

PPM1D Is a Potential Therapeutic Target in Ovarian Clear Cell Carcinomas

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Abstract Purpose: To identify therapeutic targets in ovarian clear cell carcinomas, a chemoresistant and aggressive type of ovarian cancer.

Experimental Design: Twelve ovarian clear cell carcinoma cell lines were subjected to tiling path microarray comparative genomic hybridization and genome-wide expression profiling analysis. Regions of high-level amplification were defined and genes whose expression levels were determined by copy number and correlated with gene amplification were identified. The effects of inhibition of PPM1D were assessed using short hairpin RNA constructs and a small-molecule inhibitor (CCT007093). The prevalence of *PPM1D* amplification and mRNA expression was determined using chromogenic *in situ* hybridization and quantitative real-time reverse transcription-PCR in a cohort of pure ovarian clear cell carcinomas and on an independent series of unselected epithelial ovarian cancers.

Results: Array-based comparative genomic hybridization analysis revealed regions of high-level amplification on 1q32, 1q42, 2q11, 3q24-q26, 5p15, 7p21-p22, 11q13.2-q13.4, 11q22, 17q21-q22, 17q23.2, 19q12-q13, and 20q13.2. Thirty-four genes mapping to these regions displayed expression levels that correlated with copy number gains/amplification. *PPM1D* had significantly higher levels of mRNA expression in ovarian clear cell carcinoma cell lines harboring gains/amplifications of 17q23.2. PPM1D inhibition revealed that PPM1D expression and phosphatase activity are selectively required for the survival of ovarian clear cell carcinoma cell lines with 17q23.2 amplification. *PPM1D* amplification was significantly associated with ovarian clear cell carcinoma histology ($P = 0.0003$) and found in 10% of primary ovarian clear cell carcinomas. PPM1D expression levels were significantly correlated with *PPM1D* gene amplification in primary ovarian clear cell carcinomas.

Conclusion: Our data provide strong circumstantial evidence that *PPM1D* is a potential therapeutic target for a subgroup of ovarian clear cell carcinomas.

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Ovarian clear cell carcinoma accounts for 5% to 13% of all epithelial ovarian carcinomas (1, 2). Compared with other epithelial ovarian carcinoma subtypes, ovarian clear cell carcinomas are associated with a poorer prognosis and a relatively increased resistance to platinum-based chemotherapy (1, 3). Hence, there is a need to identify alternative and/or novel therapeutic approaches for this subgroup of epithelial ovarian carcinomas.

Given its relative resistance to conventional chemotherapy, a comprehensive characterization of the molecular genetic features of ovarian clear cell carcinomas could provide clues to the mechanisms of drug resistance and identify novel therapeutic targets (4). In the context of therapeutic target discovery, inhibiting proteins whose expression is driven by gene amplification or activating genetic mutations is an effective approach (5–7). This concept is best exemplified by the successful use of trastuzumab in the treatment of *HER2*-amplified breast cancer (8).

Previous studies on the molecular features of ovarian clear cell carcinomas have been limited to comparative genomic

Translational Relevance

Ovarian clear cell carcinomas are aggressive and chemo-resistant tumors, accounting for ~13% of all ovarian carcinomas. Their management constitutes a challenge in oncology. Hence, the identification of therapeutic targets in these cancers is of paramount importance. By overlaying genome-wide data on gene copy number and expression obtained from the analysis of the largest collection of ovarian clear cell carcinoma cell lines to date, we identified a list of genes that are highly likely amplicon drivers and/or therapeutic targets in ovarian clear cell carcinoma cell lines. Using a combination of RNA interference methods and chemical inhibition, we show that PPM1D expression is required for the survival of cancer cells with 17q23.2 amplification. Amplification of this region was shown to be associated with PPM1D expression levels in primary ovarian clear cell carcinomas. Taken together, our study provides an approach for the identification of therapeutic targets in ovarian clear cell carcinoma and validates PPM1D as a therapeutic target for these aggressive cancers.

hybridization (CGH) analysis, either conventional or using low-density array platforms of small numbers of ovarian clear cell carcinomas, and yielded conflicting results. Nonetheless, frequent gains of 8q (9, 10), 17q (9, 11), which has been associated with poorer prognosis in ovarian clear cell carcinoma, and 20q (9), and loss of 9p (12) have been reported. Importantly, very few high-resolution CGH studies have been done in ovarian clear cell carcinoma (13) and no candidate therapeutic targets have hitherto been clearly identified and validated in these studies.

In recent years, it has become apparent that detailed molecular profiling of cancer cell lines can lead to the identification of better models to study specific types of cancer *in vitro* (6, 14, 15). Therefore, we hypothesized that key molecular drivers and putative therapeutic targets could be identified for ovarian clear cell carcinomas, based on a high-throughput molecular genetic profiling of ovarian clear cell carcinoma cell lines and identification of genes that are overexpressed when amplified (4, 6, 16).

Here, we have analyzed 12 ovarian clear cell carcinoma cell lines using microarray-based CGH (aCGH) and expression profiling aiming (a) to define the genetic profiles of ovarian clear cell carcinoma cell lines using high-resolution tiling-path bacterial artificial chromosome (BAC) aCGH and (b) to identify genes whose expression is driven by gene amplification and which, therefore, could be exploited as potential therapeutic targets for subgroups of ovarian clear cell carcinomas. Our results have led to the identification of potential drivers of several amplicons and validation of PPM1D as a putative therapeutic target in a subgroup of ovarian clear cell carcinomas harboring 17q23.2 amplification.

Materials and Methods

Ovarian clear cell carcinoma cell lines. We studied 12 ovarian clear cell carcinoma cell lines (Table 1). TOV21G and ES2 were obtained from the American Type Culture Collection. SMOV-2 (17), RMG-1

(18), KOC7C (19), HCH1 (20), OVAS (21), OVISe (22), OVTOKO (22), OVMANA (23), OVSAYO (23), and KK (24) were courtesy of Dr. Hiroaki Itamochi (Tottori University School of Medicine, Yonago, Japan; Table 1). Ovarian clear cell carcinoma cell lines were grown in RPMI 1640 with 10% FCS. Table 1 summarizes the characteristics of these cell lines. To determine whether these cell lines would harbor transcriptomic profiles similar to those of ovarian clear cell carcinomas, we built a ovarian clear cell carcinoma predictor based on the microarray data set from Hendrix et al. (25) using the Prediction Analysis for Microarrays in R.⁸ This Affymetrix data set includes gene expression data from primary clear cell ($n = 8$), endometrioid ($n = 37$), mucinous ($n = 13$), and serous epithelial ovarian carcinomas ($n = 41$) and normal ovaries ($n = 4$; Supplementary Methods).

DNA and RNA extraction. DNA from cell lines was extracted using the DNeasy Kit (Qiagen Ltd.) according to the manufacturer's recommendations. RNA was extracted using the Trizol method (Invitrogen). RNA concentrations were measured with NanoDrop and quality was determined using Agilent Bioanalyser (Agilent Technologies Limited) according to manufacturer's instructions.

Microarray CGH. aCGH was done with the Breakthrough Breast Cancer Research Centre 32K BAC array platform, which comprises ~32,000 BAC clones tiled across the genome and has a resolution of ~50 kb. Labeling, hybridization, washes, and image acquisition were carried out as previously described (26). Log₂ratios were normalized for spatial and intensity-dependent biases using a two-dimensional loess regression followed by a BAC-dependent bias correction as previously described (26). This left a final data set of 28,301 clones with unambiguous mapping information according to the March 2006 build (hg18) of the human genome.⁹ Data were smoothed using a local polynomial adaptive weights smoothing (aws) procedure for regression problems with additive errors (27).

Threshold values (Log₂ratio of ± 0.12) were chosen to correspond to 3 SDs of the normal ratios obtained from the filtered clones mapping to chromosomes 1-22, assessed in multiple hybridizations between pooled male and female DNA as previously described (26). A categorical analysis was applied to each clone on the array after classification as gain, loss, or no-change according to their smoothed (aws) Log₂ratio values. Low-level gain was defined as aws Log₂ratios between 0.12 and 0.4, corresponding to ~3 to 5 copies of the locus, whereas gene amplification was defined as having a Log₂ratio >0.4, corresponding to >5 copies. These figures were obtained by comparison with interphase fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) data for markers at different chromosomal locations (26, 28). Based on FISH analysis of ovarian clear cell carcinoma cell lines (PPM1D:CEP17 ratio >2.0 corresponding to aws Log₂ratio >0.8; see below), which are predominantly aneuploid, high-level amplifications were defined as aws Log₂ratio >0.8. Data processing and analysis were carried out in R 2.0.1,¹⁰ making extensive use of modified versions of the packages aCGH, marray, and aws (26).

FISH analysis. To validate the results of our aCGH analysis, we determined the copy number status of PPM1D in ovarian clear cell carcinoma cell lines by means of FISH and CISH. Dual-color FISH was done using an in-house generated digoxigenin-labeled probe for PPM1D (17q23.2) and a commercially available biotin-labeled chromosome 17 centromere (CEP17) probe (Zymed, Invitrogen) according to previously described protocols (29). CISH probes encompassing the PPM1D gene were generated as previously described (29) using two BACs, RP11-738N12 and RP11-653P10, which map to 55,871-56,051 kb and 56,004-56,234 kb, respectively, on chromosome 17q23.2. The specificity and genomic position of both BACs were validated on normal metaphase spreads and verified by end-sequencing

⁸ <http://rss.acs.unt.edu/Rdoc/library/pamr/html/00Index.html>

⁹ <http://www.ensembl.org>

¹⁰ (<http://www.r-project.org/>) and BioConductor 1.5 (<http://www.bioconductor.org/>).

Table 1. Characteristics of 12 ovarian clear cell carcinoma cell lines

Cell lines	Reference	Origin	Pretreatment	P53 status	Source
KK	23	Ascites	None	Wild-type	Dr. H. Itamochi
KOC-7C	18	Pleural effusion	None	Wild-type	Dr. H. Itamochi
HCH-1	19	Solid tumor	Unknown	Wild-type	Dr. H. Itamochi
SMOV-2	16	Primary tumor	None	Wild-type	Dr. H. Itamochi
OVAS	20	Ascites	Unknown	Wild-type	Dr. H. Itamochi
OVISE	21	Metastasis	5× Carboplatin/paclitaxel	Wild-type	Dr. H. Itamochi
OVTOKO	21	Metastasis	6× Carboplatin/paclitaxel	Wild-type	Dr. H. Itamochi
OVMANA	22	Primary tumor	3× i.p. Cisplatin	Wild-type	Dr. H. Itamochi
OVSAYO	22	Primary tumor	None	Wild-type	Dr. H. Itamochi
RMG-I	17	Ascites	None	Wild-type	Dr. H. Itamochi
ES2	—	Primary tumor	Unknown	c.722C>T, p.S241F	ATCC
TOV21	—	Primary tumor	Unknown	Wild-type	ATCC

Abbreviation: ATCC, American Type Culture Collection.

(data not shown). Hybridizations and washes were done as previously described (29). The probes were visualized using a Zeiss Axioplan 2 microscope equipped with a charge-coupled device camera, and analyzed with Cytovision software version 2.81 (Applied Imaging International). *PPM1D* and *CEP17* signals were counted in 60 nonoverlapping nuclei of neoplastic cells. Amplification was defined as a *PPM1D*:*CEP17* ratio ≥ 2.0 , whereas copy number gains were defined as a *PPM1D*:*CEP17* ratio >1.5 and <2.0 (30).

Gene expression profiling. mRNA gene expression profiling was done using the Illumina Human ref 6 gene expression assay. Raw gene expression values were cubic-spline normalized using the Illumina Beadstudio software. Only Illumina transcript probes with detection *P* values of <0.01 in at least one ovarian clear cell carcinoma cell line were included. Gene expression data are publicly available at ArrayExpress¹¹ (accession number: E-TABM-540).

Correlation of aCGH and gene expression. All genes, and relevant gene expression data, found within regions of high-level amplification ($\text{Log}_2\text{ratio} > 0.8$) were identified. Pearson's correlation tests were done for each gene within each of the high-level amplicons to identify genes whose expression was significantly correlated with ams ratios across all 12 cell lines. To identify the genes in the region with increased mRNA levels that were significantly associated with copy number gains/amplification, a two-tailed unpaired *t* test was also done for each gene within each region by comparing the mean gene expression of cell lines with copy number gains ($\text{Log}_2\text{ratio} > 0.12$) versus those without copy number gains in the region. Statistical analyses were computed using the Clustering Algorithm and Distance Integratory Statistic Tools software (ref. 31).¹²

The Ingenuity Pathway Analysis software¹³ was used to analyze pathways and networks that were significantly enriched for genes whose expression correlated with copy number gains/amplifications and losses in ovarian clear cell carcinoma cell lines. The list of genes overexpressed when gained or amplified and down-regulated when lost were independently mapped to networks and canonical pathways available in the Ingenuity database and ranked by score. The score indicates the likelihood of the genes in a network being found together due to chance. Using a 99% confidence level, scores of ≥ 3 are significant. In this study, a score of 30 was used as the cutoff for identifying gene networks significantly up-regulated or down-regulated in ovarian clear cell carcinoma cell lines.

Western blotting. Total protein lysates from cell lines (100 μg) were separated by SDS-PAGE according to standard protocols, and

immunoblotting was carried out using previously validated primary antibodies; anti-PPM1D (32), anti-p38 rabbit polyclonal antibody (Cell Signaling), and anti- β -tubulin (Sigma). p38 kinase activity following treatment with CCT007093 and H_2O_2 (positive control) in ovarian clear cell carcinoma cell lines was assessed by Western blotting for phosphorylation of p38.

Plasmids. Previously validated pSUPER constructs for RNA interference were generated as previously described (32). Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Colony formation assays. Colony formation assays for short hairpin RNA (shRNA)-mediated PPM1D knockdown and the CCT007093 small-molecule PPM1D inhibitor were done as previously described (32). Briefly, cells were transfected with a pSUPER plasmid and an additional plasmid expressing the blasticidin resistance gene (*pEFBsd*, Invitrogen) in a molar ratio of 10:1. Cells were plated in 6-well plates 24 h after transfection. Blasticidin selection (5 $\mu\text{g}/\text{mL}$) was initiated 48 h posttransfection and replenished every 3 d. For chemical inhibition of PPM1D, cells were plated in six-well plates and treated with CCT007093. Medium and inhibitor were replenished every 3 d. Colonies were fixed in methanol, stained with crystal violet, and quantified after 14 d using a Colcount machine (Oxford Optronix). Surviving fractions were calculated as previously described (33). All assays were done in triplicate.

TP53 mutation analysis in ovarian clear cell carcinoma cell lines. Sequencing of known mutation hotspots of *TP53* on exons 5 to 9 (34) in all 12 ovarian clear cell carcinoma cell lines was performed. The primers used for *TP53* sequencing have previously been described (34). Primer sequences and sequencing conditions are described in Supplementary Methods.

Tissue microarray construction. Local ethical approval for this study was granted by the Royal Marsden Hospital Research and Ethics Committee on May 11, 2006 (RMH REC Committee Ref: 06/Q801/23). Representative paraffin blocks of ovarian clear cell carcinoma tumors were obtained from The Edinburgh Royal Infirmary (A.W.), The Royal Hospital Group (W.G.M.) in Belfast, Royal Marsden Hospital (C.J.), and Hammersmith Hospital (M.E.B.) in London. All ovarian clear cell carcinoma tumors were reviewed and selected by pathologists with an interest in ovarian cancer at respective hospitals before undergoing a second central review and selection process by two gynecologic pathologists (C.J., W.G.M.). None of the selected patients had received any systemic anticancer therapy before primary debulking surgery. Eighty-eight archival surgical resection specimens of primary, pure ovarian clear cell carcinoma tumors were identified, reviewed, and arrayed into tissue microarrays (TMA) blocks containing two representative replicate 0.6-mm cores from each ovarian clear cell carcinoma.

Further validation of identified amplicons was done on another independent set of TMAs comprising 167 unselected ovarian

¹¹ <http://www.ebi.ac.uk/microarray-as/ae/>

¹² <http://rock.icr.ac.uk/software/cladist.jsp>

¹³ <http://www.ingenuity.com>

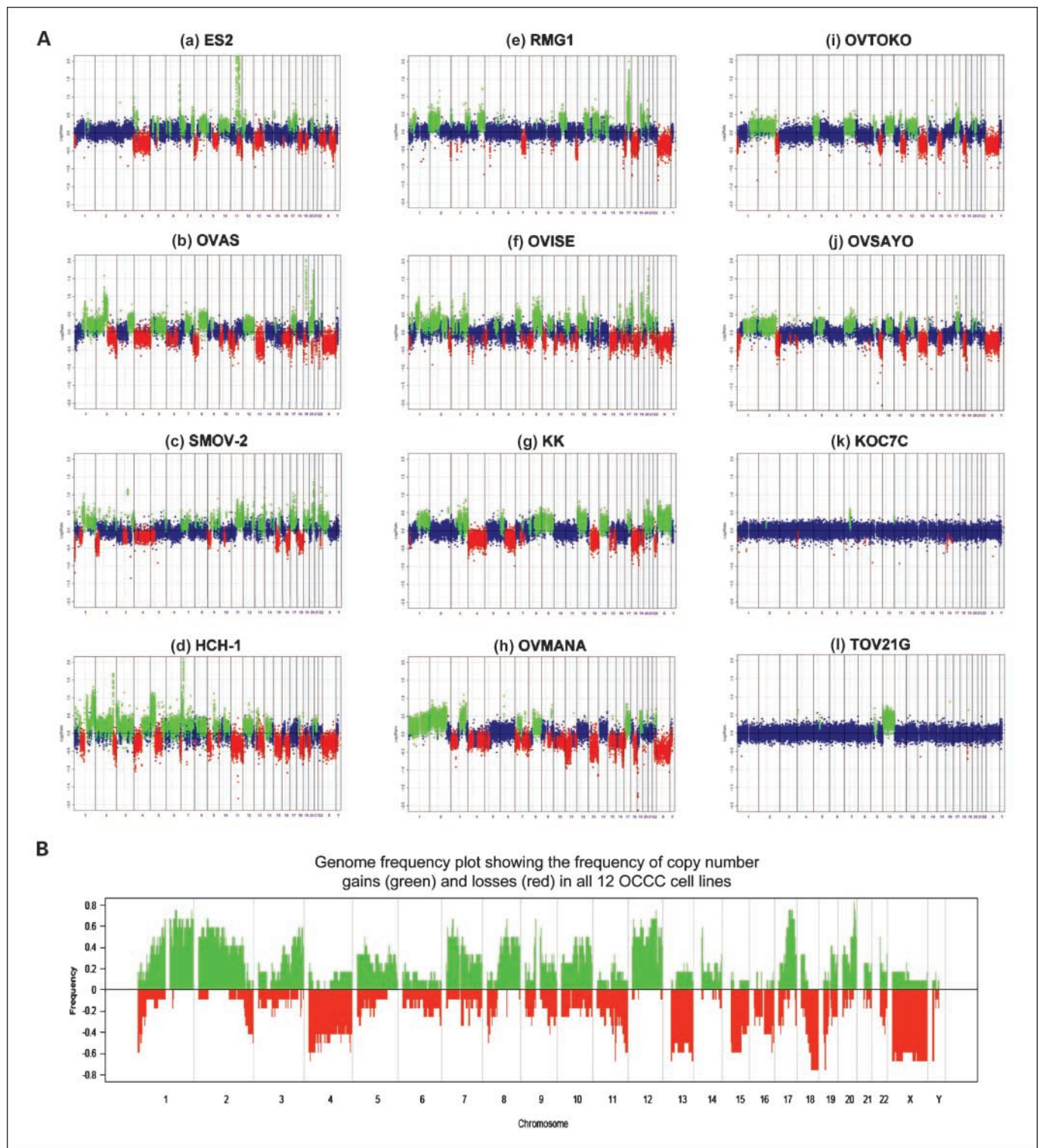


Fig. 1. Molecular genetic profiles of 12 ovarian clear cell carcinoma cell lines. *A*, molecular genetic profiles of all 12 ovarian clear cell carcinoma cell lines. *B*, frequency plot of copy number gains ($\text{Log}_2\text{ratio} > 0.12$) and losses ($\text{Log}_2\text{ratio} < -0.12$) in 12 ovarian clear cell carcinoma (OCCC) cell lines.

tumors (48 serous, 30 endometrioid, 30 clear cell, 10 mucinous, 23 mixed-epithelial, and 7 malignant mixed-Mullerian invasive ovarian tumors, as well as 5 borderline-serous and 14 borderline-mucinous tumors) to establish the prevalence of candidate therapeutic targets across the spectrum of epithelial ovarian carcinoma subtypes. The

construction and clinicopathologic details of this TMA have already been described (35).

CISH analysis. CISH was done on both TMAs using an in-house biotin-labeled probe for *PPM1D* (RP11-738N12 and RP11-653P10) according to previously described protocols (29) and analyzed by two

of the authors (C.M. and J.R.F.) on a multihanded microscope. Unequivocal signals were evaluated at X400 and X630 magnification and counted in nonoverlapping nuclei of at least 60 morphologically unequivocal neoplastic cells. Amplification was defined as >5 signals per nucleus in >50% of cancer cells, or when >50% of cells harbored large gene copy clusters (36).

Quantitative real-time reverse transcription-PCR. All ovarian clear cell carcinoma tumors with interpretable CISH results were reviewed by two of the authors (C.M. and J.R.F.) and those with >50% of tumor cells as defined by histopathologic analysis of the most representative section of the tumor were subjected to RNA extraction. RNA from ovarian clear cell carcinoma tumors was extracted using the RNeasy FFPE RNA Isolation Kit (Qiagen) followed by an additional DNase treatment as previously described (14). RNA from ovarian clear cell carcinoma cell lines was extracted as described above.

Quantification, quality control, and reverse transcription were done as previously described (14). In total, all cell lines and 30 primary ovarian clear cell carcinomas with interpretable PPM1D gene copy number status rendered optimal results. Reverse transcription was done with Superscript III (Invitrogen) using 400 ng of RNA per reaction. Triplicate reactions were done for each sample (reverse transcriptase positive), in addition to a reverse transcriptase-negative reaction to check for the absence of detectable DNA contamination.

Quantitative reverse transcription-PCR (qRT-PCR) was done using TaqMan chemistry on the ABI Prism 7900HT (Applied Biosystems) using the standard curve method (14, 37). Assays were purchased from Applied Biosystems. Three reference genes (*TBP*, *TFRC*, and *MRPL19*) were used, having been previously selected as effectively normalizing for degradation of RNA (14). PPM1D expression levels were normalized to the geometric mean of these reference genes. Additional details of the qRT-PCR analysis are described in the Supplementary Methods. A two-tailed, unpaired *t* test was used to determine whether PPM1D mRNA expression levels were significantly associated with PPM1D gene amplification.

Results

Characterization of ovarian clear cell carcinoma cell lines using gene expression profiles from primary epithelial ovarian carcinomas. The ovarian cancer data set from Hendrix et al. (25) was used to devise a predictor (based on 126 genes) for the phenotypes of the cell lines used in this study (Supplementary Methods). When this predictor was applied to expression data from the 12 ovarian clear cell carcinoma cell lines (Table 1), all 12 were predicted to have the phenotype of ovarian clear cell carcinoma tumors with highly significant posterior class probabilities (Supplementary Fig. S1).

Table 2. List of genes in highly amplified regions ($\text{Log}_2\text{ratio} > 0.8$) significantly correlated with aws ratios (Pearson's *r*, $P < 0.05$) and associated with copy number gains (unpaired *t* test, $P < 0.05$)

Gene	Amplicon	Mean expression-gain*	Mean expression-no gain †	Pearson's <i>r</i>	Pearson's <i>P</i>	<i>t</i> test <i>P</i>
<i>CENPF</i>	1q32.3-q41	998.0143	627.3786	0.6670	0.0078	0.0304
<i>TARBP1</i>	1q42.2-q42.3	236.5295	114.6858	0.8713	0.0000	0.0049
<i>REV1L</i>	2q11.2	360.2726	217.2823	0.8962	0.0000	0.0057
<i>ABTB1</i>	3q21.3	200.4706	93.3106	0.6300	0.0131	0.0217
<i>CP</i>	3q24-q25.1	2554.7366	337.4073	0.7289	0.0027	0.0061
<i>MTRR</i>	5p15.31-p15.2	467.3246	197.1642	0.9393	0.0000	0.0325
<i>MYO10</i>	5p15.2-p15.1	3346.4812	1609.3099	0.8510	0.0001	0.0441
<i>ZNF622</i>	5p15.2-p15.1	1535.9665	1056.1256	0.9300	0.0000	0.0192
<i>RICTOR</i>	5p13.2-p13.1	362.3871	237.1880	0.6435	0.0109	0.0349
<i>LOC285636</i>	5p13.1-p12	783.3502	554.8136	0.7856	0.0007	0.0111
<i>FLJ20323</i>	7p22.1-p21.3	410.1639	219.5463	0.8759	0.0000	0.0313
<i>ORAOV1</i>	11q13.2-q13.4	1143.1820	254.4139	0.8760	0.0000	0.0047
<i>CTTN</i>	11q13.2-q13.4	2102.2700	730.9370	0.8977	0.0000	0.0110
<i>IGHMBP2</i>	11q13.2-q13.4	86.1350	41.0003	0.9467	0.0000	0.0004
<i>TPCN2</i>	11q13.2-q13.4	454.9718	121.3160	0.9421	0.0000	0.0000
<i>FADD</i>	11q13.2-q13.4	1933.2216	672.2786	0.8969	0.0000	0.0073
<i>DHCR7</i>	11q13.2-q13.4	2565.4100	617.9808	0.8608	0.0001	0.0002
<i>NADSYN1</i>	11q13.2-q13.4	1038.2966	412.3342	0.9339	0.0000	0.0012
<i>PPFIA1</i>	11q13.2-q13.4	736.2234	273.1476	0.9349	0.0000	0.0002
<i>YAP1</i>	11q22.1-q22.3	2975.7735	1398.3403	0.8712	0.0000	0.0033
<i>DCUN1D5</i>	11q22.1-q22.3	2198.2705	1196.4108	0.8916	0.0000	0.0332
<i>MMP3</i>	11q22.1-q22.3	536.2950	6.2510	0.9002	0.0000	0.0162
<i>MMP1</i>	11q22.1-q22.3	8328.3999	134.6685	0.8933	0.0000	0.0177
<i>WNT3</i>	17q21.31-q21.32	31.1229	6.0125	0.5142	0.0441	0.0253
<i>RSAD1</i>	17q21.33	289.6785	155.8246	0.8098	0.0004	0.0127
<i>PCTP</i>	17q22	95.3235	32.7408	0.8841	0.0000	0.0463
<i>PPM1D</i>	17q23.2	297.4662	180.4771	0.6791	0.0065	0.0364
<i>C19ORF2</i>	19q12	3546.0705	2224.0712	0.8616	0.0000	0.0395
<i>C19ORF12</i>	19q12	445.7022	180.9905	0.9439	0.0000	0.0204
<i>LOC390927</i>	19q13.12-q13.13	15.6653	5.6050	0.6525	0.0097	0.0096
<i>SIPA1L3</i>	19q13.12-q13.13	55.4741	45.0967	0.6306	0.0130	0.0468
<i>PSMC4</i>	19q13.2	1104.0261	593.2772	0.8743	0.0000	0.0247
<i>FBL</i>	19q13.2	4054.2982	2452.8417	0.7684	0.0011	0.0200
<i>SUPT5H</i>	19q13.2	1159.5021	681.1589	0.8591	0.0001	0.0309

*Arithmetic average of Illumina expression values in cell lines with copy number gain/amplification (aws ratios > 0.12).

†Arithmetic average of Illumina expression values in cell lines with no copy number gain/amplification (aws ratios = 0.12).

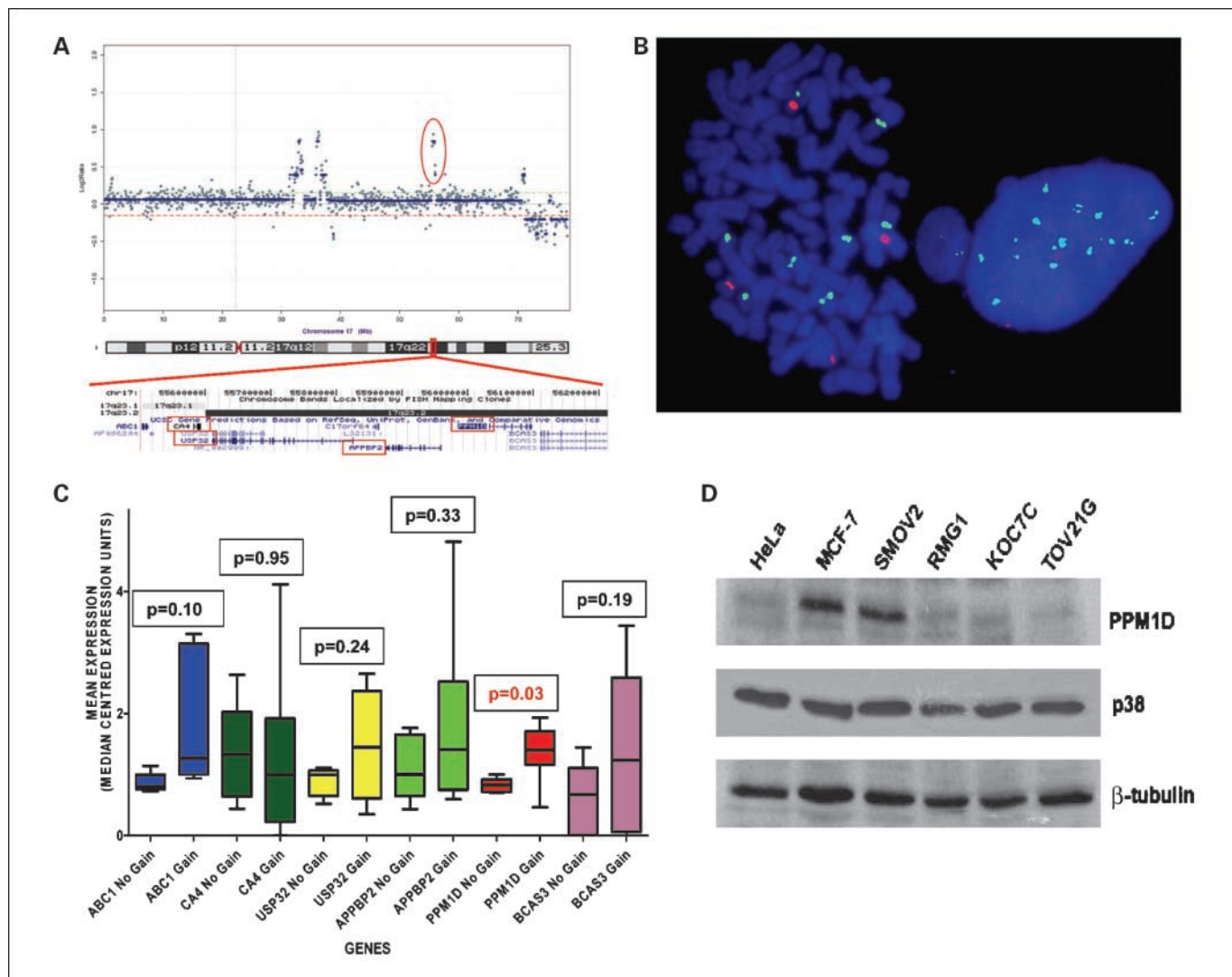


Fig. 2. 17q23.2 amplification in ovarian clear cell carcinoma cell lines. *A*, SMOV-2 genome plot of chromosome 17 showing focal high-level amplification of 17q23.2. *B*, FISH validation of aCGH results showing amplification of 17q23.2 in SMOV-2 ovarian clear cell carcinoma cell line using in-house generated *PPM1D* probes (green) composed of BACs RP11-653P10 and RP11-738N12. *C*, differential expression of genes in 17q23.2 for 12 ovarian clear cell carcinoma cell lines between those with copy number gains/amplifications versus no gains in the region (P values calculated using unpaired t test). *D*, Western blots for *PPM1D* showing overexpression in the *PPM1D*-amplified SMOV-2 and MCF-7 (positive control) cell lines. *PPM1D* was not overexpressed in RMG1 (copy number gain), TOV21G (no copy number change), KOC7C (no copy number change), and HeLa (negative control) cell lines. SMOV-2 is the only ovarian clear cell carcinoma cell line that shows *PPM1D* overexpression.

Copy number alterations in ovarian clear cell carcinoma cell lines. To characterize the molecular genetic profiles of ovarian clear cell carcinoma cell lines and delineate regions of amplification that may harbor potential therapeutic targets, we did high-resolution (~ 50 kb) aCGH analysis on DNA extracted from 12 ovarian clear cell carcinoma cell lines (Table 1). Having established the genetic profiles of the ovarian clear cell carcinoma cell lines, we sought to determine whether the genetic profiles of ovarian clear cell carcinoma cell lines and primary tumors would be similar. In fact, numerous concordant regions of copy number gains and losses were found in primary ovarian clear cell carcinomas and ovarian clear cell carcinoma cell lines, e.g., gains of 8q, 17q, and 20q and losses on 9p, 11q, 16p/q, and 19p (1, 9, 11–13). Figure 1B shows the frequency plot of copy number gains and losses for all 12 cell lines. A summary of gains ($\text{Log}_2\text{ratio} > 0.12$), losses (Log_2ratio

< -0.12), amplifications ($\text{Log}_2\text{ratios} > 0.4$), and high-level amplifications ($\text{Log}_2\text{ratio} > 0.8$) for each ovarian clear cell carcinoma cell line is included in Supplementary Table S1. Recurrent regions [at least 4 of 12 ($> 30\%$) of cell lines] of gain and loss are described in Supplementary Table S2. All genes within regions of high-level amplification found in ovarian clear cell carcinoma cell lines and known copy number variations are shown in Supplementary Table S3. The smallest region of overlap in two or more amplified regions ($\text{Log}_2\text{ratio} > 0.4$) and known copy number variations are shown in Supplementary Table S4.

Integrated analysis of aCGH and Illumina gene expression data in ovarian clear cell carcinomas. After mapping each gene to its respective genomic region and identifying the BACs mapping to each locus, we compiled a table containing copy number and expression values for $> 25,000$ genes. The t test was applied to

determine the genes whose expression correlated with copy number gains/amplifications and losses using the thresholds described above.

This analysis revealed that 879 transcripts were overexpressed when gained or amplified (Supplementary Table S5). Ingenuity Pathway analysis of these genes revealed seven networks that were significantly enriched for these genes (Supplementary Table S6). Canonical pathways significantly enriched for genes overexpressed when gained/amplified included (a) protein ubiquitination pathway, (b) purine metabolism, (c) IGF-I signaling, (c) starch and sucrose metabolism, (d) lysine biosynthesis, (e) arginine and proline metabolism, (f) VEGF signaling, (g) inositol metabolism, and (h) phosphatidylinositol 3-kinase/AKT signaling.

We identified 1,007 genes that were significantly down-regulated when lost (Supplementary Table S7). Ingenuity Pathway analysis of these genes revealed nine networks significantly enriched for these genes (Supplementary Table S8). In addition, the following canonical pathways were significantly enriched for genes down-regulated when lost: (a) purine metabolism, (b) ubiquinone biosynthesis, (c) nicotine and nicotinamide metabolism, (d) mitochondrial dysfunction, (e) protein ubiquitination pathway, (f) oxidative phosphorylation, (g) estrogen receptor signaling, (h) inositol phosphate metabolism, (i) IGF-I signaling, (j) arginine and proline metabolism, (k) nucleotide excision repair pathway, (l) PTEN signaling, (m) Fcγ receptor-mediated phagocytosis, (n) T-cell receptor signaling, (o) pentose phosphate pathway, (p)

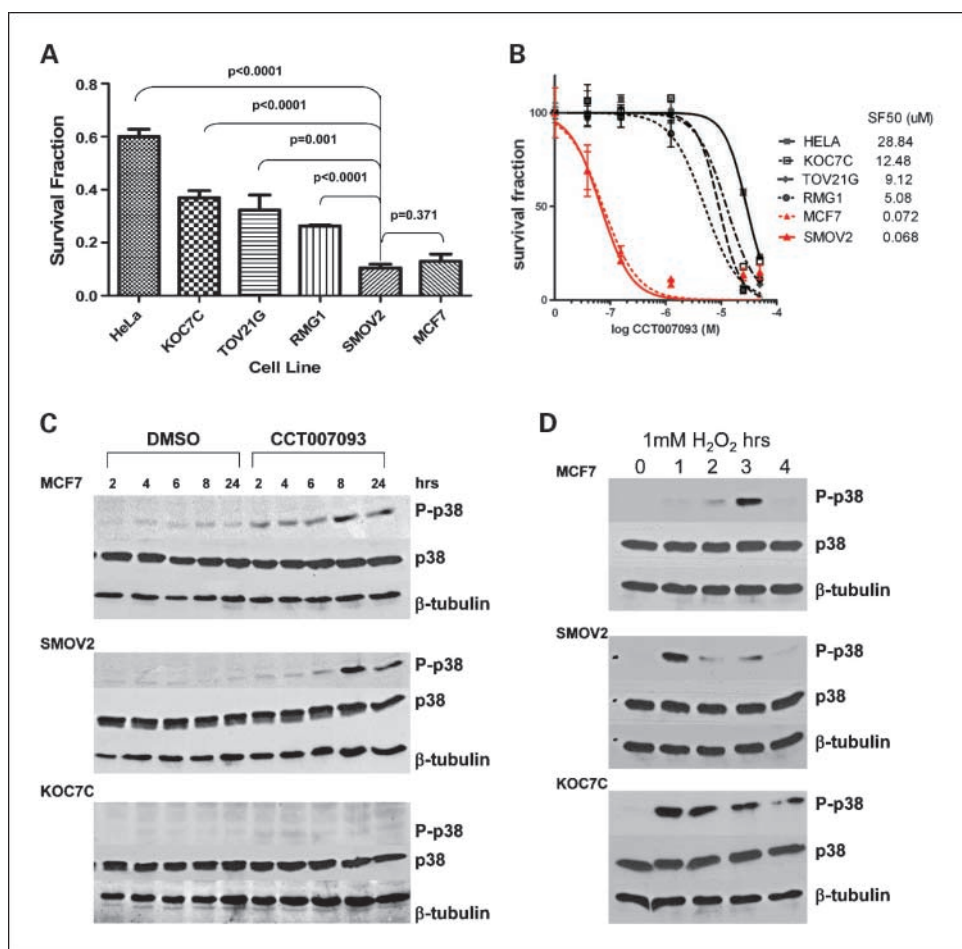


Fig. 3. PPM1D expression and phosphatase activity is required for the survival of cells with 17q23.2 amplification. **A**, PPM1D shRNA 14-d colony formation assay. The surviving fraction was calculated for each cell line using the equation surviving fraction (%) = (number of colonies from cells transfected with pSUPER-PPM1D / number of colonies from cells transfected with pSUPER-control) × 100. Columns, mean from three independent experiments; error bars, 2 SE. *P* values (unpaired *t* test statistic) represent significance of difference in survival fraction for SMOV2 relative to other cell lines. There was no significant difference between SMOV-2 and MCF7 cells in their sensitivity to PPM1D inhibition. **B**, PPM1D inhibitor (CCT007093) 14-d colony formation assay of four ovarian clear cell carcinoma cell lines (SMOV-2, RMG1, KOC7C, TOV21G) with SF50 inhibitor concentrations shown on the right of graph. The SMOV2 (PPM1D overexpressed and amplified) cell line showed increased sensitivity to CCT007093 compared with the other ovarian clear cell carcinoma cell lines without PPM1D overexpression. MCF7 (positive control) cells have previously been shown to be sensitive to CCT007093 and PPM1D shRNA inhibition whereas HeLa cells (negative control) have been shown to be resistant to PPM1D inhibition by both methods (32, 45). Black curves, cell lines without 17q23.2 amplification; red curves, cell lines with 17q23.2 amplification. **C**, p38 phosphorylation in MCF7, SMOV-2, and KOC7C cell lines treated with CCT007093. Cells were treated with CCT007093 and lysates were collected at the time points shown. Western blotting to detect phosphorylated p38 (P-p38), total p38, and β-tubulin was done as described in Materials and Methods. DMSO was used as the negative control. In CCT007093-sensitive cell lines (MCF7 and SMOV-2), p38 phosphorylation is observed at 4 h posttreatment, peaking at 8 h before decreasing by 24 h. p38 phosphorylation does not occur in CCT007093-resistant cell lines (KOC7C). **D**, p38 phosphorylation in response to H₂O₂ (1 mmol/L) in PPM1D-amplified (MCF7 and SMOV-2) and nonamplified (KOC7C) cell lines. Western blotting to detect phosphorylated p38, total p38, and β-tubulin was done as described in Materials and Methods. Note that despite the distinct response patterns to H₂O₂ stimulation, these cell lines displayed intact p38 pathway activation.

glutamate receptor signaling, (*q*) cell cycle: G₁-S checkpoint regulation, (*r*) pyrimidine metabolism, and (*s*) chondroitin sulfate biosynthesis.

Correlation of aCGH and Illumina gene expression data in highly amplified regions reveals likely amplicon drivers in ovarian clear cell carcinomas. In total, 65 regions of high-level amplification (Log₂ratio >0.8) were identified (Supplementary Table S3) and mRNA levels for all genes in these regions were retrieved from Illumina gene expression profiling of all 12 ovarian clear cell carcinoma cell lines. Given that genes that are overexpressed when amplified are the likeliest amplicon drivers (4), a Pearson's correlation was done for each gene within each of the 65 high-level amplicons to identify genes whose expression was significantly correlated with copy number states (as defined by aws ratios) across all 12 cell lines (Supplementary Table S9). A subsequent analysis where the differential expression of each gene in each amplicon was compared between cell lines with gains/amplifications versus cell lines without gains/amplifications using the unpaired *t* test (Supplementary Table S9) to identify genes whose increased expression was significantly associated with copy number gains/amplification. All genes that were significantly correlated with aws ratios and associated with copy number gain for Pearson's correlation test ($P < 0.05$) and the unpaired *t* test ($P < 0.05$), respectively, are shown in Table 2 and Supplementary Table S10. Of this list of amplicons, the 17q21-q24 region is of particular interest given that copy number gains of this region have previously been reported to be associated with poor outcome in ovarian clear cell carcinoma (11). Our analysis identified four genes whose expression correlated with copy number gains/amplifications on the 17q21-24 amplicon: *WNT3* (17q21.31-q21.32), *RSAD1* (17q21.33), *PCTP* (17q22), and *PPM1D* (17q23.2) (Table 2). Hirasawa et al. (11) previously reported that only *PPM1D* and *APPBP2* expression levels correlated with gains of 17q21-q24 in ovarian clear cell carcinoma. By overlaying our results with those of Hirasawa et al. (11), the only concordant gene was *PPM1D*, a known oncogene (38) associated with poorer outcomes in ovarian clear cell carcinoma when overexpressed at the mRNA level (11), for which a small-molecule inhibitor CCT007093 has recently been described (32). Taken together, these results led us to hypothesize that the *PPM1D* gene could be a potential therapeutic target in ovarian clear cell carcinomas with 17q23.2 amplification.

Defining the smallest region of amplification in cell lines with gains of 17q21-q24. Recurrent gains of genomic material were observed on 17q21-q24.1 in seven cell lines (SMOV-2, RMG1, ES2, OVAS, OVSAYO, OVTOKO, and OVMANA). Only SMOV-2 harbored a high-level focal amplification of 17q23.2 (Log₂ratio = 0.84; Fig. 2A; Supplementary Tables S1 and S2), which was validated by FISH. Our results confirmed the presence of high-level 17q23.2 amplification (*PPM1D*:CEP17 ratio >2.0 corresponding to aws Log₂ratio >0.8 and CISH >5 copies/nucleus in >50% of cells) in the SMOV-2 cell line (Fig. 2B), and gains in RMG1, ES2, OVAS, OVSAYO, OVTOKO, and OVMANA.

The smallest region of amplification in the 17q21-q24.1 region mapped to 17q23.2 in SMOV-2 cells spanned 642 kb from 55,503 to 56,145 kb and contained seven genes: amplified in breast cancer 1 (*ABC1*), carbonic anhydrase 4 precursor (*CA4*), ubiquitin-specific-processing protease

32 (*USP32*), chromosome 17 open reading frame 64 (*C17orf64*), the amyloid β precursor protein-binding protein 2 (*APPBP2*), the protein phosphatase magnesium-dependent 1 δ (*PPM1D*), and breast cancer amplified sequence 3 (*BCAS3*). Of these seven genes, *C17orf64* expression was not significantly detected in any of the ovarian clear cell carcinoma cell lines and was therefore excluded from subsequent analysis. Of the remaining genes, only *PPM1D* displayed expression levels that significantly correlated with gene copy number ($R = 0.68$, $P = 0.006$, Pearson's correlation) and were significantly associated with gains/amplification of 17q23.2 ($P = 0.03$, two-tailed unpaired *t* test; Fig. 2C; Table 2). These findings suggest that *PPM1D* is one of the 17q23.2 amplicon drivers (Table 2; Supplementary Table S3).

As expected, SMOV-2 cells, which harbor a high level of amplification of 17q23.2, displayed the highest level of *PPM1D* mRNA expression. Consistent with these results, Western blot analysis revealed higher levels of *PPM1D* protein expression in *PPM1D*-amplified SMOV-2 (17q23.2 amplified) cells compared with other ovarian clear cell carcinoma cell lines, including RMG1 (17q23.2 gained), KOC7C (no gain), and TOV21 (no gain) cells (Fig. 2D).

RNA interference-mediated *PPM1D* silencing and CCT007093-mediated *PPM1D* inhibition result in reduced survival of SMOV-2 cells. Given that *PPM1D* has oncogenic properties (38), is significantly overexpressed when gained/amplified in ovarian clear cell carcinoma cell lines, and is associated with poorer survival in ovarian clear cell carcinomas (11), we tested whether *PPM1D* expression and phosphatase activity would be required for the survival of *PPM1D*-overexpressing cells harboring 17q23.2 amplification. RNA interference-mediated *PPM1D* silencing and CCT007093 cell survival assays were done on four ovarian clear cell carcinoma cell lines: SMOV-2 (17q23.2 amplified), RMG1 (17q23.2 gained), KOC7C (no gain), and TOV21 (no gain). Negative and positive controls included HeLa and MCF7 cell lines, respectively. MCF7 cells were chosen as a positive control given that these cells harbor 17q23.2 amplification, overexpress *PPM1D*, and are dependent on *PPM1D* expression for their survival (32).

Transfection of the pSuper-*PPM1D* shRNA into ovarian clear cell carcinoma cell lines led to a significant reduction of *PPM1D* mRNA and protein levels 24 and 48 hours after transfection (Supplementary Fig. S2). Colony formation assay comparing cell lines transfected with *PPM1D* shRNA and scramble control revealed significant inhibition of cell survival in SMOV-2 cells compared with TOV21 ($P = 0.001$), KOC7C ($P < 0.0001$), and RMG1 cell lines ($P < 0.0001$; all two-tailed unpaired *t* test, Fig. 3A). In fact, the reduction in the survival fraction of SMOV-2 mediated by *PPM1D* shRNA was comparable with that observed in MCF7 breast cancer cells (32). We next tested whether *PPM1D* phosphatase activity would be required for the survival of SMOV-2 cells by treating these cells and nonamplified ovarian clear cell carcinoma cell lines with the *PPM1D* inhibitor CCT007093 (Fig. 3B; ref. 32). SMOV-2 cells displayed an increased sensitivity to CCT007093 compared with RMG1, KOC7C, and TOV21G. Notably, the survival fraction 50% (SF₅₀) for SMOV-2 (SF₅₀ = 0.068 μ m) was remarkably similar to that of MCF7 (SF₅₀ = 0.072 μ m; Fig. 3B), which has previously been shown to display an exquisite sensitivity to *PPM1D* inhibition by CCT007093 (32).

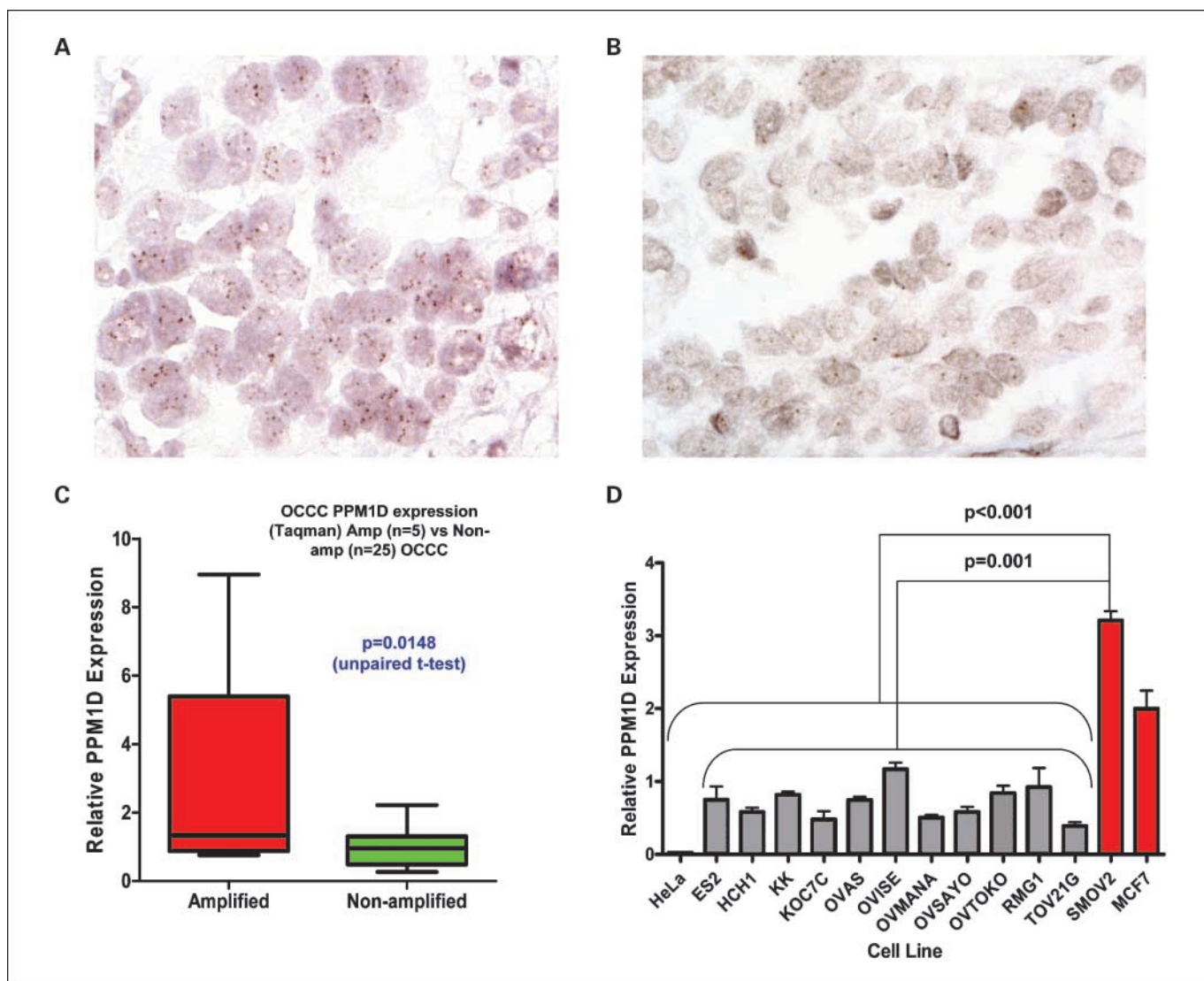


Fig. 4. PPM1D is overexpressed in primary ovarian clear cell carcinomas and ovarian clear cell carcinoma cell lines harboring 17q23.2 amplification. *A*, primary ovarian clear cell carcinoma harboring *PPM1D* gene amplification with *PPM1D*-CISH probe. *B*, primary ovarian clear cell carcinoma lacking *PPM1D* gene amplification with *PPM1D*-CISH probe. *C*, qRT-PCR results showing significantly higher levels of PPM1D mRNA expression in *PPM1D*-amplified versus nonamplified ovarian clear cell carcinoma tumors ($P = 0.0148$, unpaired t test). *D*, qRT-PCR analysis demonstrating that like primary ovarian clear cell carcinomas, a primary ovarian clear cell carcinoma cell line harboring *PPM1D* gene amplification displays *PPM1D* mRNA levels significantly higher than those found in nonamplified ovarian clear cell carcinoma cell lines ($P < 0.001$, unpaired t test, comparing only ovarian clear cell carcinoma cell lines; $P < 0.001$, unpaired t test, when ovarian clear cell carcinoma cell lines and HeLa cells were included) and similar to the levels of a breast cancer cell line harboring *PPM1D* gene amplification (MCF7).

Inhibition of PPM1D by CCT007093 is associated with enhanced p38 kinase activity in SMOV-2 cell lines. The reduction in cell survival of MCF-7 cells mediated by CCT007093 is dependent on reacquisition of p38 mitogen-activated protein kinase activity (32). To test whether the same phenomenon occurs in CCT007093-sensitive ovarian clear cell carcinoma cell lines, we assessed SMOV-2 and KOC7C cell lines for p38 phosphorylation following CCT007093 treatment. We showed that CCT007093 induces p38 phosphorylation at 4 hours posttreatment in MCF-7 and SMOV-2 cells (CCT007093 sensitive), increasing to a peak at 8 hours and then decreasing toward basal levels by 24 hours (Fig. 3C). Consistent with its previously reported selectivity in *PPM1D*-amplified cell lines (32), CCT007093 did not induce p38 phosphorylation in KOC7C cells (relatively resistant to

CCT007093; Fig. 3B). Importantly, MCF7, SMOV-2, and KOC7C displayed an intact p38 response to H_2O_2 (Fig. 3D).

SMOV-2 does not harbor TP53 mutations. Given that the oncogenic activity of PPM1D seems to be mediated via negative regulation of the p38-P53 pathway (38), we did sequencing of known *TP53* mutation hotspots on exons 5-9 for all cell lines. Of 12 cell lines, only ES2 had a missense mutation in exon 7 (c.722C > T, p.S241F; Table 1).

Amplification and overexpression of PPM1D is found in primary ovarian clear cell carcinomas. To determine the prevalence of *PPM1D* gene amplification in epithelial ovarian carcinoma, we performed CISH on a TMA composed of 88 pure ovarian clear cell carcinomas, and an independent unselected series of 167 epithelial ovarian carcinoma tumors in a separate TMA. *PPM1D* gene copy number analysis by CISH revealed

PPM1D gene amplification in 6 of 59 (10%) assessable tumors in the ovarian clear cell carcinoma TMA (Fig. 4A and B) and in 3 of 30 ovarian clear cell carcinomas (10%) in the TMA of unselected epithelial ovarian carcinoma tumors. The presence of *PPM1D* amplification was significantly associated with pure ovarian clear cell carcinomas ($P = 0.0003$, Fisher's exact test), being observed in 9 of 89 pure ovarian clear cell carcinoma samples and in none of the other epithelial ovarian carcinoma subtypes (0 of 137). Given that follow-up information was available only for the samples included in the TMA containing pure ovarian clear cell carcinomas and that only 10% of cases harbored gene amplification, the numbers were insufficient for survival analysis to be done.

We subsequently determined *PPM1D* mRNA expression levels in ovarian clear cell carcinomas by qRT-PCR analysis and investigated whether *PPM1D* expression levels would correlate with amplification of 17q23.2, as defined by CISH. This analysis revealed that *PPM1D* mRNA levels were significantly higher in cases with 17q23.2 amplification than those of ovarian clear cell carcinomas lacking amplification of this genomic locus ($P = 0.01$, two-tailed unpaired t test; Fig. 4C). qRT-PCR analysis also confirmed that the 17q23.2-amplified SMOV-2 cell line displayed significantly higher levels of *PPM1D* mRNA expression than ovarian clear cell carcinoma cells lacking 17q23.2 amplification (Fig. 4D). In summary, our results suggest that *PPM1D* may constitute a potential therapeutic target in a subset of pure ovarian clear cell carcinomas.

Discussion

We present data from the first integrated genomic and transcriptomic characterization of ovarian clear cell carcinoma cell lines. Although ovarian clear cell carcinoma cell lines had heterogeneous genomic profiles (Fig. 1), they consistently displayed a transcriptome similar to that of primary ovarian clear cell carcinomas. By overlaying aCGH and gene expression data, we identified several key canonical pathways (Supplementary Tables S7 and S9) in ovarian clear cell carcinoma cell lines whose activity may at least in part be mediated by copy number changes. Further studies on the role of these pathways in the development and progression of ovarian clear cell carcinomas are warranted.

The frequency, type, and pattern of genomic changes observed in our cell lines (Fig. 1B) seem to mirror those previously reported in primary ovarian clear cell carcinoma tumors using conventional comparative genomic hybridization (1). In particular, we observed gains of 8q, 17q, and 20q and losses on 9p, 11q, 16p/q, and 19p in >30% of the cell lines. Similar gains and losses in these regions with frequencies of >20% have previously been reported in ovarian clear cell carcinomas (9, 11, 12). Indeed, cell lines and phenotypically matched primary tumors have been shown to have similar molecular features (15), and the selection of optimal *in vitro* models of specific subtypes of primary cancers can be achieved by matching the aCGH and gene expression profiles of these tumors with those of the well-characterized cell lines (6, 14).

To identify candidate therapeutic targets, we focused our analysis on genes identified within regions of high-level amplification significantly associated with increased expression when gained in our ovarian clear cell carcinoma cell lines

(Table 2). In a previous conventional CGH study (11), although recurrent gains of 17q21-24 were observed at a similar frequency as in our cell lines, high-level copy number gains of the region, which spans a relatively large (~32.9 Mb) and gene-rich region harboring a host of other putative oncogene candidates (39), including *PPM1D*, was identified in only 1 of 20 ovarian clear cell carcinomas. Interestingly, the study also reported a correlation between gains of 17q21-24 and mRNA expression levels of *PPM1D* and *APPBP2*. Here, the high resolution of our aCGH platform has allowed us to clearly identify a small bona fide region of amplification (17q23.2) in the 17q21-q24 region and the genes within it. In addition, our analysis revealed that *PPM1D* was the only gene within the amplicon whose expression levels correlated with gene copy number ($P = 0.006$, Pearson's Correlation) and whose overexpression was associated with copy number gains/amplification ($P = 0.03$, two-tailed unpaired t test; Table 2). Although gains of 17q21-24 and *PPM1D* overexpression were previously shown to be associated with reduced survival in a cohort of 11 patients with ovarian clear cell carcinomas (11), these associations could not be validated in our cohort of primary pure ovarian clear cell carcinomas given the small number of events and the prevalence of *PPM1D* gene amplification. Larger cohorts of patients with ovarian clear cell carcinoma are required to assess the prognostic effect of 17q23.2 amplification and *PPM1D* overexpression.

PPM1D is a protein phosphatase with established oncogenic functions (38). Current evidence suggests that *PPM1D* inhibits p38 mitogen-activated protein kinase, resulting in suppression of p53 activation (40, 41), and is therefore a negative feedback regulator of the p38-p53-*PPM1D* signaling pathway following genotoxic stress (40). This antiapoptotic function of *PPM1D* may be particularly pertinent in driving the pathology of tumors with wild-type *TP53*. The relevance of this hypothesis to ovarian clear cell carcinoma is given further credence by the absence of *TP53* mutations in all but one ovarian clear cell carcinoma cell line in our study and those of previous studies showing that *TP53* mutations are remarkably uncommon in ovarian clear cell carcinomas compared with other epithelial ovarian carcinoma subtypes (42). In addition, *PPM1D* amplification and overexpression have previously been observed in 11% to 16% of breast cancers, the majority of which are *TP53* wild type (43), suggesting that an inverse correlation between the presence of *TP53* mutations and *PPM1D* amplification may exist in tumors. *PPM1D* has already been shown to be a promising therapeutic target in breast cancer (32).

We identified the SMOV-2 cell line as a model for ovarian clear cell carcinomas harboring the 17q23.2 amplicon. This cell line was originally derived from a primary ovarian clear cell carcinoma and xenograft studies in nude mice have revealed histopathologic features consistent with the original tumor, including hobnail cells and clear cells (17). shRNA-mediated *PPM1D* knockdown and *PPM1D* phosphatase activity inhibition were selectively lethal in cells harboring 17q23.2 amplification (i.e., SMOV-2 and MCF7), indicating that the survival of these cells is dependent on *PPM1D* expression and phosphatase activity. This suggests that *PPM1D* is one of the drivers of the 17q23.2 amplicon in ovarian clear cell carcinoma cell lines. In addition, we have shown that 17q23.2 amplification occurs in at least 10% of primary pure ovarian clear cell carcinomas in two large independent series of ovarian clear

cell carcinoma and epithelial ovarian carcinoma. The observed absence of *PPM1D* amplification in other epithelial ovarian carcinoma subtypes suggests that the phenomenon of *PPM1D* amplification is significantly more prevalent in primary pure ovarian clear cell carcinomas ($P = 0.0003$, Fisher's exact test). Furthermore, we provide direct evidence that *PPM1D* amplification (rather than gains of 17q21-q24; ref. 11) is significantly associated with *PPM1D* mRNA overexpression in primary pure ovarian clear cell carcinomas (Figs. 2C, D and 4D). Although *PPM1D* has previously been functionally validated as an amplicon driver in other tumor types (44, 45), this is the first study to provide strong circumstantial evidence to suggest that *PPM1D* is one of the drivers of the 17q23.2 amplicon in primary ovarian clear cell carcinomas, and that this serine/threonine phosphatase is a potential novel therapeutic target for a subgroup of ovarian clear cell carcinomas harboring 17q23.2 amplification. Finally, given the predictive nature of 17q23.2 amplification and sensitivity to a *PPM1D* inhibitor in ovarian clear cell carcinoma cell lines, *PPM1D* gene status, like *HER2* gene status assessment in breast cancer (8), may constitute a predictive marker for future clinical studies.

Our study highlights the value of an integrated genomic, transcriptomic, and functional approach in the study of ovarian clear cell carcinoma tumors. Based on our results, it is highly likely that the other genes significantly associated with increased copy number within regions of high-level amplifica-

tion identified in our study (Table 2) may be additional key molecular drivers that could, with appropriate functional validation, emerge as therapeutic targets in these tumors as well. Included in this list are genes with previously reported oncogenic properties that have also been found to be overexpressed when amplified in other tumor types, such as cortactin (*CTTN*) and oral cancer overexpressed 1 (*ORAOV1*) on 11q13.2-q13.4 (46, 47), and matrix-metalloproteinase 1 (*MMP1*) and yes-associated protein 1 (*YAP1*) on 11q22.1-q22.3 (48, 49).

Further integrated high-throughput molecular analysis approaches designed to characterize ovarian clear cell carcinomas and cell lines at the genetic, transcriptomic, and epigenetic level, followed by a correlative functional interrogation of potential molecular drivers, are likely to expedite the development of novel therapeutic strategies and identification of therapeutic targets in the management of this enigmatic tumor type.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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