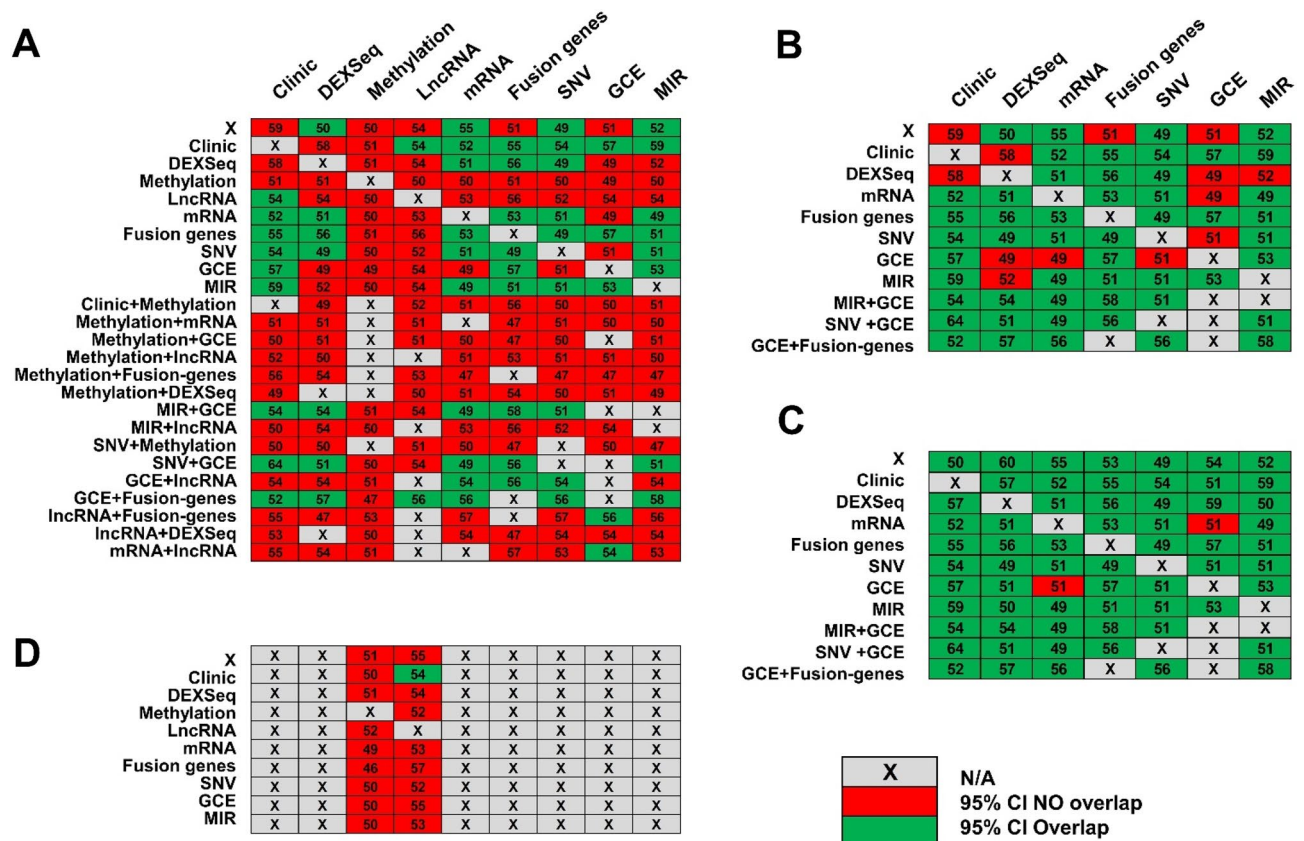


Figure 4. Validation of UI prediction models of response to treatment in TCGA datasets. The columns represent different types of clinical and molecular data: DEXSeq (individual exon expression), Methylation (DNA methylation), LncRNA (long non-coding RNA expression), mRNA (gene expression), Fusion genes (presence of fusion genes), SNV (Single Nucleotide Variation), GCE (gene copy estimation), and MIR (microRNA expression). The rows also represent different types of data, either individually or in combination. (A) Validation of all prediction models of response in TCGA: Models containing DNA methylation and LncRNA data underperformed: AUC 95% CIs of TCGA-validation models did not overlap with AUC 95% CIs of UI-built training models (red cells in the graphic). Green cells represent those AUC 95% CIs of TCGA-validation models that overlap with UI-built AUC 95% CIs. (B) When we removed models containing DNA methylation and LncRNA data, 80% of CIs from UI-built training models overlapped with CIs from TCGA-validation models. (C) When prediction models without DNA methylation and LncRNA data were adjusted with genotyping data (as detailed in Supplementary Methods), 97% of CIs from UI-built models overlapped with CIs of TCGA-validation models. (D) TCGA-validation performance did not improve when adjusting for different genotypes between UI and TCGA. This was tested in models with 1 and 2 types of data (18 models).

expression in UI HGSC data, we used data from RNA sequencing that was carried out on the Illumina HiSeq 4000 genome sequencing platform using 150 bp paired-end sequencing by synthesis (SBS) chemistry³². LncRNA expression was extracted from TCGA HGSC sequenced with the Illumina HiSeq 2000 genome sequencing platform that uses 75 bp paired-end SBS chemistry. Differences between 150 and 75 bp sequencing products may have contributed to differences in background noise and total LncRNA counts. Indeed, differences between the platforms may contribute to the decrease in performance validation with LncRNA data. Other technical differences between both databases, like libraries preparations between both sets, may influence critically overall prediction model performance.

A strength of this study is that we used diverse databases of genomic and clinical variables to build prediction models of response. We postulated that a complete database containing all variables involved in malignant cell functions would make prediction models more accurate^{22,27,31,65}. Therefore, we extracted as much information from the HGSC specimens as possible to improve our models. Likewise, with clinical data we extracted as much baseline clinical information that could affect the primary outcome of interest. These variables may have been known previously to affect the outcome, or not. Public databases not designed specifically for prediction assessment, like TCGA, may lack of some characteristics that result in important discrepancies of model performance. In the present study, we were able to adjust for these discrepancies in some of the models, except for those containing DNA methylation and/or LncRNA data. Another strength is the outcome definition. Progression-free survival of at least 6 months after the first platinum-based treatment, or responders, is an standard definition of response to chemotherapy^{2,5,6}. Indeed, patients that do not respond to initial standard treatment, platinum-resistant, or progressed during treatment, platinum-refractory, are considered a different population

when chemotherapy or clinical trials are considered^{4,8}. Treatment response was reviewed in all UI patients, and any patient that did not meet the inclusion criteria was excluded from the analysis. Also, to be included in validation analyses all TCGA patients had to meet treatment response definition criteria. Finally, validation of all models of prediction in a public, well known, independent database (TCGA) also strengthens our study. One of the inherent limitations of the study comes from its design: to build prediction models, initially, the outcome must be known, so the initial step of the analysis and validation is retrospective. The advantage of extracting data from patients of a single institution is the uniformity of diagnosis, outcome definition, specimen collection and processing, treatment philosophy and surveillance. This resulted in a homogenous population with quality biological and clinical data. The selection process may have some disadvantages, though, and the selected samples may lack diversity and it may be limited by the number of HGSC eligible patients. We are the largest of only two Gynecological Oncologic practices that serves around 80–90% of women with gynecological cancer in the state of Iowa (USA). Thus, the samples we have studied represents the female racial composition of the State of Iowa: 95.5% white, 1.1% black, 3.4 other (Latina, Asian, Pacific)⁵⁴. We adjusted these differences in genetic variation with TCGA during validation (see [Results](#)). In future studies, it may be especially important that every center that treats women with HGSC knows exactly the racial and genetic composition of the population they treat, so they can correct or adjust for these differences. We acknowledge the limited sample size used to construct prediction models, but the necessity of having an accurate outcome definition and homogeneous population is even more critical. Previous prediction studies are plagued with patients with heterogenous clinical characteristics and outcome definitions, and with different histological types of ovarian cancers that made generalizability even more difficult^{2,13,14,21}.

Before these models can be applied clinical, they must be validated prospectively. Despite internal and external validation, models of prediction still may have biases due to overfitting. In the prospective model those biases could be detected and corrected before clinical application. Then, for models of prediction to be applied, there is a process that must be followed. After a biopsy is taken (either CT-guided or during surgery) and HGSC histologic type has been confirmed, we will determine all components of the best prediction model validated prospectively. As long as CT-guided biopsies have enough tumor cellularity (over 2/3), and not too much necrosis (< 30%), it would be enough and comparable to the initial model. Sequencing is rapidly evolving with single cell RNA-seq technology⁶⁶. Each of these components will be transformed as they were formatted in the initial analysis (i.e., log transformed, coding values, etc.) and the values would be applied to the weight of each variable in the selected model. The addition of all values will give us a final score. That score, and where it is located with respect to a chosen threshold, will assess the risk of the patient to fail initial therapy. We could design customized assays, PCR-based, for the genomic features of the model that would reduce costs and complexity and would improve the turnaround time so the results could be used even before surgery. With this information, clinicians could have a good sense of which patients would respond to treatment, and they would be better informed to plan initial treatment. For example, if a patient has a higher score for response, the surgeon would take that into consideration to balance the effort in cytoreduction with possible complications. Knowing that the patient may not respond as well to initial treatment may increase the surgical effort to minimize residual disease. Also, if a patient has a lower score for response, we may want to involve them in clinical trials that add targeted treatment to the initial chemotherapy backbone to improve outcomes.

Conclusions

Based on our results and previous reporting from other prediction studies^{22,27,31,65} we can conclude that our hypothesis holds true: integrating comprehensive clinical and genomic data from patients with HGSC results in accurate and robust prediction models of treatment response. We have described high performance prediction models of response for initial treatment in HGSC. Based on these performances, some of these prediction models could be useful to provide clinical decision support that will differentiate responders to non-responders. Furthermore, these models were validated in an independent, trusted, well known database.

Data availability

Data for the prediction model has been submitted to the GEO at NCBI website: <https://www.ncbi.nlm.nih.gov/geo/>. Datasets with methylation data can be browsed by their accession number: GSE133556. Datasets with RNA-seq can be browsed by their accession number: GSE156699. The validation part of this study was performed in silico, with de-identified publicly available data. All data from TCGA is available at their website: <https://portal.gdc.cancer.gov/>. Software utilized by this study is also publicly available at Bioconductor website: <http://bioconductor.org/>.

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Author contributions

J.G.B., A.M.N., E.J.D., and B.J.S. conceived of the project and contributed to study design; J.G.B., A.M.N., and E.J.D. performed data collection; J.G.B., and B.J.S. performed computational analyses; J.G.B., E.J.D., B.J.S., and T.A.B. analyzed and interpreted the data; J.G.B., K.W.T., K.K.L., and B.J.S. wrote the manuscript with input from all authors; J.G.B., E.J.D., A.M.N., B.J.S., D.P.B., M.J.G., M.E.M., T.A.B., K.W.T. and K.K.L. read and approved the final version of the manuscript.

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Competing interests

K.W.T. is a cofounder of and holds an equity stake in Immortagen, Inc. All other authors: J.G.B., E.J.D., A.M.N., B.J.S., D.P.B., M.J.G., M.E.M., T.A.B., K.K.L. certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent/licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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TP53 Sequencing and p53 Immunohistochemistry Predict Outcomes When Bevacizumab Is Added to Frontline Chemotherapy in Endometrial Cancer: An NRG Oncology/ Gynecologic Oncology Group Study

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abstract

PURPOSE The status of p53 in a tumor can be inferred by next-generation sequencing (NGS) or by immunohistochemistry (IHC). We examined the association between p53 IHC and sequence and whether p53 IHC alone, or integrated with *TP53* NGS, predicts the outcome.

METHODS From GOG-86P, a randomized phase II study of chemotherapy combined with either bevacizumab or temsirolimus in advanced endometrial cancer, 213 cases had p53 protein expression data measured by IHC and *TP53* NGS data. An analysis was designed to integrate p53 expression by IHC with the presence or absence of a *TP53* mutation. These variables were further correlated with progression-free survival (PFS) and overall survival (OS) in the chemotherapy plus bevacizumab arms versus the chemotherapy plus temsirolimus arm.

RESULTS In the analysis of p53 IHC, the most striking treatment effect favoring bevacizumab was in cases where p53 was overexpressed (PFS hazard ratio [HR]: 0.46, 95% CI, 0.26 to 0.88; OS HR: 0.31, 95% CI, 0.16 to 0.62). On integrated analysis, patients with *TP53* missense mutations and p53 protein overexpression had a similar treatment effect on PFS (HR: 0.41, 95% CI, 0.22 to 0.83) and OS (HR: 0.28, 95% CI, 0.14 to 0.59) favoring bevacizumab plus chemotherapy relative to temsirolimus plus chemotherapy. Concordance between *TP53* NGS and p53 IHC was 88%. Concordance was 92% when cases with *TP53* mutations and *POLE* mutations or mismatch repair deficiency were removed.

CONCLUSION IHC for p53 alone or when integrated with sequencing for *TP53* identifies a specific, high-risk tumor genotype/phenotype for which bevacizumab is particularly beneficial in improving outcomes when combined with chemotherapy.

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ASSOCIATED CONTENT

Appendix

Data Sharing Statement

Data Supplement

Protocol

Author affiliations and support information (if applicable) appear at the end of this article.

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INTRODUCTION

Cancer of the uterine corpus, or endometrial cancer, is the most common gynecologic malignancy in the United States.¹ Furthermore, endometrial cancer incidence and mortality are on the rise worldwide.¹⁻³ Nearly 66,000 cases are expected in 2022, and 13,000 lives will be lost to this pervasive and understudied disease.^{4,5} In response to these alarming statistics, cooperative groups such as NRG Oncology and its antecedent organization, the Gynecologic Oncology Group (GOG), have sought to improve outcomes for women with advanced and recurrent diseases.

GOG-86P was one of the first studies to combine molecular inhibitors such as bevacizumab or temsirolimus

with chemotherapy in patients with advanced endometrial cancer.⁶ GOG-86P was a three-arm, randomized phase II study of paclitaxel/carboplatin/bevacizumab, paclitaxel/carboplatin/temsirolimus, or ixabepilone/carboplatin/bevacizumab as the initial therapy for measurable advanced or recurrent endometrial cancer.⁶ The conclusion of that study was that the primary end point, grouped progression-free survival (PFS), was not significantly increased in any experimental arm compared with historical controls from GOG-209 who were treated with chemotherapy alone.⁷ However, a recently published follow-up exploratory analysis assessed outcomes on the basis of mutations in the tumor suppressor *TP53*,⁸ the most commonly mutated gene in advanced/recurrent endometrial cancer.⁹ When

CONTEXT

Key Objective

Clinical management of advanced endometrial cancer is transitioning from histologic subtype to molecular stratification. The most aggressive molecular subtype is abnormal p53; however, a major debate is how to appropriately determine p53 status. We performed a retrospective analysis of p53 status by immunohistochemistry (IHC) and next-generation sequencing (NGS) using specimens from a completed clinical trial, GOG-86P.

Knowledge Generated

p53 expression by IHC strongly correlated with mutational status in > 90% of cases. p53 overexpression by IHC identified patients with improved outcomes when bevacizumab is added to the chemotherapy backbone, with an overall survival hazard ratio of 0.31.

Relevance

Determining p53 status by IHC is a fast, cost-effective, and reliable method that can be easily integrated into clinical management of advanced endometrial cancer. The integration of sequencing with IHC may allow better stratification of cases in which the p53 immunostaining reveals an unusual pattern, such as cytoplasmic or highly heterogeneous p53 expression.

the overall impact of *TP53* mutations determined by next-generation sequencing (NGS) was assessed on all experimental arms of the study combined, those patients whose tumors harbored mutated *TP53* did worse, both by PFS and by overall survival (OS), compared with patients whose tumors had wild-type (WT) *TP53*.⁸ This is not surprising given the association between mutant p53 and aggressive histology¹⁰⁻¹⁴ and highlights the acute need to develop more effective therapies for patients with mutated *TP53* tumors.^{15,16}

However, when cases with *TP53* mutations were evaluated and compared on the individual experimental arms of GOG-86P, a picture of differential sensitivity emerged. The conclusion of the original GOG-86P report showed no PFS benefit compared with historical controls treated with chemotherapy alone. However, this more recent exploratory analysis indicates that bevacizumab combined with chemotherapy is superior to temsirolimus plus chemotherapy, specifically in patients with *TP53*-mutated tumors as determined by NGS,⁸ although a chemotherapy-only reference arm was not included in the GOG-86P trial design.⁶ Indeed, the risks of progression and/or death for those patients were significantly decreased in the bevacizumab arms (PFS hazard ratio [HR]: 0.48, 95% CI, 0.31 to 0.75; OS HR: 0.61, 95% CI, 0.38 to 0.98). On the other hand, patients with WT *TP53* did not experience improved outcomes on either arm. Since the original GOG-86P included a third arm of bevacizumab in combination with a different microtubule spindle disruptor (ixabepilone) in place of paclitaxel, this retrospective study combined the two bevacizumab-containing arms into a single group to increase power to detect differences. However, the improvement in PFS and OS with p53-mutated tumors persisted when the bevacizumab-containing arms were analyzed independently.⁸

Given the identification of mutated *TP53* as a relevant biomarker for improved outcomes in the bevacizumab plus chemotherapy arms of GOG-86P, we wished to determine how best to assess tissues for p53 status. Fortunately, translational end points for 213 patients on GOG-86P included both immunohistochemistry (IHC) for p53 and NGS of *TP53*, enabling the current analyses. Herein, we performed a comparison between IHC and NGS and determined the association of both variables with histology and clinical outcomes. As with the previous exploratory study of GOG-86P,⁸ we combined data from the two bevacizumab-containing arms into a single group and compared outcomes with those of patients in the temsirolimus plus chemotherapy group.

METHODS

Detailed methods are provided in the Data Supplement (online only).

Study Cohort

GOG-86P (ClinicalTrials.gov identifier: [NCT00977574](https://clinicaltrials.gov/ct2/show/study/NCT00977574)) was a three-arm, randomized phase II study of paclitaxel/carboplatin/bevacizumab (NSC#704865, IND#7921), paclitaxel/carboplatin/temsirolimus (NSC#683864, IND#61010), or ixabepilone/carboplatin/bevacizumab (NSC#710428, IND#59699) as initial therapy for measurable stage III or IVA, stage IVB, or recurrent endometrial cancer. Biospecimens were collected from patients who consented to participate in the translational research component of the study. The collection of archival formalin-fixed paraffin-embedded tissue and tumor DNA was coordinated by the NRG Oncology Biospecimen Bank. From these cases, 213 tumors were analyzed using IHC for p53 and NGS for *TP53*. NGS methodology has been previously reported in detail.⁸ The distribution of p53 expression (IHC)

and mutational status (*TP53* NGS) by race and ethnicity are provided in the Data Supplement.

***TP53* Mutation Classification Criteria**

TP53 mutations by NGS were grouped into four categories: (1) WT: mutations that did not result in an amino acid substitution; (2) truncating: frameshift or splice site mutations that result in a truncated protein; (3) gain of function (GOF): missense mutations that are canonical gain-of-function mutations (P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L, and R282W¹¹⁻¹³); and (4) unclassified missense mutations or indels, referred to herein as somatic variants of unknown function (sVUFs).

p53 IHC

IHC for p53 (DO-7, Dako) was performed as previously described,¹⁷ and cases were assigned to one of three categories (Fig 1A): (1) WT: 1%-80% of tumor cell nuclei staining positive, usually with variable intensity; (2) null: no tumor cell nuclear staining with a positive internal control; and (3) overexpressed (OE): uniform and intense nuclear staining in at least 80% of tumor cell nuclei (estimated). Some cases did not fall into these simplified groups; examples are shown in Figure 1A. Cases were scored as equivocal when there was no nuclear signal in the absence of a positive internal control. Cases scored as focal had overall WT staining but with focal areas of clear p53 overexpression, indicating heterogeneity with respect to p53 expression.

Statistical Analyses

An exploratory analysis of biomarkers on GOG-86P was performed. Patients were grouped based on tumor *TP53* mutational status, histology, and p53 IHC results. Outcomes (PFS and OS) were compared between treatment arms or between groups using proportional hazards models. Data from the two bevacizumab-containing arms (arms 1 and 3) were combined and compared with the temsirolimus-containing arm (arm 2).

RESULTS

IHC and Relationship to Histology

Of the 213 cases, 63% were WT by IHC, 28% were OE, and 8% were null (Fig 1B). The p53 null and OE cases were generally restricted to higher-grade endometrioid and serous histologies (Fig 1C).

IHC and Relationship to *TP53* Sequence

We next examined the relationship between *TP53* mutational status and p53 protein expression by IHC. The overall concordance rate between p53 protein expression and *TP53* mutational status was 88%. Among tumors with WT p53 by IHC, 88% also had no mutations in the *TP53* gene (Fig 1D). Among the cases with mutated *TP53*, cases with truncating mutations were generally p53 null (78%) and cases with missense mutations were OE by IHC (92%, Fig 1E). No case with a predicted truncating mutation in *TP53* was OE by IHC. The discordance rate was

low—in only 26 of 213 cases (12%), the IHC did not predict the *TP53* sequence (Table 1). Some of the discordant cases can be potentially explained by concurrent mutations in polymerase epsilon (*POLE*) or by mismatch repair deficiency (MMRd; Table 1). For other cases, p53 staining was either focal or equivocal, which confounds categorical binning. A secondary review also identified two data entry errors and one *TP53* variant (P72R) that is a well-documented polymorphism.¹⁸ We therefore re-evaluated the concordance of p53 IHC with *TP53* sequencing after removing the cohort with *TP53* mutations and a concurrent *POLE* mutation and/or MMRd; we also moved the three misclassified cases from the discordant group to the concordant group. The updated concordance was 92%.

We did not observe a relationship between p53 status (IHC and NGS) and race or ethnicity (Data Supplement), which may be due to the high representation of White (80%) and non-Hispanic (84%) patients in the study cohort.

Relationship of Sequence and IHC as Single Discriminators of Clinical Outcomes

***GOG-86P* HRs and CIs for NGS status alone.** Since *TP53* mutations predominate in the serous subtype of endometrial cancer,⁹ we first analyzed serous histology as a predictor of response (Data Supplement). Analysis of outcomes by histology indicates improvement in PFS in nonserous cases with bevacizumab (HR: 0.74, 95% CI, 0.56 to 0.98) and a trend toward improvement in the bevacizumab arms in serous cases (HR: 0.61, 95% CI, 0.36 to 1.05).

We recently reported that the PFS HR for bevacizumab plus chemotherapy versus temsirolimus plus chemotherapy in cases with mutated *TP53* is 0.48 (95% CI, 0.31 to 0.74) compared with 0.87 (95% CI, 0.58 to 1.30) for WT tumors.⁸ For OS, the HR was 0.61 (95% CI, 0.38 to 0.96) for *TP53*-mutated cases versus 1.05 (95% CI, 0.64 to 1.74) for WT cases.⁸ We further divided the mutated cases into three categories: (1) truncating; (2) known oncogenic or GOF; and (3) missense mutations of unknown function, which we term herein sVUF. The presence of a *TP53* GOF mutation was prognostic, meaning regardless of therapy such a mutation portended a worse outcome with a significantly lower PFS and OS as compared with cases with WT p53 (Data Supplement). The low number of cases in each category did not provide adequate power to make a definitive statement as to the impact of bevacizumab versus temsirolimus for each type of *TP53* mutation. Nevertheless, we observed a trend toward improved PFS and OS with bevacizumab in all groups, most notably cases with missense GOF or sVUF mutations (Fig 2, Data Supplement).

***GOG-86P* treatment HRs and CIs for IHC status alone.** Evaluation of IHC as a prognostic factor suggests that patients with overexpression of p53 by IHC had a worse OS than cases with WT p53 (Fig 3A). However, overexpression of p53 portended longer outcomes on the bevacizumab-containing arm as compared with temsirolimus (Figs 2 and 3B). Specifically, the

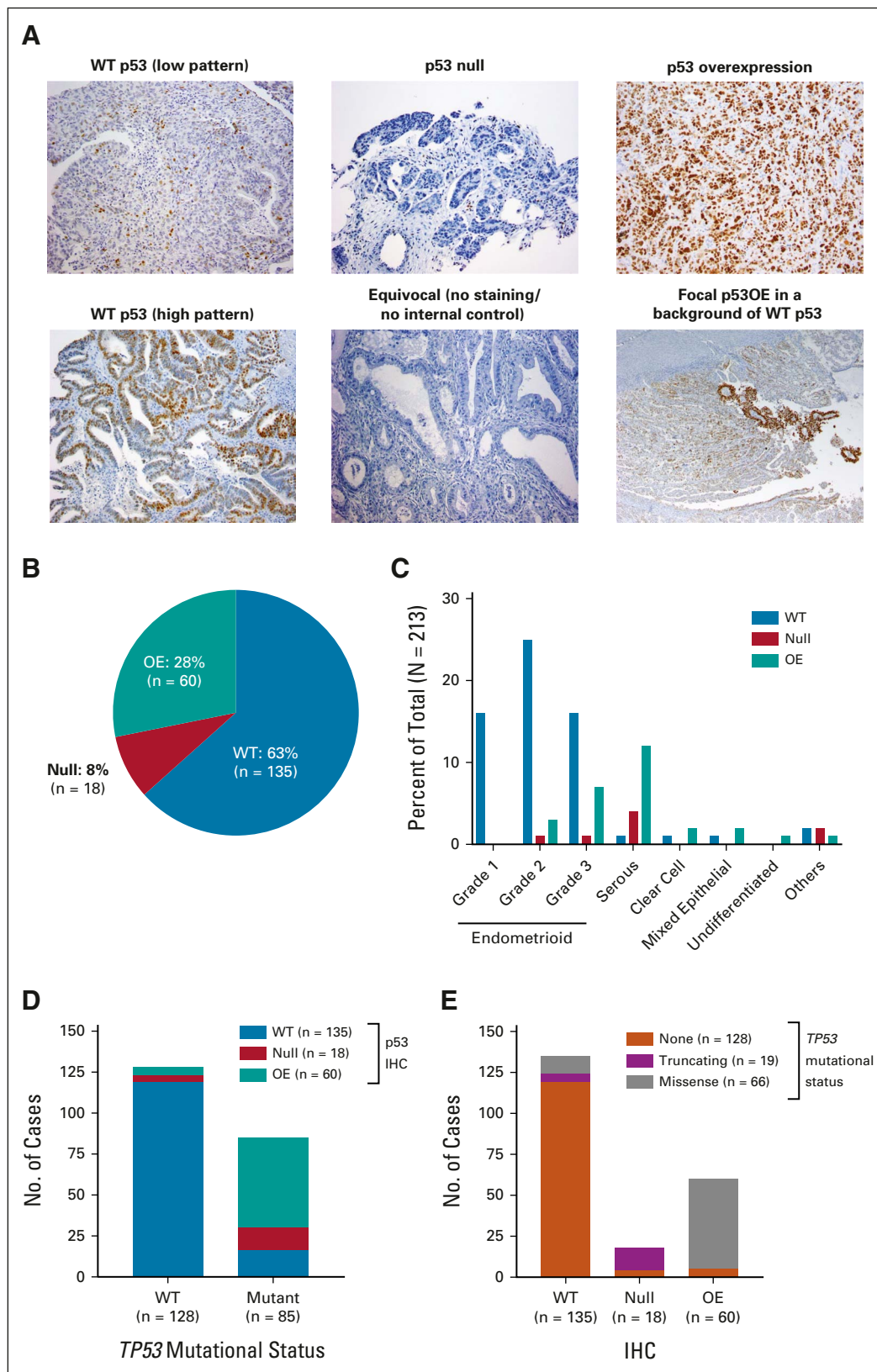


FIG 1. Concordance between p53 expressions by IHC with mutation in *TP53*. (A) Examples of WT p53 staining (both low and high patterns), null p53, and overexpression of p53. Also shown are cases with equivocal staining for p53 and focal regions of p53 overexpression. (B) Overall distribution of p53 expression by IHC in cases with available data. (C) Percent of cases with WT p53 staining, null staining, or OE by histology. (D) Concordance on the basis of (continued on next page)

FIG 1. (Continued). the absence (WT) or presence of a *TP53* mutation with p53 expression by IHC. (E) Concordance on the basis of the specific type of *TP53* mutation. IHC, immunohistochemistry; OE, overexpressed; WT, wild-type.

HR and 95% CIs for PFS on the basis of IHC alone, bevacizumab plus chemotherapy versus temsirolimus plus chemotherapy, are 0.46 (0.26 to 0.88) for OE IHC, 0.71 (0.24 to 2.34) for null IHC, and 0.74 (0.49 to 1.12) for WT by IHC (Fig 2A). For OS on the basis of IHC, the HR for OE = 0.31 (0.16 to 0.62), null = 1.26 (0.35 to 5.87), and WT = 1.05 (0.65 to 1.77, Fig 2B). There was no difference in outcomes by treatment arm for cases with a WT p53 expression pattern by

IHC, and the number of cases in the p53-null category was too small to discern differences between groups (Data Supplement).

Integrated Analysis of Sequence and IHC as Discriminators of Clinical Outcomes

To improve on a p53-based classifier of patients who most benefited from bevacizumab, we designed an integrated

TABLE 1. Discordant p53 IHC Versus *TP53* Next-Generation Sequencing Cases in GOG-86P

GOG_ID	p53 IHC		<i>TP53</i> Mutation	MMRd	<i>POLE</i> Mutation	Secondary Concordance Analysis
	Original Interpretation	Secondary Interpretation				
GOG86P-176	WT	Slide not available	Truncating	P47Rfs*76 ^a	PMS2 loss	Excluded
GOG86P-178	WT	WT	Truncating	V73Wfs*50 ^a	PMS2 loss	Excluded
GOG86P-183	WT	WT	GOF	R248W	PMS2 loss	Excluded
GOG86P-175	WT	Equivocal ^b	GOF	R248W	A252V, A31S	Excluded
GOG86P-39	Null	Equivocal ^b	WT		E1949K	Discordant
GOG86P-173	WT		SNP	P72R ^c		Concordant
GOG86P-188	WT	WT	sVUF	R181H	R2225H	Excluded
GOG86P-172	WT	WT	Truncating	R333Vfs*12 ^a	R53W	Excluded
GOG86P-174	WT	WT	Truncating	G244D,R196fs* ^a		Discordant
GOG86P-177	WT	Slide not available	Truncating	S90Pfs*33 ^a		Discordant
GOG86P-179	WT	WT	sVUF	G244S		Discordant
GOG86P-180	WT	WT	sVUF	Y234H		Discordant
GOG86P-181	WT	Microscopic focus of overexpression; WT background	sVUF	A159P		Discordant
GOG86P-182	WT	Equivocal	Truncating	P153Afs*28 ^a		Discordant
GOG86P-184	WT		Truncating	X307_splice ^d		Discordant
GOG86P-185	WT	WT	sVUF	R267W		Discordant
GOG86P-186	WT	WT	Truncating	X307_splice ^d		Discordant
GOG86P-187	WT	WT	sVUF	C135Y		Discordant
GOG86P-127	OE (data entry error)	WT	WT			Concordant
GOG86P-140	OE (data entry error)	WT	WT			Concordant
GOG86P-106	OE	Endometrium is WT; core biopsy is OE	WT			Discordant
GOG86P-166	OE	OE tumor is very small and noninvasive	WT			Discordant
GOG86P-51	OE	OE tumor is very small	WT			Discordant
GOG86P-34	Null	Equivocal	WT			Discordant
GOG86P-47	Null	Equivocal	WT			Discordant
GOG86P-48	Null	Equivocal	WT			Discordant

Abbreviations: GOF, gain of function; IHC, immunohistochemistry; MMRd, mismatch repair deficiency; OE, overexpressed; SNP, single-nucleotide polymorphism; sVUF, somatic variants of unknown function; WT, wild-type.

^afs* indicates a frameshift mutation that results in a premature stop codon.

^bEquivocal denotes a null staining pattern but no internal control.

^cSplice indicates a mutation in a splice site that results in a truncated protein.

^dP72R is a common TP53 polymorphism.

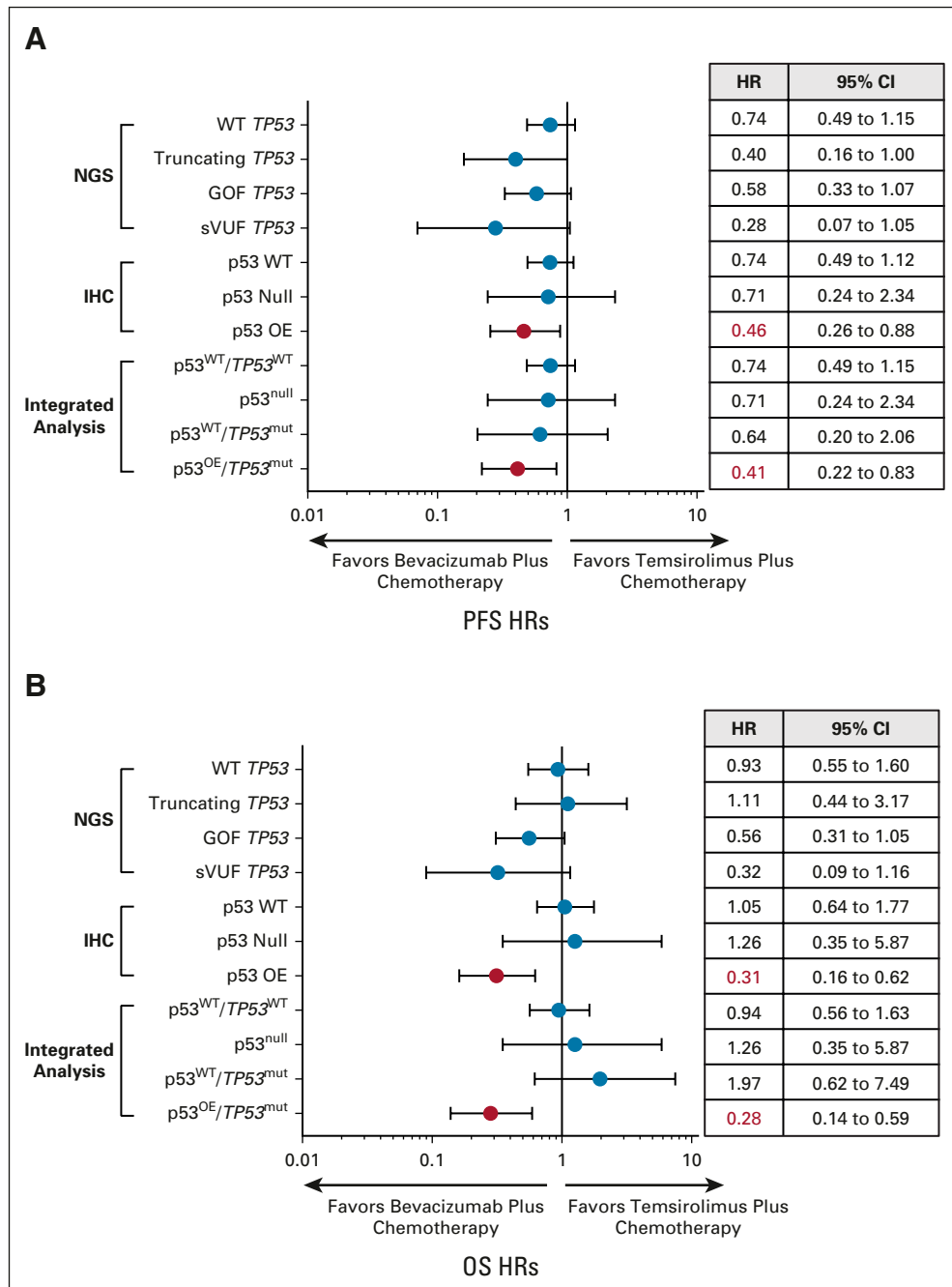


FIG 2. Comparison of HRs for bevacizumab plus chemotherapy versus temsirolimus plus chemotherapy by *TP53* mutational status, expression by IHC, or integrated sequencing/IHC analysis. Forest plots of (A) PFS and (B) OS. Also show are HR and 95% CI. Significantly improved HRs are indicated in red. GOF, gain of function; HR, hazard ratio; IHC, immunohistochemistry; NGS, next-generation sequencing; OS, overall survival; PFS, progression-free survival; sVUF, somatic variants of unknown function; WT, wild-type.

analysis scheme to account for both IHC and *TP53* sequence variables (Fig 4A).

Similar to an analysis by IHC alone, the integrated category with OE p53 by IHC and mutation in *TP53*, designated the p53^{OE}/*TP53*^{mut} category, had worse OS as compared with cases with WT p53, although there was not a significant difference in PFS (Fig 4B, compare blue and teal lines; HR

PFS: 0.75, 95% CI, 0.53 to 1.08; HR OS: 0.58, 95% CI, 0.39 to 0.86). However, both PFS and OS were increased for cases in the p53^{OE}/*TP53*^{mut} category when treated with bevacizumab plus chemotherapy versus temsirolimus plus chemotherapy: PFS HR = 0.41 (95% CI, 0.22 to 0.83) and OS HR = 0.28 (95% CI, 0.14 to 0.59, Fig 4C). The improvement noted for this group of patients remains

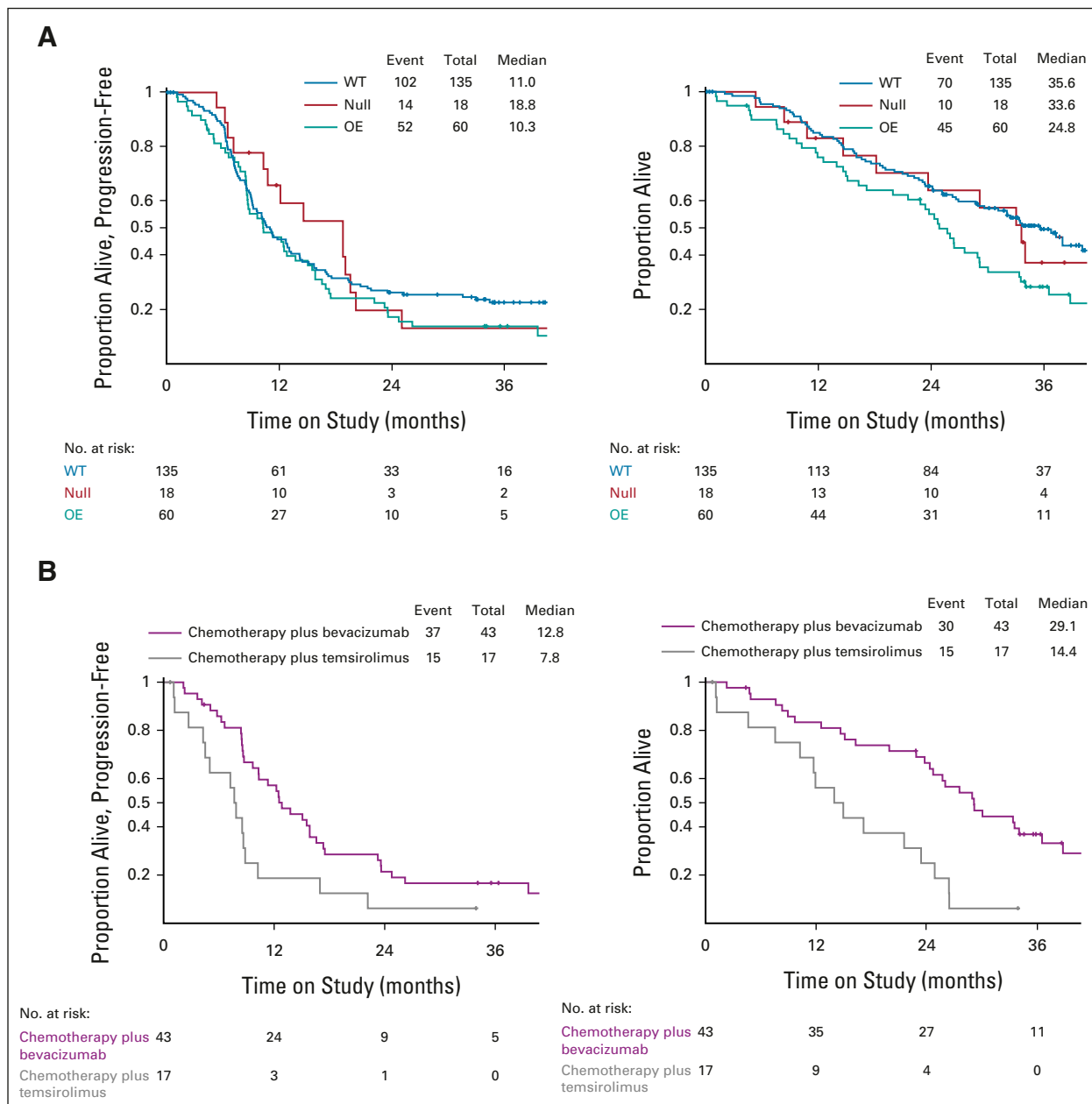


FIG 3. p53 overexpression by IHC predicts for response to bevacizumab plus chemotherapy. (A) Kaplan-Meier curves for PFS (left) and OS (right) on the basis of p53 status by IHC. (B) Kaplan-Meier curves for PFS (left) and OS (right) for cases with p53 overexpression by IHC by treatment arm. IHC, immunohistochemistry; OE, overexpressed; OS, overall survival; PFS, progression-free survival; WT, wild-type.

significant whether the two bevacizumab arms (arms 1 and 3) are combined when compared with the temsirolimus arm (arm 2; Fig 4C and Data Supplement).

DISCUSSION

To date, it has been unclear which patients with endometrial cancer benefit from the addition of bevacizumab to chemotherapy. Patients whose tumors harbor mutated p53 have worse outcomes in general,^{8,9,19,20} and better treatments are needed for these individuals. We have previously reported in the GOG-86P study that while overall there was

no statistically significant PFS benefit of adding bevacizumab to chemotherapy compared with historical controls, patients whose tumors harbored mutant *TP53* benefited significantly from bevacizumab when added to chemotherapy in the upfront setting when compared with the nonbevacizumab experimental arm.⁸ But what is the best test to identify the cases with mutated p53? This question is especially timely given the integration of molecular stratification of endometrial cancer to prospectively assign patients to treatments in the PORTEC-4a trial.^{21,22} To our knowledge, this trial is a landmark trial for endometrial cancer because it is the first to integrate clinical and

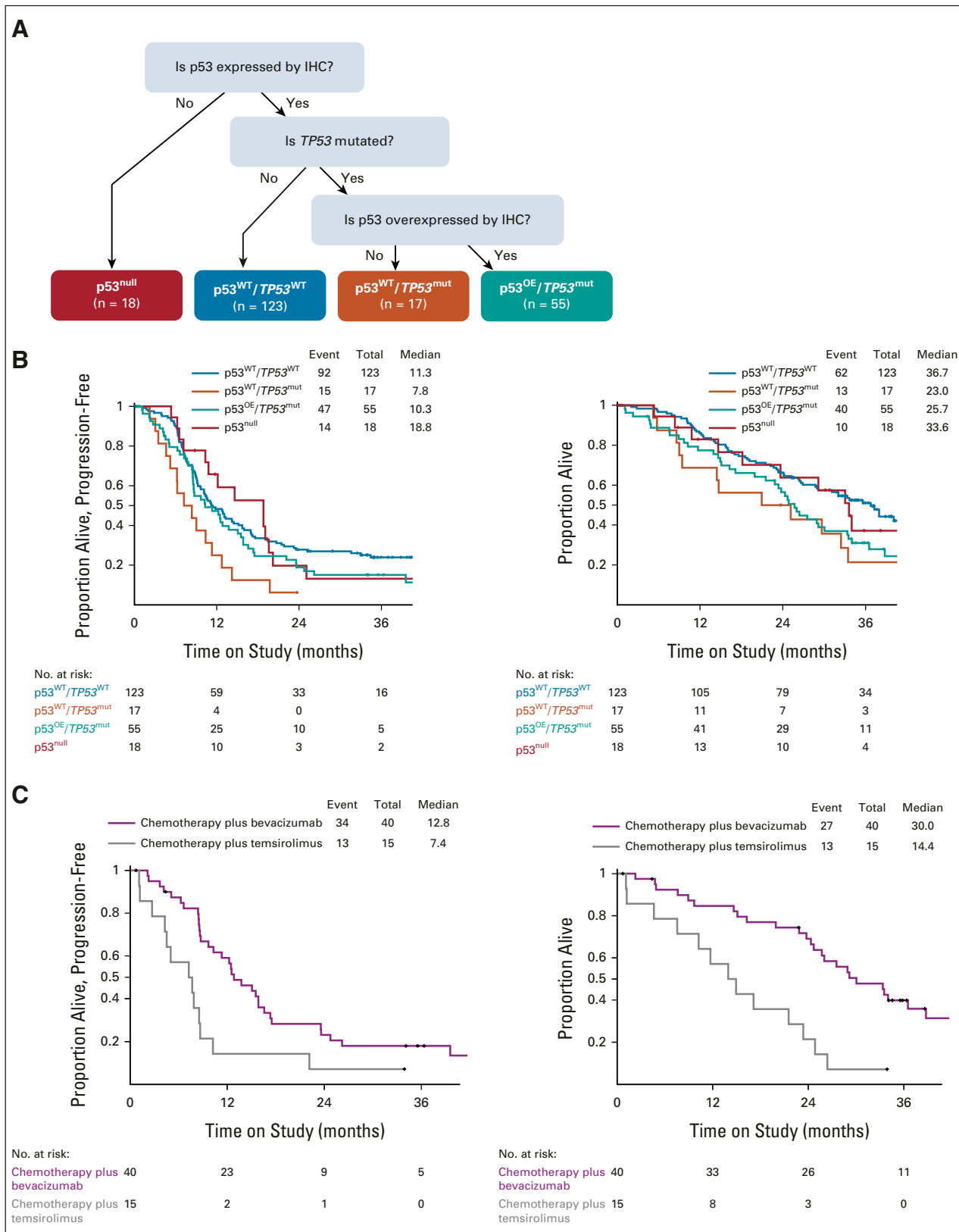


FIG 4. Integrated analysis of p53 using IHC and sequencing data improves the prediction of response to bevacizumab plus chemotherapy. (A) Decision tree for integrated analysis of p53 by IHC and sequencing. Kaplan-Meier curves for (B) PFS and (C) OS for the integrated categories independent of treatment. Kaplan-Meier curves for (D) PFS and (E) OS for the integrated category of p53^{OE}/TP53^{mut} by treatment arm. IHC, immunohistochemistry; OE, overexpressed; OS, overall survival; PFS, progression-free survival.

molecular prognostic factors to select adjuvant therapy, and p53 status is one of four molecular markers. The functional status of p53 in a tumor can be inferred by sequence analysis and/or by IHC.²³ Herein, we sought to determine whether p53 IHC was predictive of *TP53* mutations using sequence analysis and whether p53 IHC alone or integrated with *TP53* sequencing was similarly predictive of outcome. By so doing, we hope to guide the most predictive yet feasible methodology to assess the p53 status of gynecologic tumors given the growing evidence that tumors with mutated p53 deserve special consideration with respect to therapy.^{8,19,20}

The most important finding from this study was the identification of a potential new biomarker (p53 status) identifying a subset of patients on GOG-86P who benefited significantly from the addition of bevacizumab to upfront chemotherapy. p53 IHC alone had significant discriminatory power, with a PFS HR of 0.46 and an OS HR of 0.31 favoring bevacizumab arms in those with overexpression. On integrated analysis using both NGS and IHC platforms, patients whose tumors harbored a *TP53* mutation by NGS accompanied by p53 protein overexpression by IHC (p53^{OE}/*TP53*^{mut}) were found to derive substantial benefits. In particular, the OS HR of 0.28 reflects a durable, long-term improvement in survival when bevacizumab is added to chemotherapy as a frontline treatment, with a median OS of 30.0 months as compared with 14.4 months in the chemotherapy plus temsirolimus arm.

Overall, concordance between *TP53* mutation status and IHC was very good at 88% overall. Several studies in endometrial and other cancer types have demonstrated a strong association of p53 staining and mutational status, with a concordance of over 90%.²⁴⁻²⁶ There are a number of legitimate biologic explanations for why sequence and IHC results may not be 100% concordant in 24 discordant cases, including the presence of a *POLE* mutation in the tumor or MMRd (see also the Data Supplement). Indeed, the concordance rate was enhanced to 92% when the six discrepant cases with a *TP53* mutation and either a *POLE* mutation or MMRd were excluded from the analysis.

Our findings are in line with the molecular stratification of endometrial cancer as set forth in the ProMisE algorithm (Proactive Molecular Risk Classifier for Endometrial Cancer)¹⁹ and a similar classifier developed by McAlpine and colleagues.²⁷ Cases are first assessed for MMRd by IHC for MMR proteins PMS2 and MSH6, followed by identification of *POLE* hotspot mutations by NGS. The remaining cases are subjected to IHC for p53 and binned as either abnormal (p53abn) or WT. If IHC for p53 is not definitive, then NGS for mutations is performed whereby cases with mutated p53 are classified as p53abn.¹⁹ WT p53 cases that have no *POLE* mutations or MMRd are designated as no specific molecular profile or NSMP. The ProMisE algorithm treats both null/truncating p53 cases the same as OE/missense mutations. Our analysis of p53 goes a step

further and subdivides the p53abn cases by the expression level of p53 by IHC (null, WT, or OE) and the presence or absence of a *TP53* mutation, whether it be a canonical GOF mutation or an sVUF. Note that truncating mutations were mutually exclusive from p53 overexpression by IHC (Fig 1E).

A limitation of our study is the inability to comment on the specific impact of truncating mutations in *TP53* and/or null cases by IHC because of the low number of such cases. Subsequent studies of p53 null cases are warranted, given that previous studies in ovarian cancer demonstrated that loss of p53 portends poorer outcomes.^{28,29} Other limitations of this study include that not all patients on the trial had available IHC and/or NGS data, which creates a potential for bias. In addition, the GOG-86P Protocol (online only) did not include a chemotherapy-only arm, and no IHC or NGS data are available from the trial GOG-209, which served as the historical chemotherapy alone control for PFS and OS.

While these data are the product of a retrospective, secondary analysis and require prospective confirmation, our findings lend support to a protocol for the assessment of tumor p53 status. A stepwise approach to tumor evaluation could be instituted as outlined in Figure 5, wherein advanced endometrial cancers are first subjected to IHC for p53. If p53 is OE, a patient with the potential to benefit significantly from bevacizumab plus chemotherapy upfront is identified. However, the integration of sequence and IHC provides a slightly better prediction if both tests can be performed, particularly in those rare cases where immunostaining reveals an unusual pattern such as cytoplasmic or highly heterogeneous p53 expression. In the event that sequencing is not available as an option to the patient (eg, lack of insurance reimbursement), p53 IHC alone may be sufficient to predict for sensitivity to bevacizumab-containing chemotherapy. The ease of rapid implementation of our findings is bolstered by the standardized reporting guidelines for p53 IHC. Although the minimum percentage of tumor cells stained for a diagnosis of overexpression has varied from study to study (from 60% to > 90%), most studies emphasize the importance of the uniformity of staining intensity. Further studies have suggested that diffuse staining of uniform intensity (usually strong) is very well correlated with mutation. We selected a high threshold for this publication, using 80% or more positive nuclear staining for p53 as the definition of OE. Despite excellent concordance between mutational data and p53 immunophenotype, concordance is not perfect. We also acknowledge that the interpretation of p53 staining results can be subject to intra- and interobserver variation. A recent multi-institutional study of 50 cases stained and interpreted in three different laboratories identified eight cases, or about 16%, without uniform p53 results.³⁰ In addition, four cases from one institution were weakly stained but diffuse in distribution, leading to an erroneous diagnosis of WT p53 expression. This type of error was mitigated in this study by having all stains performed in a single laboratory. Future studies will

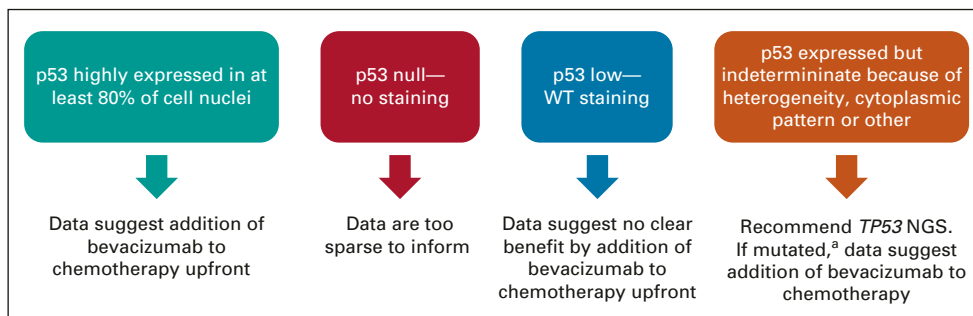


FIG 5. A potential clinical management schema supported by the findings from GOG-86P. For ease and feasibility, tumor testing for p53 status can begin with standard IHC. If p53 is highly expressed in the nuclei of 80% or more of tumor cells, our data indicate that bevacizumab, when added to chemotherapy in the upfront setting, significantly improves PFS and OS. If the immunostaining is negative entirely, or null, we cannot make a strong recommendation because of the low number of cases, but we are aware that some p53 null cases appeared to benefit on the bevacizumab arms of GOG-86P. Therefore, we recommend weighing the risk of bevacizumab, including hypertensive complications, in each individual patient before adding bevacizumab to upfront chemotherapy. If immunostaining is modest and not uniform, this suggests a WT p53 expression pattern. Our data suggest no clear benefit by adding bevacizumab to chemotherapy upfront. If p53 immunostaining is indeterminate, which may occur due to high cellular heterogeneity of p53 expression, cytoplasmic instead of nuclear staining or other conditions, we recommend sequencing *TP53*. If results reveal a mutation (other than the known SNP at P72), consideration should be given for adding bevacizumab to chemotherapy upfront. Note that tumors that coexpress *POLE* and *TP53* mutations or potentially those with microsatellite instability or mismatch repair deficiency comprise a special category, and at this time, the data do not inform management. IHC, immunohistochemistry; OS, overall survival; PFS, progression-free survival; SNP, single nucleotide polymorphism; WT, wild-type.

need to refine how cases with high cytoplasmic staining or focal nuclear overexpression of p53 in the setting of WT staining should be binned (Fig 1A). The functional consequence of these expression patterns as they relate to treatment prediction remains undefined. However, if sequencing can be performed specifically on cases with indeterminate p53 by IHC, and a missense mutation in *TP53* is identified, patients could be offered bevacizumab plus chemotherapy upfront. Hence, if not available for all cases, sequencing may be a useful and predictive second-tier test specifically when IHC is indeterminate.

In conclusion, our findings provide a logical framework for the assessment of p53 by IHC and NGS in routine

molecular stratification of endometrial cancer. Our study has the potential to change practice for advanced endometrial cancer by emphasizing (1) the strong correlation between highly overexpressed p53 protein on IHC and the presence of a missense mutated *TP53* sequence; (2) the usefulness of IHC as a cost-saving initial study that is predictive of bevacizumab plus chemotherapy benefit in the majority of cases; and (3) the use of *TP53* sequencing as a means to understand p53 status when immunostaining is indeterminate. As these findings are the result of a retrospective analysis of tissues from GOG-86P, we look forward to the opportunity to confirm our findings in future prospective clinical trials.

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CLINICAL TRIAL INFORMATION

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**TP53 Sequencing and p53 Immunohistochemistry Predict Outcomes When Bevacizumab Is Added to Frontline Chemotherapy in Endometrial Cancer: An NRG Oncology/Gynecologic Oncology Group Study**

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APPENDIX 1. GYNECOLOGIC ONCOLOGY GROUP

The following Gynecologic Oncology Group member institutions participated in the primary treatment studies: University of Oklahoma Health Sciences Center, Washington University School of Medicine, Duke University Medical Center, University of California Irvine Medical Center, University of Iowa Hospitals and Clinics, Women and Infants Hospital, Ohio State University Comprehensive Cancer Center, Rush University Medical Center, Walter Reed National Military Medical Center, University of Cincinnati, Cleveland Clinic Foundation, The Hospital of Central Connecticut, UCSF-Mount Zion, Memorial Sloan Kettering Cancer Center, Mayo Clinic, Cancer Research for the Ozarks NCORP, University of Texas Southwestern Medical Center, Georgia Center for Oncology Research and Education (CORE), Northwestern University, University of North Carolina at Chapel Hill, MD Anderson Cancer Center, University of Wisconsin Hospitals and Clinics, Roswell Park Cancer Institute, University of Colorado Cancer Center – Anschutz Cancer Pavilion, Women's Cancer Center of Nevada, University of Hawaii, Abington Memorial Hospital, University of Mississippi Medical Center, State University of New York Downstate Medical Center,

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Supplemental Materials

Supplemental Methods

Supplemental Discussion

Supplemental Table S1: Distribution of cases by race and ethnicity.

Supplemental Figure S1: Kaplan-Meier curves for (A) PFS and (B) OS for serous and non-serous histologies.

Supplemental Figure S2: Kaplan-Meier curves for PFS for serous and non-serous histologies by treatment arm.

Supplemental Figure S3: Kaplan-Meier curves for PFS for serous and non-serous histologies by treatment arm.

Supplemental Figure S4: *TP53* GOF mutations portend poorer outcomes as compared to cases with WT *TP53*.

Supplemental Figure S5: Kaplan-Meier curves for PFS for *TP53* mutations by treatment arm.

Supplemental Figure S6: Kaplan-Meier curves for OS for *TP53* mutations by treatment arm.

Supplemental Figure S7: Kaplan-Meier curves for PFS for p53 expression by IHC by treatment arm.

Supplemental Figure S8: Kaplan-Meier curves for OS for p53 expression by IHC by treatment arm.

Supplemental Figure S9: Hazard ratios for integrated analysis by treatment arm.

Supplemental Figure S10: Kaplan-Meier curves for PFS for the indicated integrated categories by treatment arm.

Supplemental Figure S11: Kaplan-Meier curves for OS for the indicated integrated categories by treatment arm.

SUPPLEMENTAL METHODS

Study cohort

GOG-86P (NCT 00977574) was a three-arm randomized phase II study of paclitaxel/carboplatin/ bevacizumab (NSC#704865, IND#7921), paclitaxel/carboplatin/temsirolimus (NSC#683864, IND#61010) or ixabepilone (NSC#710428, IND# 59699)/carboplatin/bevacizumab as initial therapy for measurable stage III or IVA, stage IVB, or recurrent endometrial cancer. The control group for GOG-86P was historical, from a subset of patients assigned to the paclitaxel/carboplatin (PC) arm of GOG-209 (NCT 00063999).^{1,2} The patients enrolled on GOG-209 used as historical controls had similar disease characteristics compared to those enrolled on GOG-86P.¹⁻³ The design of GOG-86P was based on PFS estimates from GOG-177 paclitaxel/doxorubicin/cisplatin (TAP) arm, in which median PFS was 8.3 months.⁴

For GOG-86P, the primary endpoint was PFS, defined as the time alive and progression-free from the date of study entry. Secondary endpoints included OS and best confirmed response using RECIST 1.1. The frequency and severity of acute adverse effects was graded according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0. For further details related to study design, please refer to Aghajanian et al.³ Biospecimens were collected from patients who consented to participate in the translational research component of the study. Collection of archival formalin fixed paraffin embedded (FFPE) tissue and tumor DNA was coordinated by the NRG Oncology Biospecimen Bank.

The total number of patients enrolled on GOG-86P was 349. From these cases, 213 tumors were analyzed using IHC for p53 and NGS for *TP53*. NGS methodology has been previously reported in detail.⁵ The distribution of p53 expression (IHC) and mutational status (*TP53* NGS) by race and ethnicity are provided in **Supplemental Table S1**. Tumor NGS data, IHC and histology were correlated with each other and with clinical outcomes in the chemotherapy + bevacizumab arms versus the chemotherapy + temsirolimus arm; tumors from historical control cases receiving chemotherapy from GOG-209 were not available for sequencing or IHC analysis.

TP53 Mutation Classification Criteria

TP53 mutations by NGS were grouped into four categories based on likely functional consequence: (1) Wild Type (WT) were those mutations that did not result in an amino acid substitution, (2) frameshift or splice site mutations that result in a truncated protein (3) missense mutations that are canonical “gain of function” (GOF) mutations, and (4) unclassified missense mutations or indels, referred to herein as “somatic variants of unknown function” (sVUFs). GOF missense mutations were identified from previous reports in the literature documenting the acquisition of an oncogenic phenotype when the mutations were expressed in preclinical models. Specifically, eight missense mutations were considered GOF according to published criteria detailed in Brachova et al.: P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L and R282W.⁶⁻⁸

Immunohistochemistry

The immunohistochemical staining method has been previously described.⁹ Briefly, antigen retrieval was performed using citrate pH 6.00 using heat (microwave oven) at 100°C for 4 min. Five-micrometer sections of FFPE sections were stained with monoclonal p53 antibody (DO-7, Dako, pre-diluted). Antibody–antigen complexes were detected using DAB I-view detection kit (Ventana, Tucson, AZ) following the manufacturer's instructions.

Three simplified categories were used to describe immunohistochemistry for p53 (**Figure 1A**): 1) wild type (WT): 1-80% of tumor cell nuclei staining positive, usually with variable intensity; 2) null: no tumor cell nuclear staining with a positive internal control; and 3)

overexpressed (OE): uniform and intense nuclear staining in at least 80% of tumor cell nuclei (estimated). Most, but not all, cases could be assigned to one of these three categories. However, as shown in **Figure 1A**, some cases did not fall into these simplified groups. Cases were scored as equivocal when there was no nuclear signal in the absence of a positive internal control. Cases scored as focal had overall WT staining but with focal areas of clear p53 overexpression, indicating heterogeneity with respect to p53 expression. A secondary pathologic review was performed by primary reading pathologist (R.A.S.), who was blinded to the original reads, for cases in which the p53 IHC data were discordant with the *TP53* sequencing data, e.g., mutated *TP53* but WT p53 by IHC.

Histology

Tumor histology and grade was determined from central pathologic review and classified as endometrioid grades 1, 2, or 3, serous, clear cell, mixed epithelial, undifferentiated, adenocarcinoma not otherwise specified, mucinous, squamous or other. The determination of serous histology was made per the NRG Central Pathology Committee protocol, which is consistent with the newer World Health Organization guidelines published in 2020 (WHO Classification of Tumours Editorial Board. Female genital tumors. Lyon (France): International Agency for Research on Cancer; 2020, (WHO classification of tumours series, 5th ed.; vol 4, page 256).

Integrated analysis of p53 IHC and *TP53* NGS

A decision tree for integrated analysis of p53 IHC and *TP53* NGS was generated (see **Figure 4A**). First, cases were analyzed by p53 IHC expression. Those with no detectable p53 staining were categorized as p53 null regardless of *TP53* mutational status (p53^{null}). Of the cases with p53 expression by IHC, cases were further divided based on the presence or absence of a mutation in *TP53*. Cases with no mutation and WT expression of p53 were categorized as p53 WT (p53^{WT}/*TP53*^{WT}). The remaining cases with mutated *TP53* were subdivided based on p53 IHC into two categories: WT p53 protein but mutated *TP53* sequence (p53^{WT}/*TP53*^{mut}) and OE p53 protein with mutated *TP53* (p53^{OE}/*TP53*^{mut}). Note that every case in the p53^{OE}/*TP53*^{mut} category contained a missense mutation or indel in *TP53*. There were no cases with a *TP53* truncating mutation and overexpression at the protein level.

Statistical Analyses

Consistent with protocol objectives, an exploratory analysis of biomarkers on GOG-86P was performed. Data from the two bevacizumab-containing arms (Arms 1 and 3) were combined in the analyses presented herein. Patients were grouped based on tumor *TP53* mutational status, histology and p53 IHC results. Mutations in *TP53* as well as p53 IHC were assessed by histologic category. For purposes of the correlation with clinical outcomes, histology was dichotomized as serous and non-serous. Progression-free survival was defined as the time from treatment randomization to documentation of disease progression, death or last contact, whichever occurred first. Survival was defined as the time from treatment randomization until death or last contact. Outcomes (PFS and OS) were compared between treatment arms or between groups using proportional hazards models. Hazard ratio (HR) estimates and 95% profile likelihood confidence limits from these models were reported. Kaplan-Meier estimates of the time to event distributions were plotted; at risk counts, medians and event counts were included in the plots. No p-values are reported. A confidence interval that excludes a hazard ratio of 1.0 was considered evidence of a statistical relationship between the endpoint and the analysis group. Confidence intervals that included 1 did not always rule out clinically significant differences. Interpretation in the situation of low precision is made with caution when neither hypothesis can be ruled out. No correction for multiple analyses was employed. Confirmation of results is

recommended. Statistical analyses were generated using SAS/STAT software, Version 9.4 of the SAS System for Windows. Copyright © 2016 SAS Institute Inc.

SUPPLEMENTAL DISCUSSION

In this study, we evaluated the association between *TP53* mutations and p53 protein expression on outcomes. Overall, concordance between *TP53* mutation status and IHC was very good at 88% overall. Several studies in endometrial and other cancer types have demonstrated a strong association of p53 staining and mutational status, with a concordance of over 90%.¹⁰⁻¹² Among the 26 discordant cases in our study, two were data entry errors, and one mutation is now known to be a polymorphism. There are a number of legitimate biologic explanations for why sequence and IHC results may not be 100% concordant in the remaining 24 discordant cases, including the presence of a *POLE* mutation in the tumor or MSI. Indeed, the concordance rate was enhanced to 92% when the six discrepant cases with a *TP53* mutation and either a *POLE* mutation or MSI were excluded from the analysis. Such tumors may have mutations in *TP53* and in many other genes that are not functionally relevant at the protein level. Heterozygosity, intra-tumor heterogeneity, overexpression of MDM2 (the p53 ubiquitin ligase), and RNA decay are other possible explanations for lack of p53 protein expression in the absence of a truncating *TP53* mutation. Indeed, overexpression of MDM2 has been observed in cancers from other tissues and is considered an oncogenic signal.¹³ Three cases with WT *TP53* were read as overexpressed by IHC. High expression of WT p53 is notable in cells that are stressed.¹⁴ WTp53 staining with a *TP53* mutation has also been previously described in high grade serous ovarian tumors.¹⁰ The reported incidence of this combination of findings is 4.1%.¹⁰

Among the cases with sequencing and IHC results, 15 cases had *POLE* mutations, with a concurrent *TP53* mutation in one-third of the cases (N=5). Similarly, 12 of the 15 *POLE*-mutated cases expressed WT levels of p53 by IHC, including one case that had a hotspot *TP53* R248W GOF mutation. For cases with MMRd, 94% expressed WT p53 by IHC and sequencing (52 of 55 cases with MMRd). In addition, the three cases with mutated *TP53* were all WT by IHC regardless of the type of mutation (i.e., two truncating mutations and one GOF mutation). The schema of the ProMiseE algorithm is to screen first for MMRd/MSI-H and then for *POLE* mutations and p53 expression by IHC.¹⁵ The implications of co-mutations in *POLE* or MMRd with *TP53* have been recently studied.^{11,16} Interestingly, the outcomes in such cases as discerned from TCGA¹¹ and PORTEC¹⁵ data indicate a prolonged recurrence-free survival. However, the sample size in many studies,^{15,17} including ours, is not sufficient to discern if concurrent mutated/overexpressed p53 in the setting of a *POLE* mutation or MMRd/MSI-H has an impact on response to treatment. Given the relative ease of IHC as compared to NGS, the order of events in the classifier could be tested in future studies.

TP53 mutations predominate in the serous subtype of endometrial cancer.¹⁸ Thus we assessed the association of treatment and PFS and OS endpoints for patients with serous and with non-serous tumor histology. In this cohort, there was no significant difference in outcome by histology (**Supplemental Figure S1**). Relative to the temsirolimus plus chemotherapy arm, the hazard of progression or death was reduced in the bevacizumab + chemo arms for non-serous tumor cases, PFS HR was 0.74 (95% CI: 0.56 – 0.98), but less so for the hazard of death, OS HR 0.88 (95% CI: 0.64 – 1.22, **Supplemental Figures S2 and S3**). For patients with serous tumor histology the PFS HR on the bevacizumab arms relative to the temsirolimus arm was 0.61 (95% CI: 0.36 – 1.05) and for OS the HR was 0.64 (95% CI: 0.36 – 1.18, **Supplemental Figures S2 and S3**). We had hoped that histology alone, either serous or non-serous, would be another indicator of cases that would benefit from bevacizumab relative to temsirolimus + chemotherapy since cases with serous histology correlate strongly with the Cancer Genome Atlas (TCGA) cluster 4 “copy number high” endometrial cancers, the same group of tumors in which *TP53* mutations are most common.¹⁸ Unfortunately, our data indicate that histology is not by itself a sufficient predictor of bevacizumab outcomes in this setting. This is explained in part by the frequency of *TP53* mutations or overexpression of p53 in high grade

endometrioid cases, which are also included in the “copy number high” TCGA cluster.¹⁸ Classifying by high grade histology may improve prediction over cell type alone.

Why are mutations in the tumor suppressor p53 potentially relevant to bevacizumab + chemotherapy sensitivity? We propose two main hypotheses. First, cell cycle checkpoint abrogation occurs - cells with mutated p53 lack the normal G1/S cell cycle checkpoint, preventing DNA repair before cells progress into S phase.¹⁹ However, an alternative pathway downstream of p38 MAPK allows maintenance of the G2/M cell cycle checkpoint and subsequent repair of damaged DNA.²⁰ This makes cancer cells potentially more resistant to chemotherapy. In addition to the loss of WT p53 function enforcing the G1/S cell cycle checkpoint, the inhibition of downstream signaling associated with bevacizumab has the potential to abrogate G2/M cell cycle checkpoints thereby amplifying the effects of chemotherapy.²¹⁻²³ Second, angiogenesis is dysregulated. Tumors with mutated p53 enhance VEGF expression and angiogenesis, potentially making cells more vulnerable to antiangiogenics such as bevacizumab.²⁴ An additional strength of this study is that we observed that even missense mutations in *TP53* that were of unknown function and classified as sVUF, yet were overexpressed, predicted for better outcomes in the bevacizumab arms. Thus, we propose that protein overexpression is a signal of oncogenic GOF activity in tumors with missense *TP53* mutations.

REFERENCES FOR SUPPLEMENTAL METHODS & DISCUSSION

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Supplemental Table S1: Distribution of cases by race and ethnicity. To examine the association between race/ethnicity and p53 categorization, global X² tests were applied. However, no p-value reached a significance level of 0.05 or even 0.10. The tables were sparse and X² tests may not be valid.

p53 protein expression (IHC) Group

<i>Race and Ethnicity by IHC Group</i>	NULL		OE		WT		Total	
	N	%	N	%	N	%	N	%
Race								
Not specified	2	11.1	1	1.7	5	3.7	8	3.8
Asian	1	5.6	1	1.7	5	3.7	7	3.3
Black/African American	5	27.8	9	15.0	11	8.1	25	11.7
Am Indian/Alaskan Native	0	0	1	1.7	1	0.7	2	0.9
White	10	55.6	48	80.0	113	83.7	171	80.3
Ethnicity								
Hispanic or Latino	1	5.6	4	6.7	8	5.9	13	6.1
Non-Hispanic	13	72.2	49	81.7	117	86.7	179	84.0
Not Specified	4	22.2	7	11.7	10	7.4	21	9.9
Total	18	100.0	60	100.0	135	100.0	213	100.0

TP53 Mutation Group

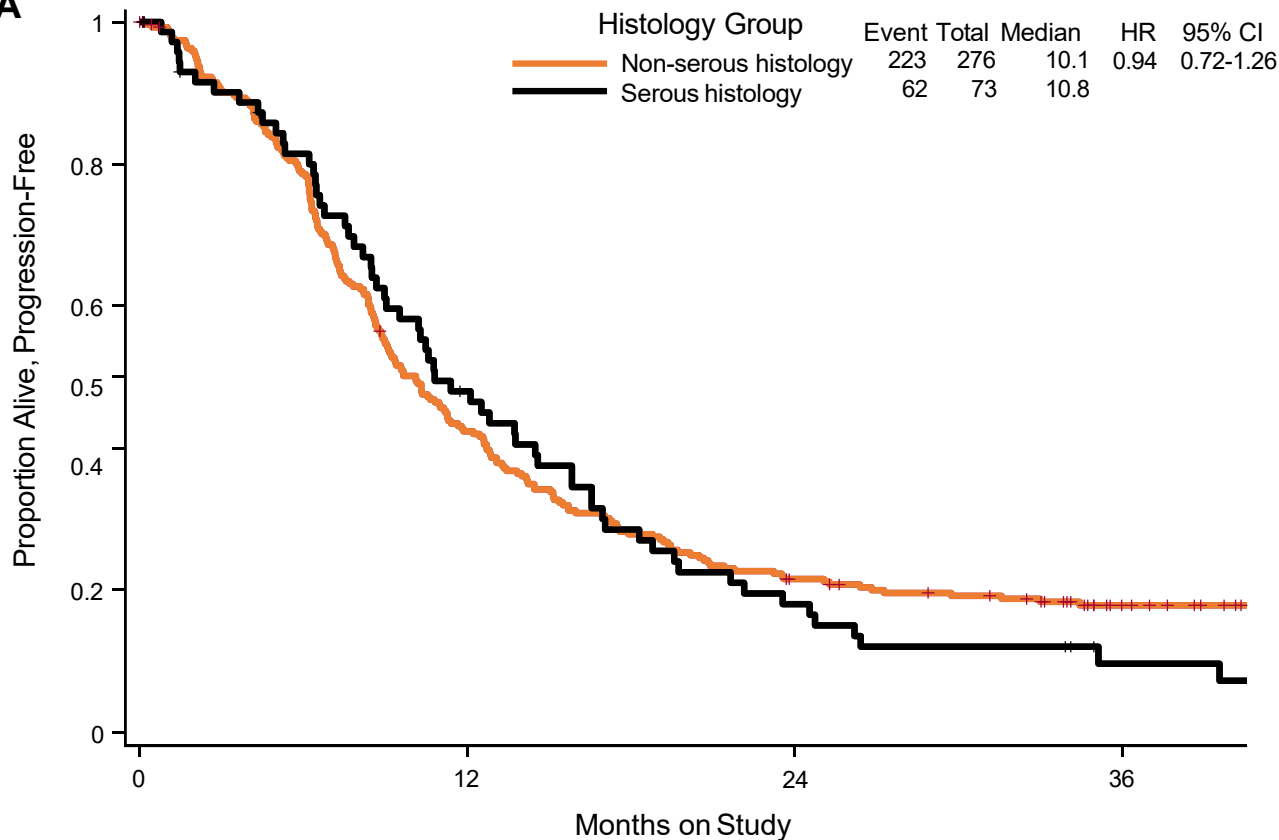
<i>Race and Ethnicity by Mutation Group</i>	GOF		Truncating		sVUF		WT		Total	
	N	%	N	%	N	%	N	%	N	%
Race										
Not specified	1	1.6	2	6.7	0	0	5	4.0	8	3.5
Asian	1	1.6	1	3.3	0	0	5	4.0	7	3.0
Black/African American	7	11.5	7	23.3	1	6.3	11	8.9	26	11.3
Am Indian/Alaskan Native	1	1.6	0	0	0	0	1	0.8	2	0.9
White	51	83.6	20	66.7	15	93.8	102	82.3	188	81.4
Ethnicity										
Hispanic or Latino	4	6.6	1	3.3	1	6.3	7	5.6	13	5.6
Non-Hispanic	50	82.0	24	80.0	11	68.8	109	87.9	194	84.0
Not Specified	7	11.5	5	16.7	4	25.0	8	6.5	24	10.4
Total	61	100.0	30	100.0	16	100.0	124	100.0	231	100.0

Integrated p53 IHC and TP53 Mutation Group

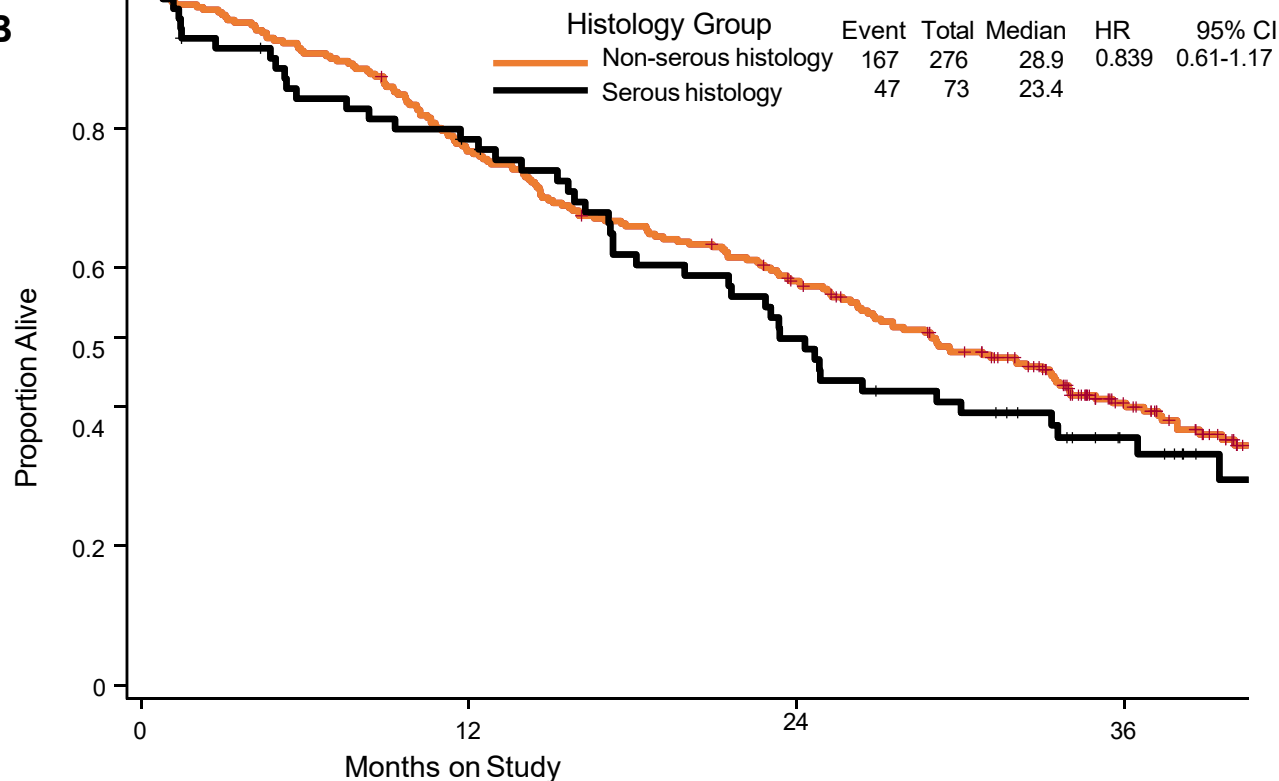
<i>Race and Ethnicity by Integrated Group</i>	p53 ^{null}		p53 ^{WT} /TP53 ^{WT}		p53 ^{WT} /TP53 ^{mut}		p53 ^{OE} /TP53 ^{mut}		Total	
	N	%	N	%	N	%	N	%	N	%
Race										
Not specified	2	11.1	5	4.1	0	0	1	1.8	8	3.8
Asian	1	5.6	4	3.3	1	5.9	1	1.8	7	3.3
Black/African American	5	27.8	11	8.9	2	11.8	7	12.7	25	11.7
Am Indian/Alaskan Native	0	0	1	0.8	0	0	1	1.8	2	0.9
White	10	55.6	102	82.9	14	82.4	45	81.8	171	80.3
Ethnicity										
Hispanic or Latino	1	5.6	7	5.7	1	5.9	4	7.3	13	6.1
Non-Hispanic	13	72.2	108	87.8	14	82.4	44	80.0	179	84.0
Not Specified	4	22.2	8	6.5	2	11.8	7	12.7	21	9.9
Total	18	100.0	123	100.0	17	100.0	55	100.0	213	100.0

Supplemental Figure S1

A

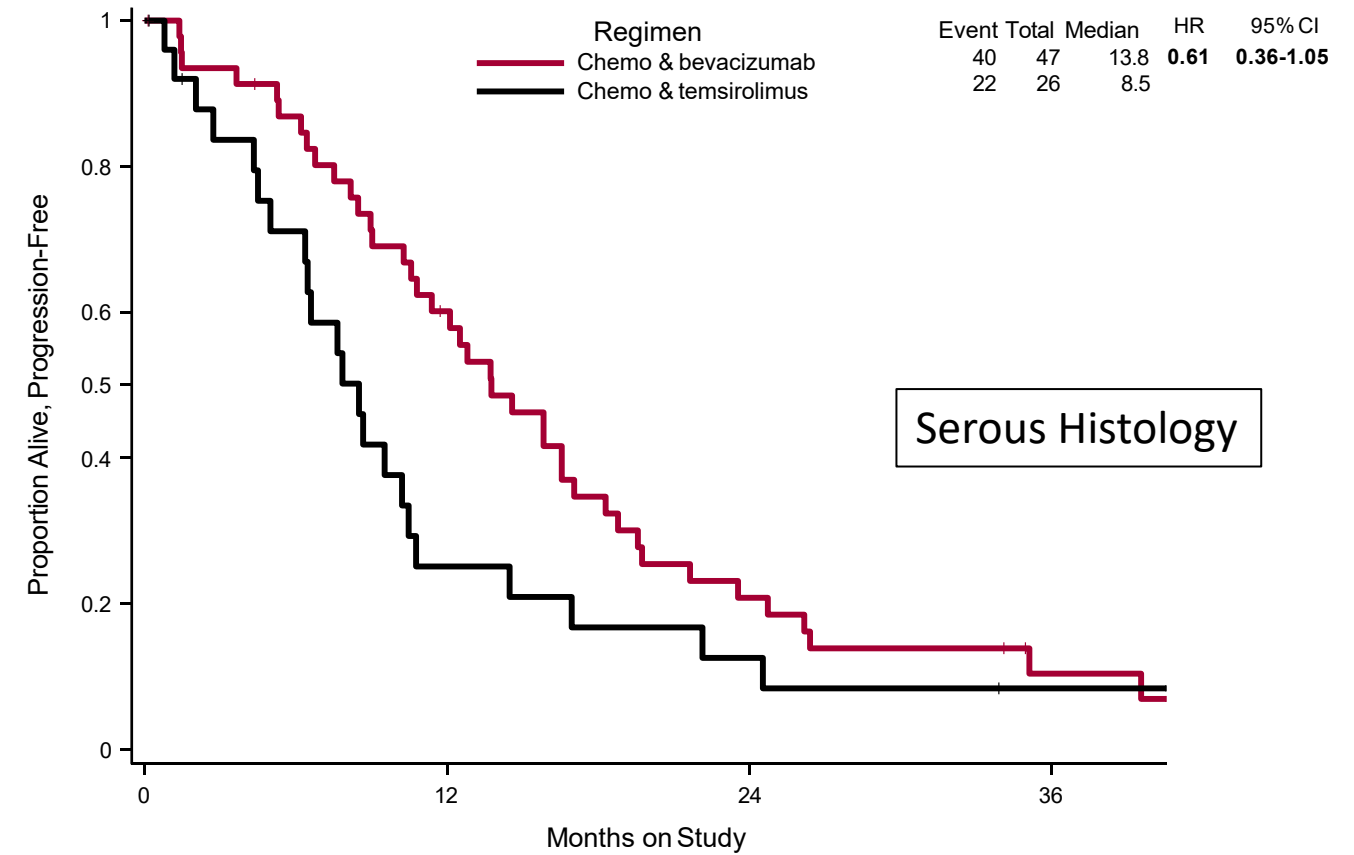
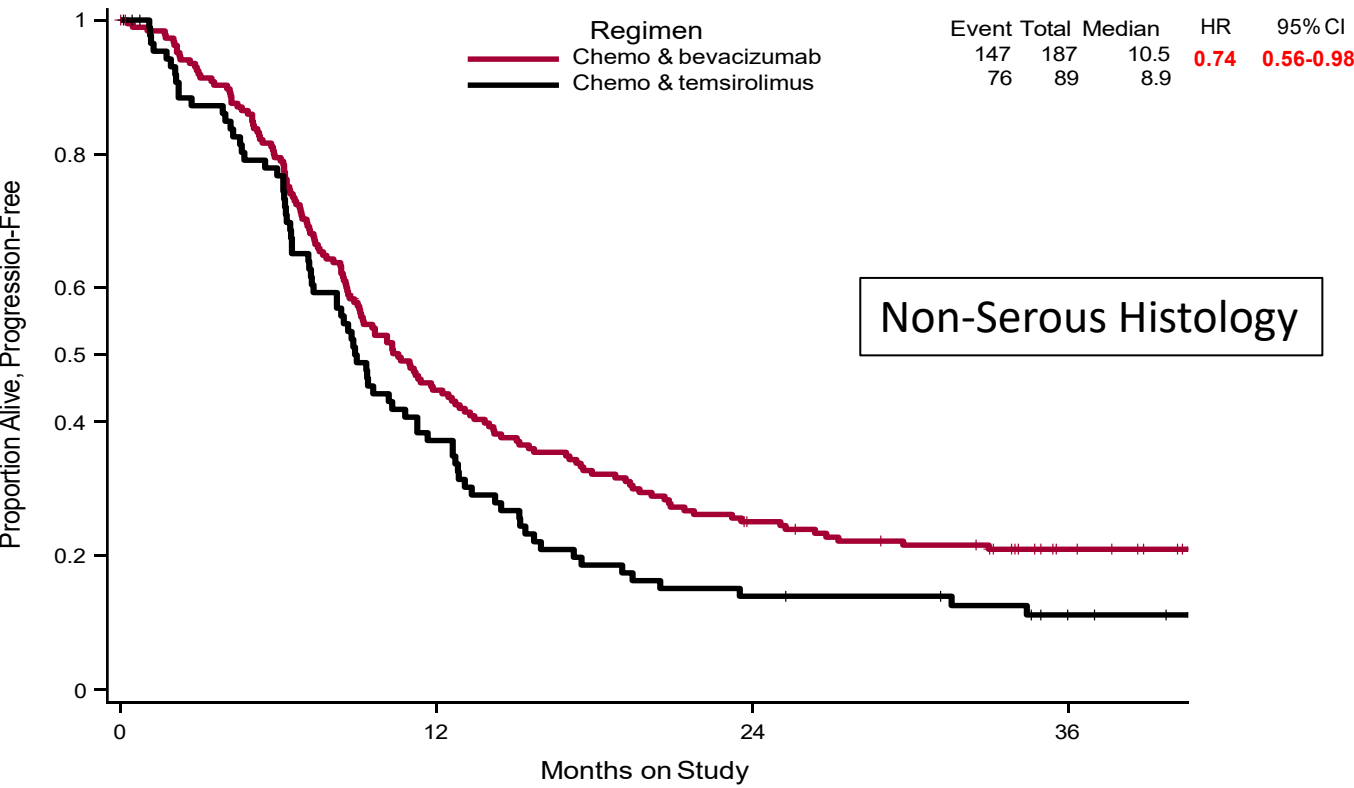


B



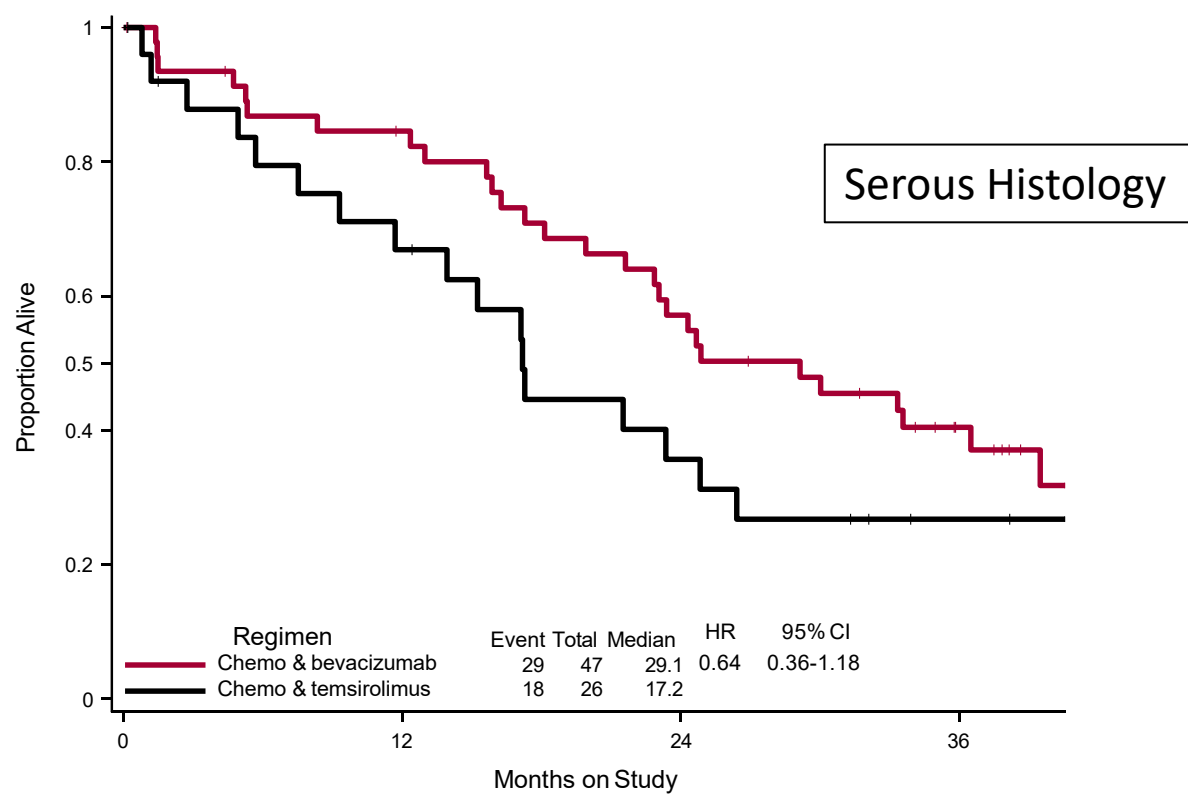
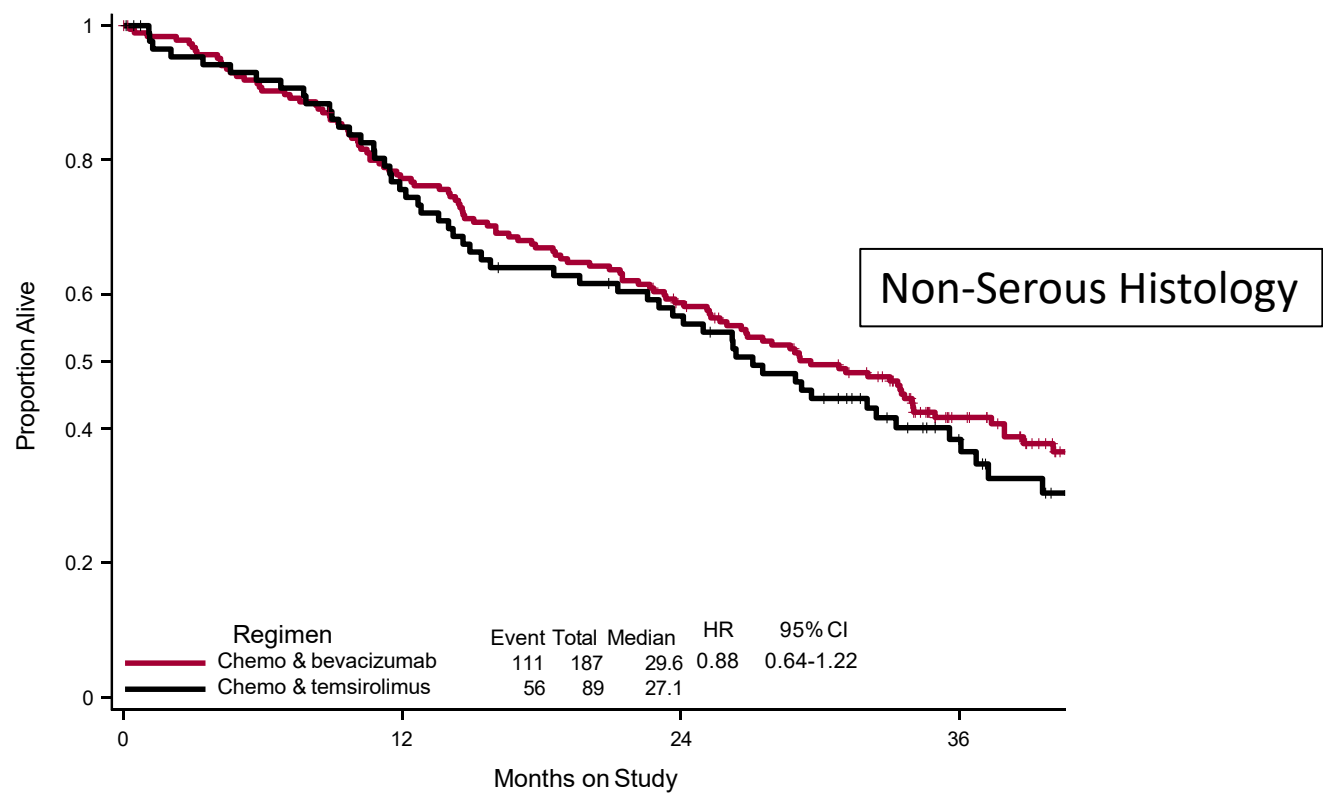
Supplemental Figure S1: Histology is not predictive of outcomes independent of treatment. Kaplan-Meier curves for (A) PFS and (B) OS for serous (*black*) and non-serous (*orange*) cases. Hazard ratios and 95% confidence intervals are provided.

Supplemental Figure S2



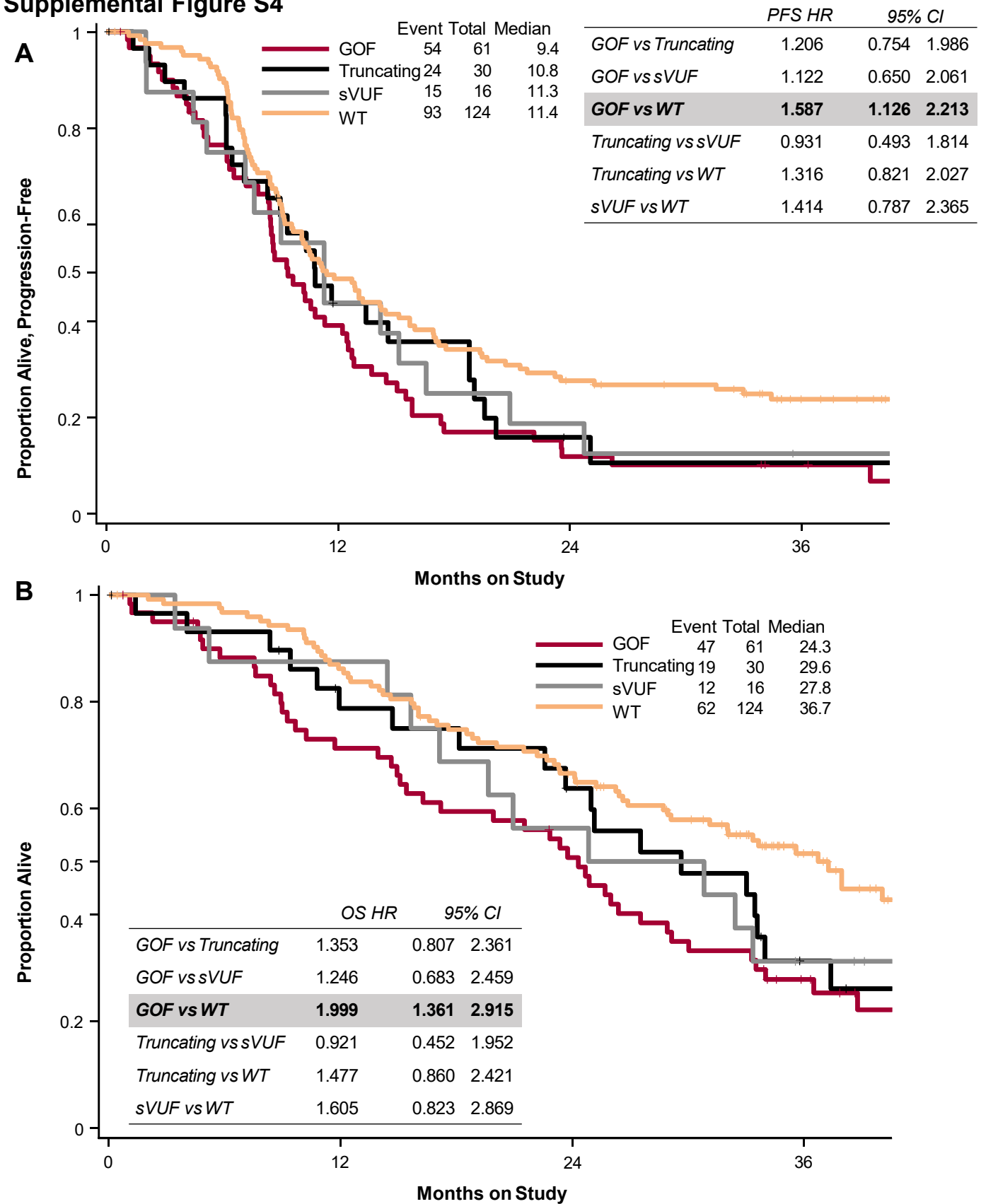
Supplemental Figure S2: Kaplan-Meier curves for PFS for serous and non-serous histologies by treatment arm.

Supplemental Figure S3



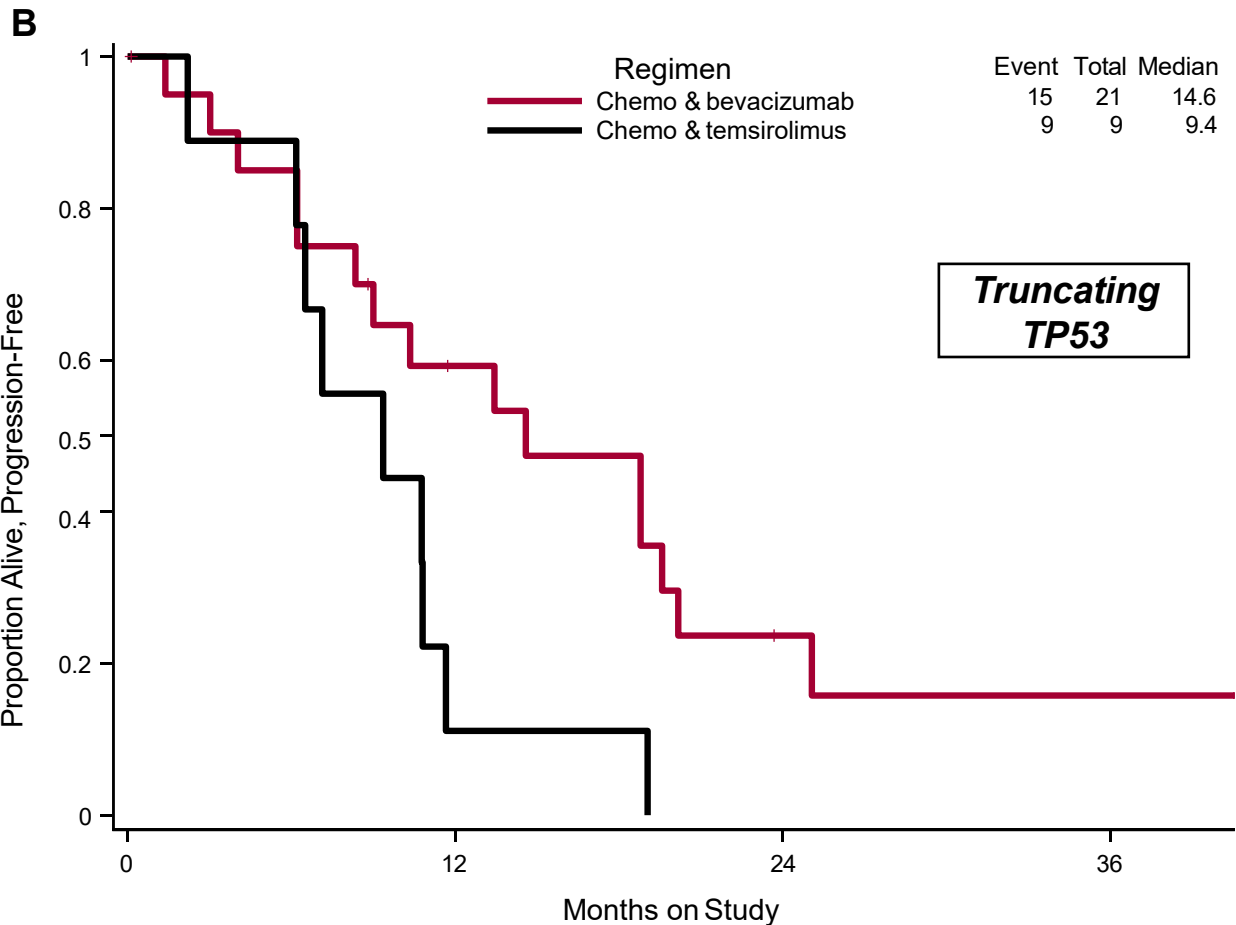
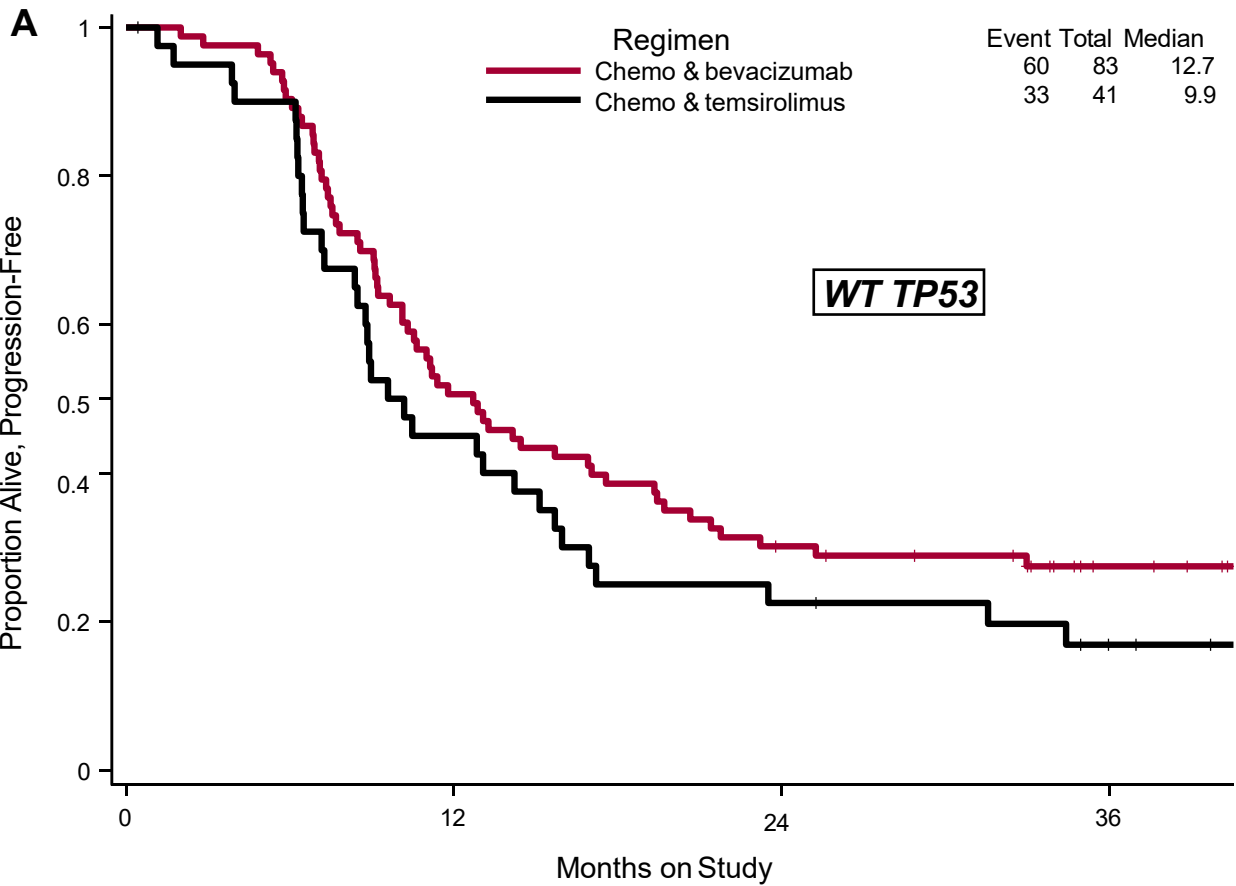
Supplemental Figure S3: Kaplan-Meier curves for OS for serous and non-serous histologies by treatment arm.

Supplemental Figure S4

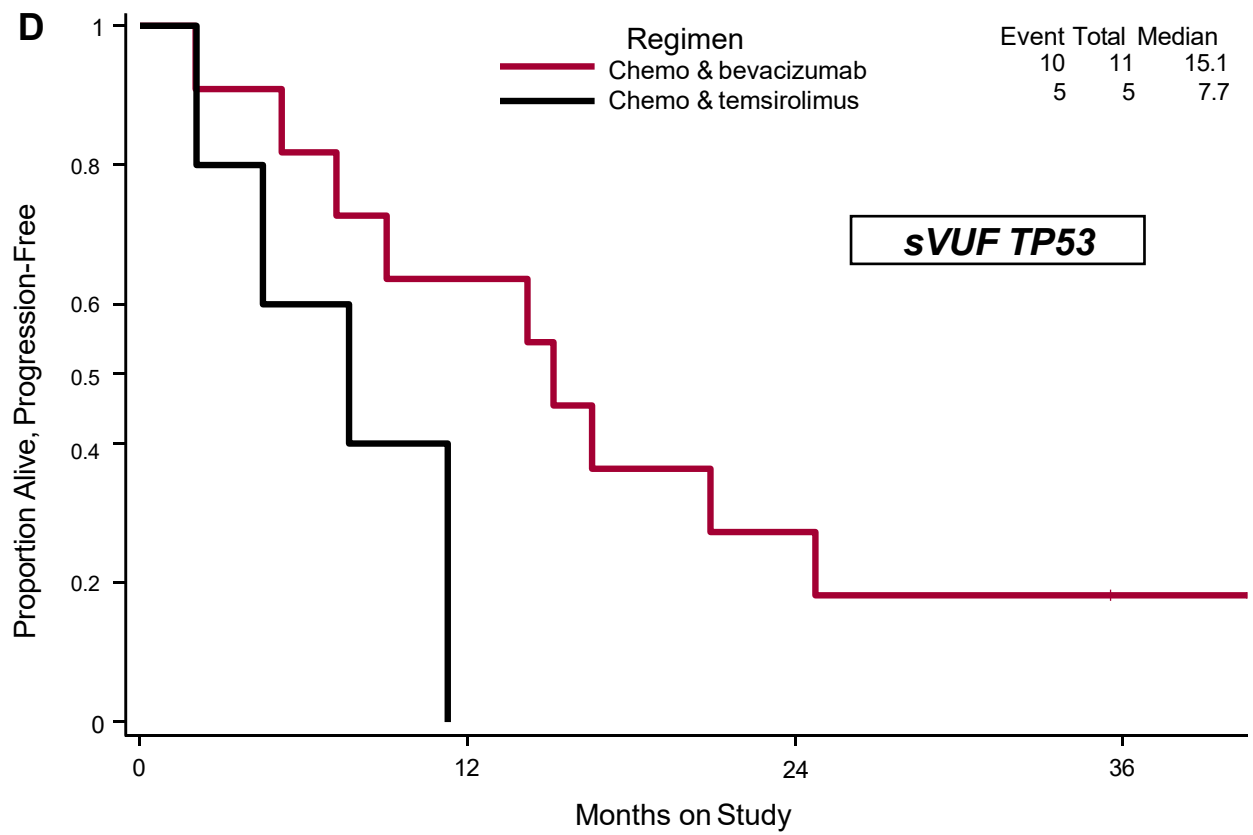
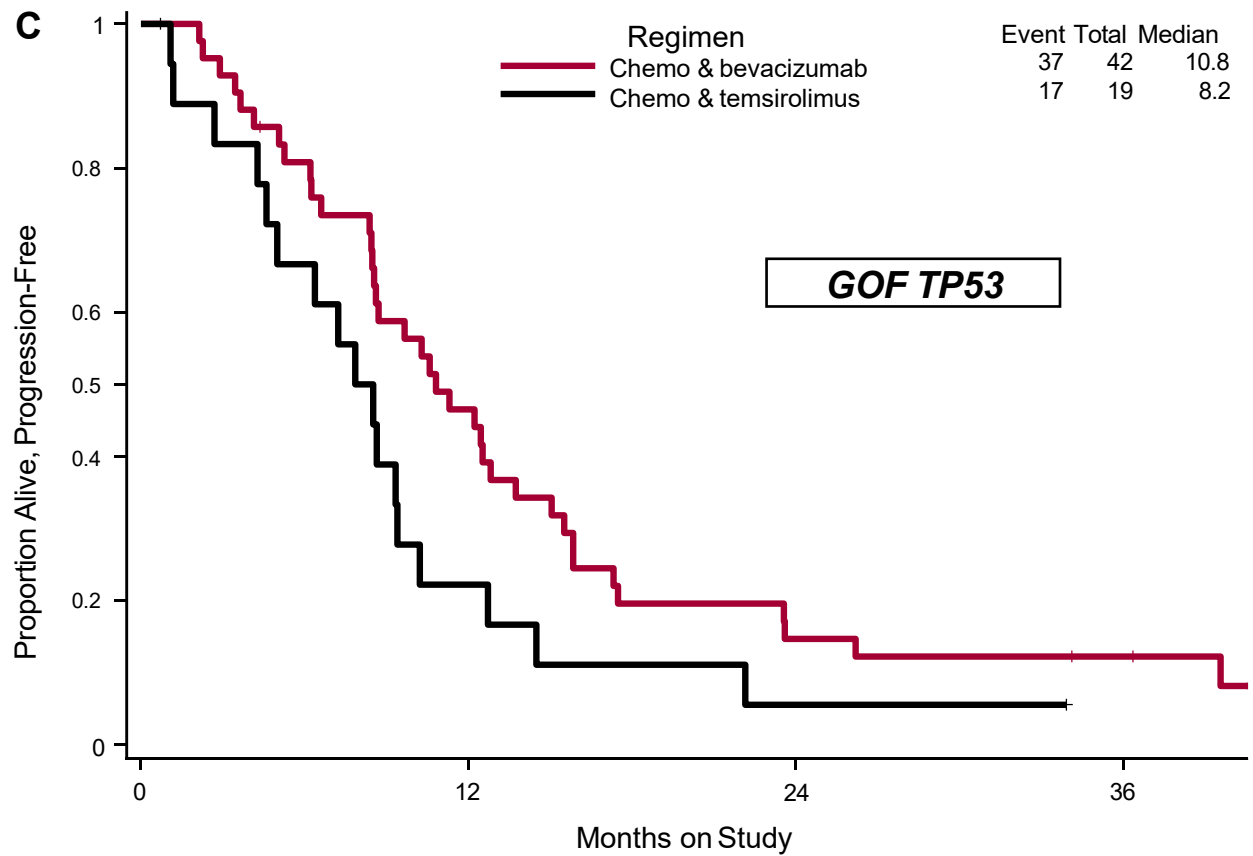


Supplemental Figure S4: *TP53* GOF mutations portend poorer outcomes as compared to cases with WT *TP53*. Kaplan-Meier curves for (A) PFS and (B) OS based on *TP53* mutational subtypes. **WT**: wild-type. **Truncating**: mutations that result in a premature stop codon and therefore loss of protein expression. **GOF**: gain-of-function missense mutation. **sVUF**: somatic variant of unknown function – a missense mutation that is not a GOF mutation.

Supplemental Figure S5



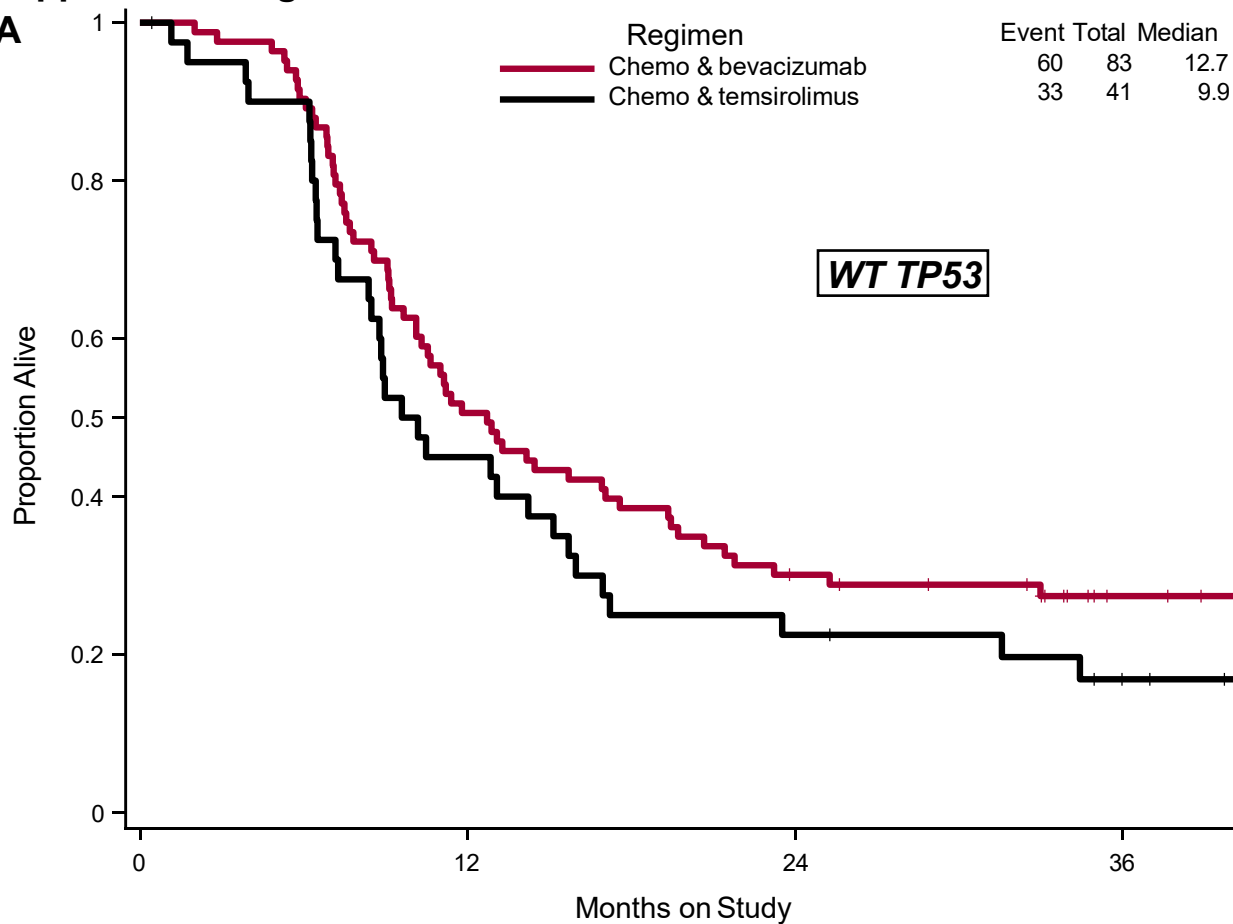
Supplemental Figure S5 (con't)



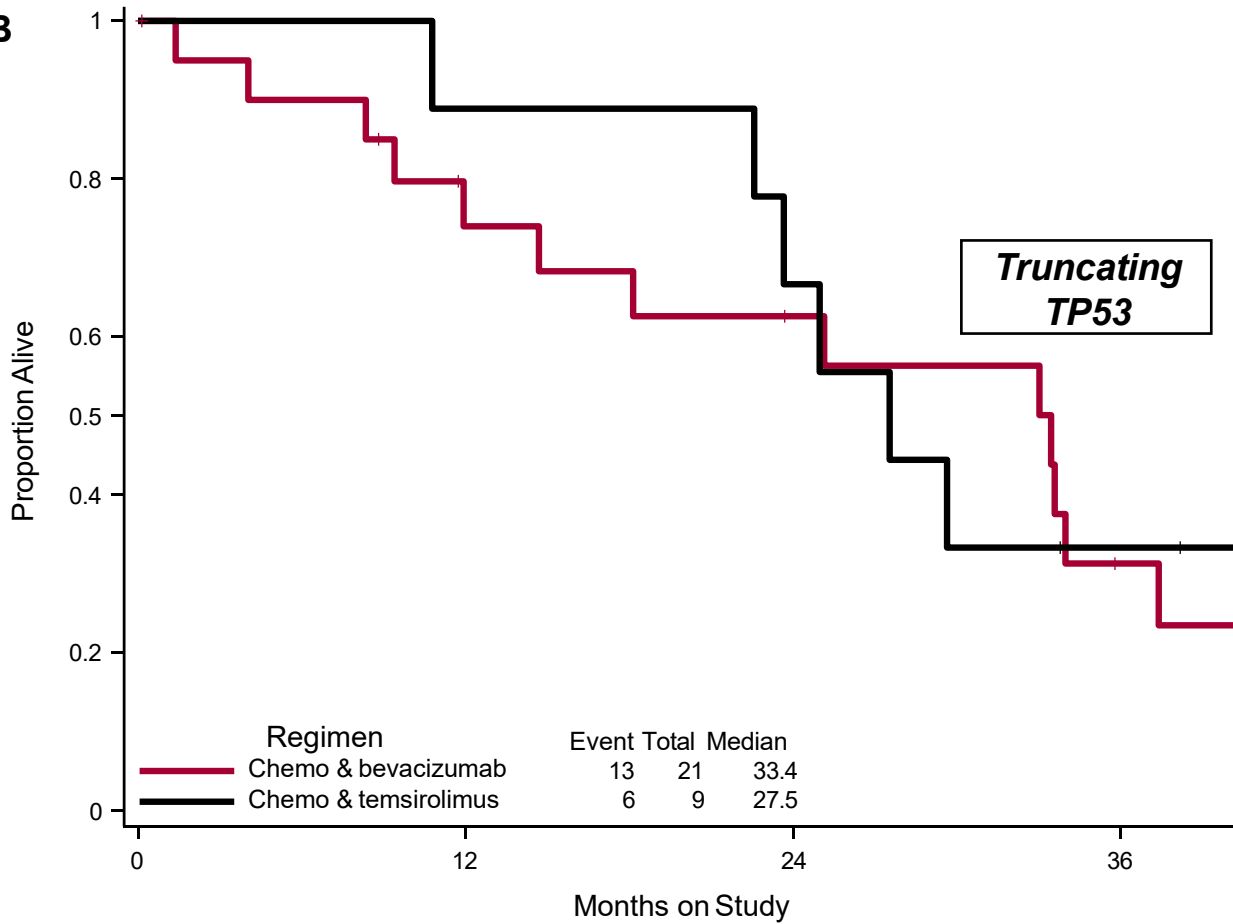
Supplemental Figure S5: Kaplan-Meier curves for PFS for *TP53* mutations by treatment arm. (A) WT *TP53* (no mutations). (B) Truncating *TP53*: mutations that result in a premature stop codon and therefore loss of protein expression. (C) GOF: gain-of-function missense mutation. (D) sVUF: somatic variant of unknown function – a missense mutation that is not a GOF mutation.

Supplemental Figure S6

A

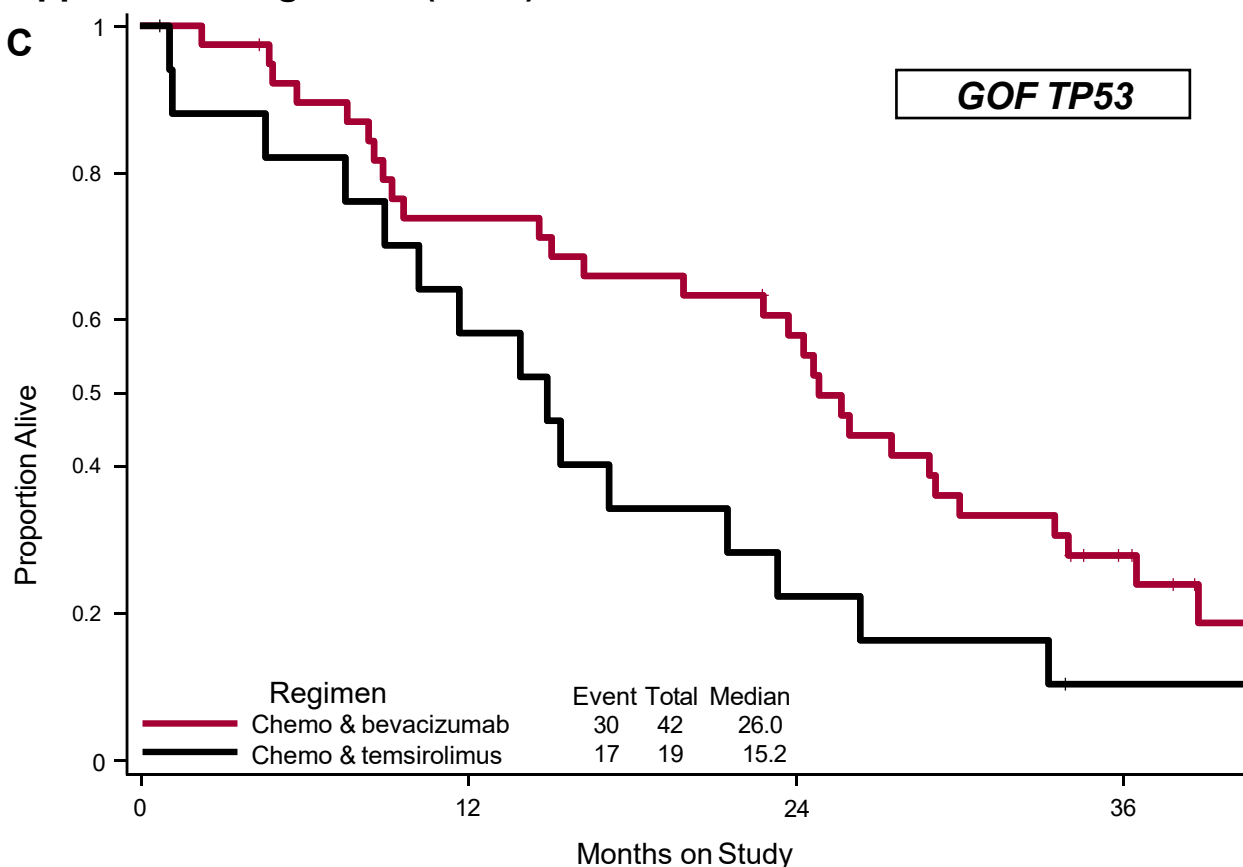


B

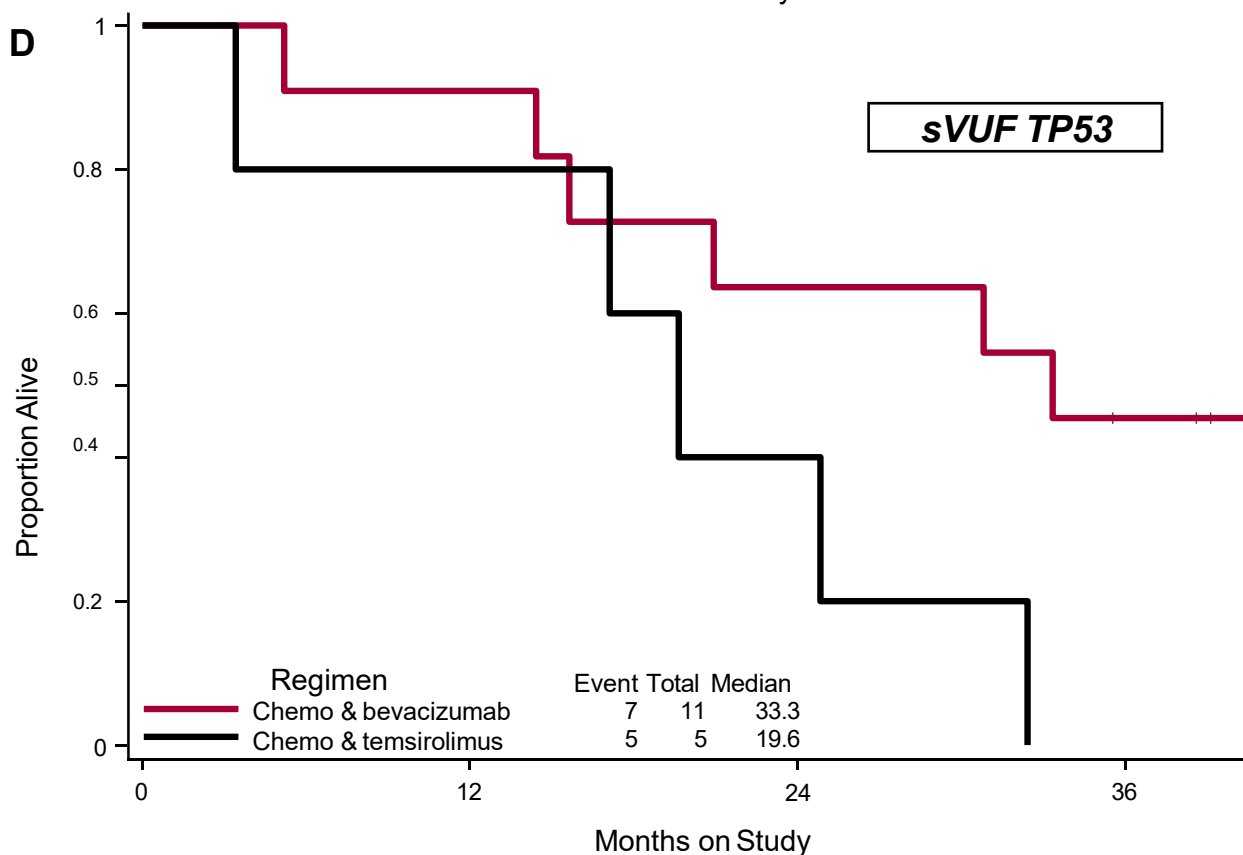


Supplemental Figure S6 (con't)

C

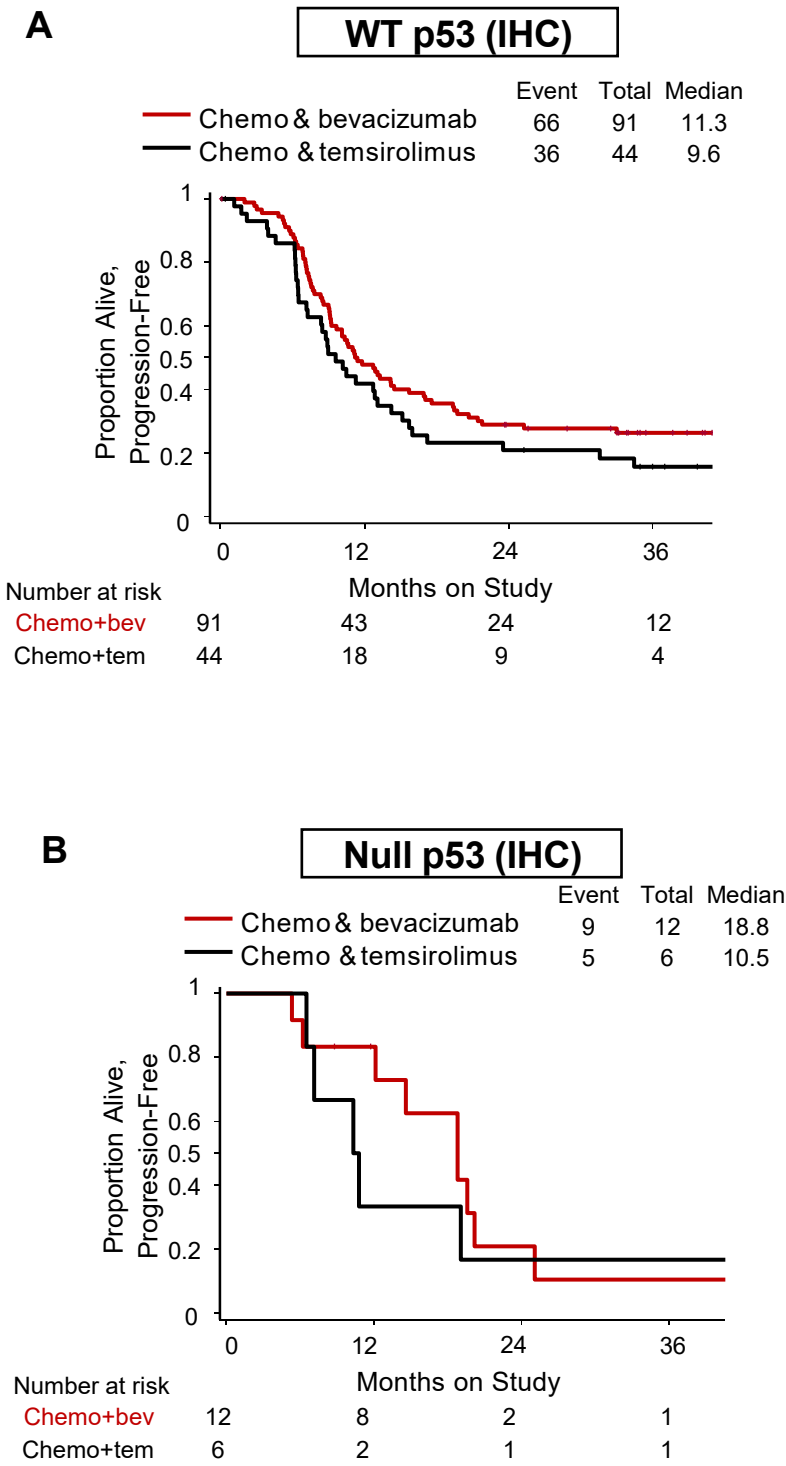


D



Supplemental Figure S6: Kaplan-Meier curves for OS for TP53 mutations by treatment arm. (A) WT TP53 (no mutations). (B) Truncating TP53: mutations that result in a premature stop codon and therefore loss of protein expression. (C) GOF: gain-of-function missense mutation. (D) sVUF: somatic variant of unknown function – a missense mutation that is not a GOF mutation.

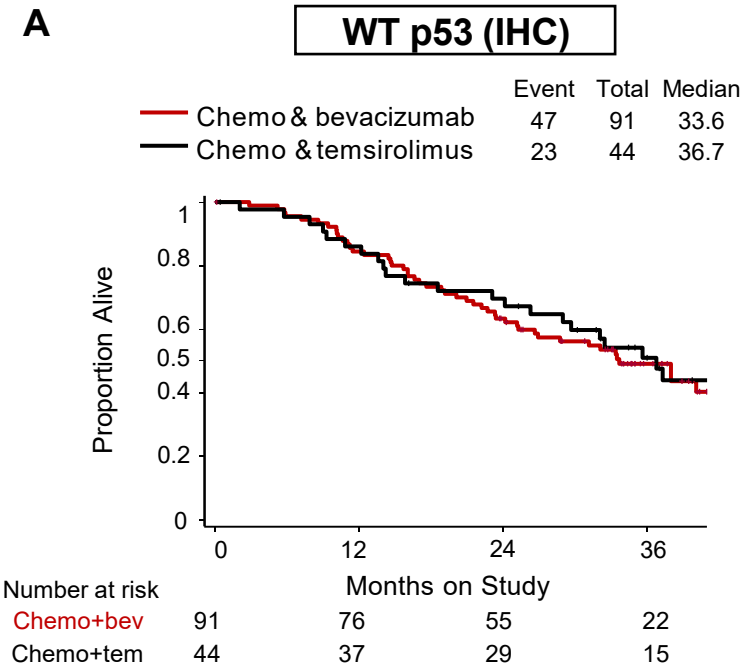
Supplemental Figure S7



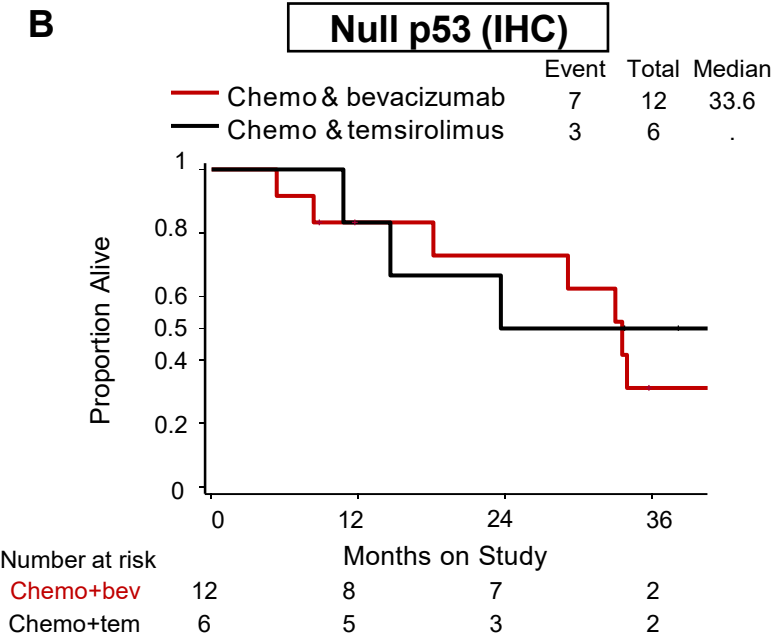
Supplemental Figure S7: Kaplan-Meier curves for PFS for p53 IHC expression by treatment arm. (A) WT p53 expression pattern by IHC. (B) null p53 expression pattern by IHC. Note: Kaplan-Meier curve for PFS for p53 overexpression (OE) by immunohistochemistry by treatment arm is presented in the main manuscript as Figure 4C.

Supplemental Figure S8

A



B



Supplemental Figure S8: Kaplan-Meier curves for OS for p53 IHC expression by treatment arm. (A) WT p53 expression pattern by IHC. (B) null p53 expression pattern by IHC. Note: Kaplan-Meier curve for OS for p53 overexpression (OE) by immunohistochemistry by treatment arm is presented in the main manuscript as Figure 4D.

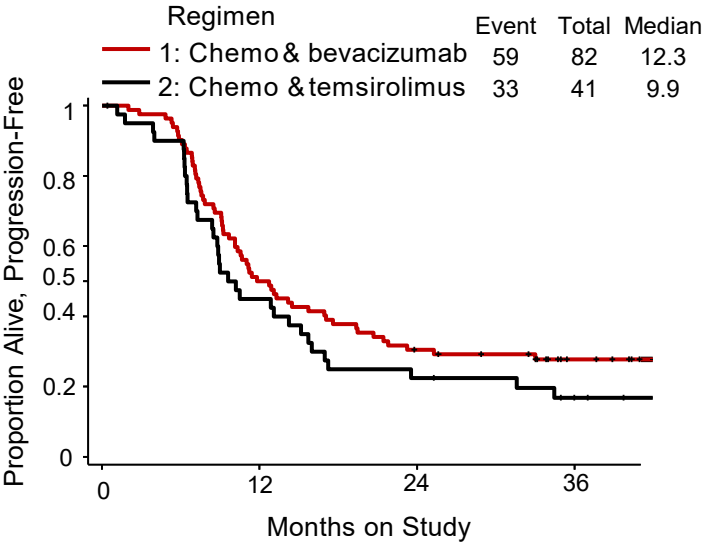
Supplemental Figure S9

Endpoint		Category	HR	95% CI	
PFS	PC + bevacizumab vs PC + temsirolimus	No IHC	5.316	1.536	16.693
		p53 ^{null}	0.843	0.231	3.075
		p53 ^{WT} /TP53 ^{WT}	0.607	0.370	0.994
		p53 ^{WT} /TP53 ^{mut}	0.977	0.283	3.506
		p53 ^{OE} /TP53 ^{mut}	0.401	0.183	0.879
	PC + bevacizumab vs IC + bevacizumab	No IHC	5.860	1.634	19.522
		p53 ^{null}	1.403	0.368	5.705
		p53 ^{WT} /TP53 ^{WT}	0.614	0.368	1.031
		p53 ^{WT} /TP53 ^{mut}	2.783	0.715	13.463
		p53 ^{OE} /TP53 ^{mut}	0.944	0.461	1.883
	PC + temsirolimus vs IC + bevacizumab	No IHC	1.102	0.461	2.670
		p53 ^{null}	1.664	0.438	6.760
		p53 ^{WT} /TP53 ^{WT}	1.012	0.612	1.685
		p53 ^{WT} /TP53 ^{mut}	2.848	0.677	14.374
		p53 ^{OE} /TP53 ^{mut}	2.356	1.124	4.789
OS	PC + bevacizumab vs PC + temsirolimus	No IHC	1.124	0.245	3.860
		p53 ^{null}	1.029	0.189	5.598
		p53 ^{WT} /TP53 ^{WT}	0.613	0.319	1.161
		p53 ^{WT} /TP53 ^{mut}	3.358	0.901	14.154
		p53 ^{OE} /TP53 ^{mut}	0.290	0.125	0.666
	PC + bevacizumab vs IC + bevacizumab	No IHC	1.126	0.245	3.921
		p53 ^{null}	0.677	0.131	3.137
		p53 ^{WT} /TP53 ^{WT}	0.397	0.209	0.737
		p53 ^{WT} /TP53 ^{mut}	2.994	0.768	14.605
		p53 ^{OE} /TP53 ^{mut}	1.053	0.481	2.279
	PC + temsirolimus vs IC + bevacizumab	No IHC	1.001	0.380	2.692
		p53 ^{null}	0.658	0.129	3.004
		p53 ^{WT} /TP53 ^{WT}	0.647	0.356	1.167
		p53 ^{WT} /TP53 ^{mut}	0.892	0.192	4.611
		p53 ^{OE} /TP53 ^{mut}	3.634	1.628	8.096

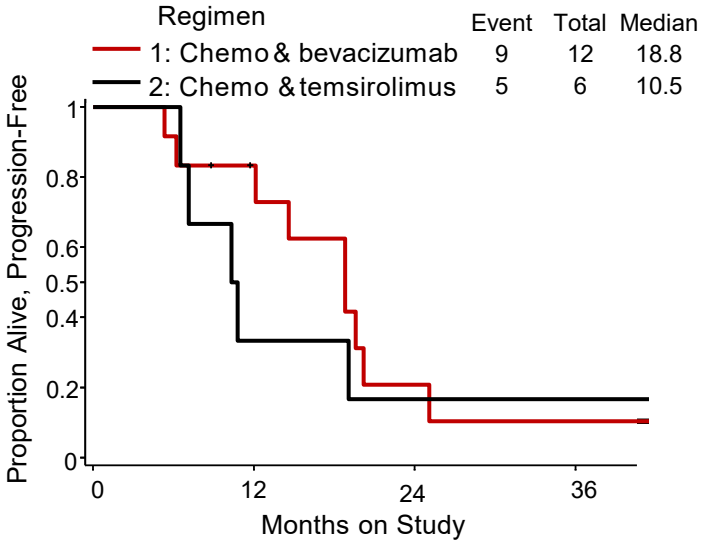
Supplemental Figure S9: Hazard ratios for integrated analysis by treatment arm. Cases were analyzed by each of the three arms on GOG-86P. Arm 1: paclitaxel (P) + carboplatin (C) + bevacizumab. Arm 2: PC+temsirolimus. Arm 3: ixabepilone (I, a mitotic spindle inhibitor) + C + bevacizumab. The number of cases in each integrated category are listed. Grey shading indicates HRs that are statistically significant.

Supplemental Figure S10

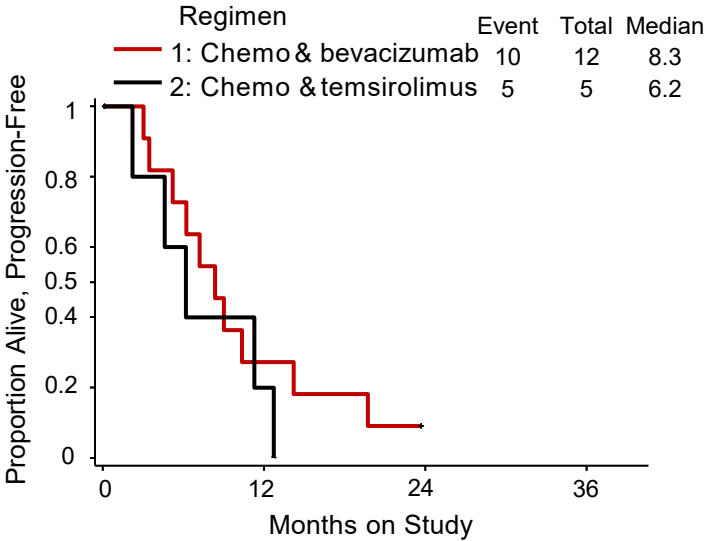
p53^{WT}/TP53^{WT}



p53^{null}



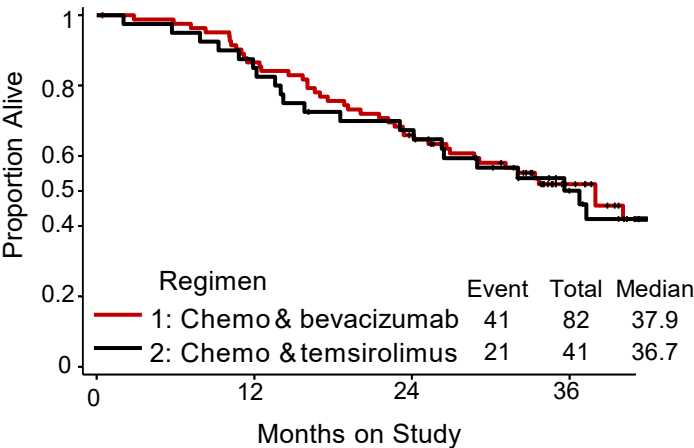
p53^{WT}/TP53^{mut}



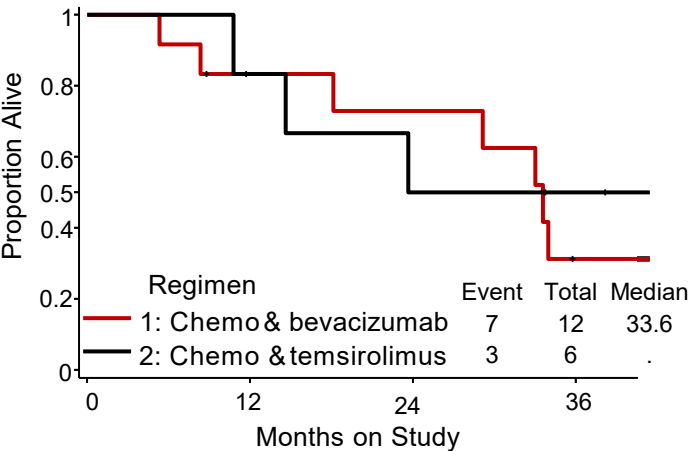
Supplemental Figure S10: Kaplan-Meier curves for PFS for the indicated integrated categories by treatment arm. p53 IHC categories: p53^{WT}= WT p53, p53 null = p53^{null}. TP53 mutational categories: TP53^{WT}= no mutation in TP53; TP53^{mut}= mutation in TP53 (all missense mutations in this cohort).

Supplemental Figure S11

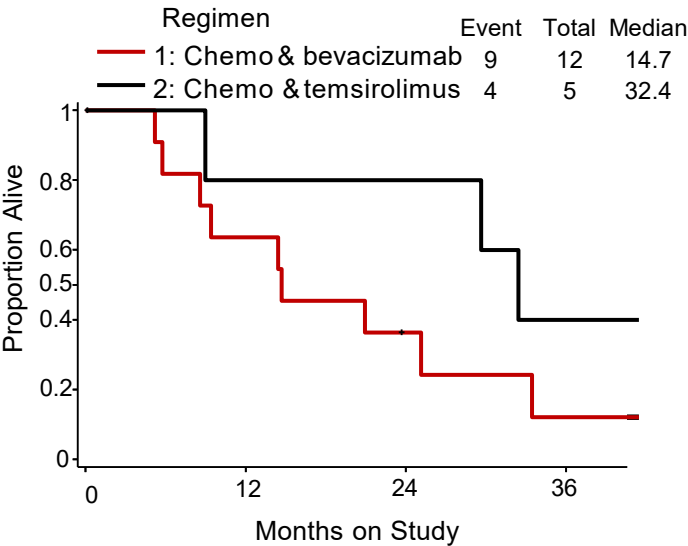
p53^{WT}/TP53^{WT}



p53^{null}



p53^{WT}/TP53^{mut}



Supplemental Figure S11: Kaplan-Meier curves for OS for the indicated integrated categories by treatment arm. p53 IHC categories: p53^{WT}= WT p53, p53 null = p53^{null}. TP53 mutational categories: TP53^{WT}= no mutation in TP53; TP53^{mut}= mutation in TP53 (all missense mutations in this cohort).