Resistance to CDK2 Inhibitors Is Associated with Selection of Polyploid Cells in CCNE1-Amplified Ovarian Cancer


Abstract

Purpose: Amplification of cyclin E1 (CCNE1) is associated with poor outcome in breast, lung, and other solid cancers, and is the most prominent structural variant associated with primary treatment failure in high-grade serous ovarian cancer (HGSC). We have previously shown that CCNE1-amplified tumors show amplicon-dependent sensitivity to CCNE1 suppression. Here, we explore targeting CDK2 as a novel therapeutic strategy in CCNE1-amplified cancers and mechanisms of resistance.

Experimental Design: We examined the effect of CDK2 suppression using RNA interference and small-molecule inhibitors in SK-OV-3, OVCAR-4, and OVCAR-3 ovarian cancer cell lines. To identify mechanisms of resistance, we derived multiple, independent resistant sublines of OVCAR-3 to CDK2 inhibitors. Resistant cells were extensively characterized by gene expression and copy number analysis, fluorescence-activated cell sorting profiling and conventional karyotyping. In addition, we explored the relationship between CCNE1 amplification and polyploidy using data from primary tumors.

Results: We validate CDK2 as a therapeutic target in CCNE1-amplified cells by showing selective sensitivity to suppression, either by gene knockdown or using small-molecule inhibitors. In addition, we identified two resistance mechanisms, one involving upregulation of CDK2 and another novel mechanism involving selection of polyploid cells from the pretreatment tumor population. Our analysis of genomic data shows that polyploidy is a feature of cancer genomes with CCNE1 amplification.

Conclusions: These findings suggest that cyclin E1/CDK2 is an important therapeutic target in HGSC, but that resistance to CDK2 inhibitors may emerge due to upregulation of CDK2 target protein and through preexisting cellular polyploidy.

Introduction

Deregulation of the cell cycle is a hallmark of cancer and is therefore an attractive therapeutic target. Despite this, the clinical usage of cell-cycle inhibitors has been disappointing to date. In contrast with the development of other targeted agents in cancer, surprisingly few cell-cycle inhibitors have involved selection of patients based on molecular features. Identifying predictive biomarkers and patient subsets that are most likely to benefit from cell-cycle inhibitors is important to the clinical development of these agents.

High-grade serous ovarian cancer (HGSC) is the most common subtype of epithelial ovarian cancer. Recent studies have identified a high frequency of TP53 mutations, BRCA dysfunction and clinically relevant gene expression subtypes. In addition, genomic instability and widespread copy number changes seem to be a mechanism of tumor evolution and may also influence treatment response. For example, genomic amplification of 19q12 incorporating cyclin E1 (CCNE1) in approximately 20% of HGSC is associated with poor overall survival and primary treatment failure.

Cyclin E1 forms a complex with CDK2 to regulate G1–S transition by phosphorylation of downstream targets including the tumor suppressor RB1. Deregulation of the cell cycle in tumors is thought to induce a hyper-proliferative phenotype, leading to genomic instability and driving malignant transformation. Recent functional studies in vitro have shown “oncogene addiction” to maintained CCNE1...
Translational Relevance

Cyclin E1 (CCNE1) is amplified in various tumor types including high-grade serous ovarian cancer where it is associated with poor clinical outcome. We show that suppression of the cyclin E1 partner kinase, CDK2, induces apoptosis in a CCNE1 amplicon-dependent manner. Little is known of mechanisms of resistance to CDK inhibitors. We therefore generated cells with reduced sensitivity to CDK2 inhibitors and identified two bypass mechanisms, one involving CDK2 upregulation and another associated with the selection of pre-existing polyploid cells from a heterogeneous parental population. Using primary tumor data, we show for the first time that polyploidy is a common and specific feature of CCNE1-amplified cancers. These findings validate CDK2 as a novel therapeutic target in CCNE1-amplified tumors and preemptively identify mechanisms of resistance that may influence clinical response.

expression when amplified, evidenced by amplicon-dependent attenuation of cell viability, clonogenic survival, induced G1 arrest, and increased apoptosis after siRNA-mediated knockdown (7, 8). Therapeutically, CCNE1 function may most readily be targeted via its partner kinase CDK2. Currently, there are more than 20 small-molecule CDK inhibitors in clinical trials for various cancer types (1, 2). These compounds are generally classified as pan-CDK or highly selective inhibitors and act by inducing cell-cycle arrest and apoptosis via inhibition of cell-cycle kinases (Cdk1,2,4,6) and/or transcriptional Cdks (Cdk7,8,9; ref. 9).

We aimed to determine whether ovarian tumor cells with CCNE1 gene amplification are selectively sensitive to inhibition of CDK2 by gene knockdown or with small-molecule inhibitors. We also explored potential mechanisms of resistance to CDK inhibition to preempt the likely emergence in patients.

Materials and Methods

Cell lines

Ovarian cell lines were obtained from the National Cancer Institute Repository (NCI, Bethesda, MD) and fingerprinted using short tandem repeat (STR) markers to confirm identity against the Cancer Genome Project database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom). Primer sequences for six STR markers (CSF1PO, TPOX, THO1, vWA, D16S539, D7S820, D5S818) and analysis have been previously described (10).

Gene suppression studies

Methods and transfection conditions for siRNA studies have been previously described (7). Microarray data from short hairpin RNA (shRNA) experiments were obtained from the Integrative Genomics Portal and analyzed using the GENE-E software (11). Cell line copy number data were obtained from the Cancer Cell Line Encyclopedia (12).

Inhibitors and drug sensitivity assays

PHA-533533 was obtained from Pfizer and dinaciclib from Merck. Cells were maintained at 37°C and 5% CO2 in RPMI-1640 containing 10% (v/v) fetal calf serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. Drug sensitivity was assessed using a 72-hour viability assay (MTS) and a 7-day clonogenic survival assay. For the viability assay, 5,000 cells were seeded in 96-well plates and allowed to attach overnight before the addition of drug at various concentrations. After 72 hours of drug incubation, cell viability was determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). For clonogenic survival assays, single cells were seeded at low density in 6-well plates, allowed to attach overnight before the addition of drug. After 7 days of growth in drug, cell colonies were washed, fixed, and stained with 20% (v/v) methanol and 0.1% (w/v) crystal violet. Cells were rinsed in water, air-dried, digitally scanned, and discrete colonies (>50 cells per colony) counted using MetaMorph (Molecular Devices). IC50 dose was approximated by fitting a four-parameter dose–response curve (Hill equation) using Prism 5 (GraphPad Software).

Molecular methods

Cell line DNA was extracted using a DNeasy Kit (Qiagen) for quantitative-PCR (qPCR) of CCNE1 DNA copy number status as described previously (5) or for single-nucleotide polymorphism (SNP) microarray analysis (below). Total RNA was extracted from cell pellets using the mirVana RNA Isolation Kit (Life Technologies) for gene expression profiling or reverse transcribed using M-MLV (Promega) before SYBR green real-time PCR. Experimental details for gene expression analysis, including primer sequences, have been described elsewhere (5).

Western blot analysis

Whole-cell protein lysates were boiled, resolved by SDS-PAGE using 12.5% (w/v) acrylamide gels, and then transferred to polyvinylidene difluoride membranes. Blots were blocked in 5% (w/v) non-fat milk powder in PBS-T (0.1% Tween 20 in PBS) and probed overnight at 4°C in primary antibody against cyclin E1 (clone HE12; Santa Cruz Biotechnology), CDK2 (clone D-12; Santa Cruz Biotechnology), p-Rb (Ser 807/811; Cell Signaling Technology), or the p89 PARP1 caspase cleavage fragment (Cell Signaling Technology), or the p89 PARP1 caspase cleavage fragment (Cell Signaling Technology). Membranes were washed in PBS-T and incubated with peroxidase-conjugate secondary antibody for 1 hour at room temperature, washed, and developed by chemiluminescence before being exposed to radiographic film. Blots were reprobed with an antibody against α-tubulin to assess protein loading.

Generation of cell lines resistant to CDK inhibitors

Subconfluent cells in 6-well plates were treated with PHA-533533 or dinaciclib at the IC50 dose (4 μmol/L and
10 nmol/L, respectively, based on a 72-hour cytotoxicity assays) for two 72-hour periods (media removed and fresh drug added) after which surviving cells were allowed to repopulate the culture for 96 hours. The process was repeated once and the remaining cells were cultured in the presence of drug for three additional passages. Selected cells (passage 4) were then maintained either in the presence or absence of drug to monitor the change in drug sensitivity over time. Cell pellets were collected on dry ice and stored at –80°C before nucleic acid extraction.

**Proliferation assays**

To determine proliferation rate, 50,000 cells were seeded in multiple wells of 6-well plates. Cells were collected from triplicate wells and counted using a Countess automated cell counter every 24 hours for 4 days.

**SNP and gene expression microarrays**

Illumina OmniExpress microarrays were run as a service from Australian Genome Research Facility according to the manufacturer’s instructions. Data were extracted using Illumina’s GenomeStudio v2010.3 with Genotyping module 1.8.4 software, with the default Illumina settings and illumina HumanOmniExpress-12v1_H manifest cluster file. Normalized log2 R ratios (log2 ratio of observed normalized signal intensity to expected intensity) were segmented using circular binary segmentation (13) and the regions of copy number change per gene estimated using the mean segment value. Regions of gain or amplification were defined as those where the mean segment log2 R ratio value was more than 0.3; losses were defined as regions of log2 R less than –0.3. Data visualization was conducted using Partek Genomics Suite 6.6 (Partek Inc.). Affymetrix Gene ST 1.0 microarrays were conducted and data normalized using the GC robust multi-array average (GCRMA) method available in the R package as described previously (14). Molecular profiles of stable and selected cells were compared with that of the parental cells and differentially expressed genes identified using empirical Bayes methods available in the R-package limma (15). A gene was selected as differentially expressed if the false discovery rate (FDR) was less than 5%. Pathway analysis was conducted in either GeneGo (Thomson Reuters) or Gene Set Enrichment Analysis (GSEA; Broad Institute of Harvard and MIT, Cambridge, MA). Complete copy number and gene expression microarray data are available from the Gene Expression Omnibus (Accession ID GSE48921). Methods for the analysis of copy number data obtained from The Cancer Genome Atlas (TCGA) are described elsewhere (16).

**Cell-cycle analysis**

All viable and dead cells were collected 48 hours after drug treatment for death assessment and cell-cycle analysis by flow cytometry. For characterization of cell ploidy, viable cells were collected from subconfluent cultures. Collected cells were fixed in ethanol and stained with propidium iodide (PI) as described previously (7). Up to 10,000 single cell events were recorded using a FACS Canto II flow cytometer (BD Biosciences). Cell-cycle profiles and percentage of cells in each cell-cycle phase for populations of each ploidy were modeled using Modfit LT (Beckman Coulter).

**Cell sorting by ploidy**

Approximately 2 × 10^6 OVCAR-3 parental cells were collected, resuspended as a single cell suspension, and filtered through a 70 μm filter to eliminate clumps and aggregates. Cells were then stained with the live-cell DNA-selective Vybrant DyeCycle Violet stain (Life Technologies), incubated at 37°C for 30 minutes, and hypotriploid (G1 subpopulation peak), and hyperpentaploid (G2 subpopulation peak) cells sorted by flow cytometry using the Aria II system (BD Biosciences). Sorted cells were expanded, then resorted, to further enrich for each population. Purity of established cultures was assessed by PI staining as described above.

**Karyotyping**

Cells were treated with colcemid (0.2 μg/mL) for 30 minutes, harvested, incubated in 0.075 mol/L hypotonic KCl at 37°C for 30 minutes, fixed in methanol:acetic acid (3:1), dropped onto glass slides, and G-banded with trypsin and Leishman stain according to standard cytogenetic techniques.

**Immunohistochemistry**

Sections from formalin-fixed paraffin embedded tissue blocks were cut to 4 μm, dried at 60°C for 30 minutes, and stained with cyclin E1-specific clone HE12 on a Ventana BenchMark ULTRA immunostainer (Ventana Medical Systems). The Ventana staining protocol using the OptiView DAB IHC Detection Kit (Catalogue Number 760-700) included pretreatment with cell conditioner 1 (pH 8.5) for 64 minutes, followed by incubation with diluted HE12 antibody (Santa Cruz Biotechnology, Inc.) at 36°C for 12 minutes. Antibody incubation was followed by counterstaining with hematoxylin II and bluing reagent for 4 minutes each. Subsequently, slides were removed from the immunostainer, washed in water with a drop of dishwashing detergent, and mounted. No chromogen was detected when primary antibody cyclin E (HE12) was omitted.

**Results**

**CCNE1-amplified tumor cells require CDK2 for survival**

We selected tumor cell lines that either had no CCNE1 copy number change (SK-OV-3), low-level gain (OVCAR-4), or high-level amplification (OVCAR-3) based on our previous analysis of 19q12 copy number (7). Copy number of CCNE1 was strongly associated with gene expression in these lines, but CDK2 expression was unrelated to CCNE1 status (Supplementary Fig. S1A). We have previously reported an amplicon-dependent decrease in cell viability after siRNA-mediated knockdown of CCNE1 (7). Consistent with this observation, we found that both CCNE1-gained and -amplified lines showed selective sensitivity to siRNA-mediated CDK2 knockdown (Fig. 1A), validated at the RNA (Supplementary Fig. S1B) and protein level (Fig. 1B). The effect was less pronounced in short-term survival...
assays, where only the CCNE1-amplified OVCAR-3 cell line showed specific sensitivity to CCNE1 or CDK2 knockdown (Supplementary Fig. S1C).

To validate our findings in a larger and diverse set of tumor cell types, we made use of data from a genome-wide shRNA screen of 102 cancer cell lines with known copy number status (12), including a high proportion of epithelial ovarian cancer (n = 25; ref. 11). Cells were infected with a pool of 54,020 shRNAs targeting 11,194 genes and grown for at least 16 doublings. The abundance of shRNA sequence relative to a reference pool was measured by microarray (11) to identify genes essential for survival. Consistent with the siRNA data, we found a statistically significant depletion of shRNAs against CCNE1 and CDK2 in CCNE1-amplified cell lines across multiple tumor types (Fig. 1C). Of the lines assayed, 23 had a copy number gain involving CCNE1 (log2 ratio > 0.3), including 11 ovarian cancer lines. The remaining 79 lines without CCNE1 amplification included 14 ovarian cancers, providing a comparison group (Supplementary Fig. S2A).

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Cyclin E1 primarily interacts with CDK2 but can also activate CDK1 and CDK3 (6); however, we found that only depletion of CDK2 shRNAs was significantly associated with reduced survival in CCNE1-amplified cells (Supplementary Table S1). shRNAs targeting CDK6 were enriched in CCNE1-amplified cells, suggesting that CCNE1 amplification protects cells from inhibition of CDK6. Alternatively, CDK6 expression may be essential in CCNE1-nonamplified cells and therefore associated with shRNA depletion.

CCNE1-amplified cells are sensitive to CDK2 small-molecule inhibitors

Two small-molecule CDK inhibitors, PHA-533533 (17) and dinaciclib (18), were obtained to examine the relative sensitivity in cell lines by CCNE1 amplification status. These compounds were selected as they show high specificity against CDK2; however, both also have in vitro activity against other kinases. PHA-533533 inhibits CDK2/A, CDK2/E, CDK5/p25, CDK1/B, GSK3B (glycogen synthase...
kinase B) and CDK4/D (IC\textsubscript{50} values of 37, 55, 65, 208, 732 nmol/L and >10 μmol/L, respectively; ref. 17), whereas the more potent inhibitor, dinaciclib, targets CDK2/E, CDK5/\textit{p}35, CDK1/B, and CDK9/T (IC\textsubscript{50} values of 1, 1, 3, and 4 nmol/L, respectively; refs. 1, 18). We observed \textit{CCNE1} amplicon-dependent sensitivity to both PHA-533533 (Fig. 2A) and dinaciclib (Fig. 2B) in clonogenic survival assays. Differential effects were less apparent in short-term viability assays (Supplementary Fig. S3), with only OVCAR-3 cells showing heightened sensitivity to dinaciclib. These results are consistent with our gene suppression experiments, where the most pronounced effects of \textit{CCNE1} and CDK2 inhibition were seen in 7-day siRNA clonogenic (Fig. 1A) and long-term shRNA culture experiments (Fig. 1C and D). Treatment with either inhibitor resulted in decreased phosphorylation of the downstream target Rb at CDK-specific serine Ser 807/811 and initiation of apoptosis, indicated by the presence of PARP cleavage products 24 hours after treatment (Fig. 2C and D). Consistent with the survival data, the strongest effects were observed in the OVCAR-3 \textit{CCNE1}-amplified cell line.

**Resistance to CDK2 inhibition generated in vitro is stable and associated with cross-resistance**

Resistance to single-agent molecularly targeted therapies is a common clinical problem (19), yet little is known of resistance mechanisms to CDK inhibitors (1). We therefore investigated resistance after extended exposure of OVCAR-3 \textit{CCNE1}-amplified cells to CDK2 inhibitors, deriving five independent cell lines that were resistant to PHA-533533 (OVCAR3-533533-R1, -R3, -R5, -R6, and -R7). Cells were pulse treated with drug at the IC\textsubscript{50} concentration (4 μmol/L) followed by recovery in media as outlined in Fig. 3A. After the selection process, the average IC\textsubscript{50} values in a 72-hour cytotoxicity assay shifted from approximately 4 to 8 μmol/L (average 2.1-fold increase in IC\textsubscript{50} value, \( P < 0.001 \); Fig. 3B) and from 0.46 μmol/L to 2.9 μmol/L in clonogenic survival assays for the R1 cell line (6.3-fold increase in IC\textsubscript{50} value; Supplementary Fig. S4A). Although more pronounced in clonogenic survival assays, the level of resistance generated was modest. We therefore also attempted to generate resistant lines after continued drug exposure (without media recovery steps). However, we found this method to be less reproducible and did not result in a higher level of resistance (data not shown). Similarly, we were unable to derive stable resistant cell lines after treatment with escalating drug doses (up to 10 μmol/L), possibly due to an increase in off-target effects at higher concentrations.

A resistant line to dinaciclib was similarly derived (Supplementary Fig. S4B). Resistance was surprisingly stable in both the 533533-R1 and dinaciclib-RD1 cell lines, and we observed little attenuation of resistance for up to 40
passages in the absence of inhibitor (Fig. 3C and Supplementary Fig. S4C). Microsatellite fingerprinting of long-term cell cultures confirmed that resistant cells were derived from the parental population, and not outgrowth of contaminating cells (see Materials and Methods). As PHA-533533 is most selective for CDK2 (20), our subsequent analyses focused mainly on lines made resistant to this drug. We next investigated whether resistance in the 533533-R1 cells also altered sensitivity to dinaciclib and other cytotoxic agents (Fig. 3D). We observed decreased sensitivity to dinaciclib ($P < 0.01$) and cisplatin ($P < 0.01$). No cross-resistance was observed with doxorubicin ($P = 0.378$). As both PHA-533533 and doxorubicin are substrates for the p-glycoprotein drug efflux pump (M. Ciomei; personal communication), the lack of cross-resistance suggests that upregulation of p-glycoprotein is unlikely to account for PHA-533533 resistance in R1 cells. Increased proliferation rates observed in resistant cells, suggest that altered drug sensitivity was not due to a reduction in growth (Supplementary Fig. S4D).

We characterized Rb-phosphorylation and induction of PARP cleavage in resistant and parental lines following PHA-533533 exposure. The degree of Rb de-phosphorylation following drug treatment was comparable in R1 and parental lines, and marginally attenuated in the R6 cell line, suggesting that resistance was unlikely to be due to decreased CDK2 signaling via Rb (Fig. 3E). In contrast, the appearance of PARP cleavage products by Western blot analysis and increased cell death, as determined by fluorescence-activated cell sorting (FACS; Supplementary Fig. S5), was only apparent at higher drug doses in resistant lines, suggesting that the induction of apoptosis was impaired downstream of Rb regulation. The observation of cross-resistance to cisplatin further supports a generalized mechanism of resistance, possibly associated with increased pro-survival signaling.
Resistance is associated with CDK2 upregulation rather than mutation

We examined potential genetic mechanisms that may confer resistance in the established cell lines, focusing initially on CCNE1 copy number and CDK2 mutation status. All resistant cell lines showed identical microarray copy number profiles at CCNE1, suggesting that resistance was not associated with a change in copy number status (Fig. 4A). In addition, no mutations in CDK2 were identified after complete exon sequencing in the parental and all five resistant cell lines (Supplementary Materials and Methods).

To broaden our genomic analysis, we compared gene expression profiles of parental with resistant cells collected immediately after drug selection or after culture in media alone (collection points T1 and T2; Fig. 3A and C). In a genome-wide analysis, we found that drug exposure resulted in substantial changes in gene expression with relatively few changes observed in the stable resistant compared with parental cells cultured in the absence of drug (Fig. 4B and C and Supplementary Data). Pathway analysis revealed significant enrichment of genes involved with AKT signaling, cell cycle, and DNA damage response in selected cells (Supplementary Table S2).

Looking specifically at CCNE1, we observed transient upregulation by exposure to PHA533533, but this was not apparent in the stably resistant lines maintained in the absence of drug (Fig. 4D). In contrast, CDK2 mRNA was upregulated in the stable resistant lines (Fig. 4D). Increased CDK2 protein was most apparent in the R6 line (Supplementary Fig. S6A). We failed to observe an alteration in the gene expression of other PHA-533533 targets including CDK1, CDK5, CDK4, or GSK3B (ref. 17; Supplementary Fig. S6B), suggesting that drug resistance was not associated with upregulation of these targets.

We also looked specifically for changes in gene copy number by SNP microarray. We identified 26 genes that were gained in at least four out of five resistant lines compared with parental cells, and 136 new deletions (Supplementary Table S3). GSEA of significantly altered positional gene sets (C1 gene set) identified 14q32 as the most significantly amplified region, incorporating 10 genes including AKT1 (Supplementary Table S4). Increased AKT1 copy

Figure 4. A, SNP microarray copy number profiles across chromosome 19 for OVCAR-3 parental and five PHA-533533-resistant cell lines. Peak amplification at 19q12 incorporating CCNE1 indicated. B, gene expression heatmap of parental and resistant cell lines after drug selection (selected) and after maintained growth in media (stable). Samples clustered by 1142 unique genes differentially expressed between each pairwise comparison (FDR-corrected P < 0.05). C, Venn diagram depicts number of significantly differentially expressed genes (FDR-corrected P < 0.05) identified in each pairwise comparison. D, dot plot of CCNE1 and CDK2 microarray gene expression in parental (n = 4) and resistant cell lines maintained in inhibitor (selected) or in media (stable; n = 5). Error bars indicate SEM. *, P < 0.05.
number is consistent with AKT1 pathway upregulation in cells cultured in the presence of drug (Supplementary Table S2), although does not seem to result in maintained gene upregulation in stable cells. The most significant regions of loss were localized to 13q12, incorporating BRCA2, and 22q13, including RBX1 (Supplementary Tables S3 and S4). Loss of RBX1 is intriguing, given its involvement with ubiquitin-mediated degradation of cyclin E1 (21), suggesting a possible mechanism of pathway deregulation. Further functional analysis is required to validate the functional significance of these and other identified changes.

**Increased DNA ploidy is associated with resistance to CDK2 inhibition**

We conducted FACS analysis to characterize the cell-cycle effects of inhibitors and noted a substantial shift in the DNA content of resistant cell lines (Fig. 5A and Supplementary Fig. S7A). Modeling of FACS data suggested the presence of two distinct populations in the parental line that were diploid or pseudo-diploid, and another with approximately double the DNA content that was possibly tetraploid or near-tetraploid. In contrast, the near-tetraploid population seemed to dominate the R1, R3, R5, and R7 cells (Fig. 5B).

Analysis of FACS data is complicated by overlapping cell-cycle profiles of multiple populations, we therefore conducted conventional karyotyping of the parental, R1 and R6 cell lines (Supplementary Table S5), with the relative frequency of karyotypes estimated across 50 metaphases (Supplementary Fig. S8A). Karyotyping confirmed the presence of two populations in parental OVCAR-3 cells, one that was hypotriploid (62–68 chromosomes) and a second that was hyperpentaploid (118–128 chromosomes; Fig. 5C and Supplementary Fig. S8B). Shared structural rearrangements between populations suggest that hyperpentaploid cells are likely to have originated after duplication of the hypotriplod genome (Supplementary Table S5). In contrast, the R1 cell line consisted almost entirely of hyperpentaploid cells (Fig. 5D and Supplementary Fig. S8C).

In the FACS analysis, the R6 line contained both hypotriploid and hyperpentaploid cells (Supplementary Fig. S8D). These findings suggested that cells with an increased DNA content had a selective advantage in the presence of CDK2 inhibitors. Upregulation of CDK2 in the R6 cell line, and a karyotype that more closely resembled the parental line, was consistent with a different mechanism of resistance.

To determine whether hyperpentaploid cells preexisting in the parental population show intrinsic resistance to CDK2 inhibitors, we used flow cytometry to isolate live cells from each population. Hypotriploid cells in G1 (A1,2) or hyperpentaploid cells in G2–M (B1,2) were collected and expanded in culture (Fig. 5E). FACS analysis of selected cells estimate an enrichment of more than approximately 90% purity of each population (Fig. 5F) and remained stable throughout the course of our experiments. Dose–response assays showed that hyperpentaploid cells had partial intrinsic resistance to the PHA-533533 inhibitor compared with the hypotriploid population, with the unsorted (parental) cells showing intermediate sensitivity (Fig. 5G). We did not see an increased resistance to cisplatin (data not shown), suggesting that the reduced sensitivity of hyperpentaploid cells to PHA-533533 is specific.

**Primary tumors with CCNE1 gene amplification are associated with polyplody**

Cancer genomes of high DNA ploidy are thought to arise as a result of discrete whole-genome-doubling (WGD) events, followed by further focal loss of chromosomal material, resulting in variations in absolute DNA ploidy values (22). Using allele-specific copy number data derived from SNP microarrays, it is possible to assess genome-doubling events and DNA ploidy bioinformatically, and we took this approach to study the relationship with CCNE1 copy number in existing data from TCGA (22).

We found that CCNE1 gain or high-level amplification was significantly associated with an increased proportion of tumors with evidence of WGD compared with unamplified tumors ($\chi^2 P < 0.0001; \text{Fig. 6A}$). Furthermore, the number of tumors with increased DNA ploidy (2+2) was higher in patients with $\text{CCNE1}$ copy number amplification ($\chi^2 P < 0.0001; \text{Fig. 6B}$ and Supplementary Fig. S9A). To determine whether the association with WGD was specific to $\text{CCNE1}$ amplification and not a generalized increase in copy number events, we assessed the proportion of genome-amplified segments in each tumor subset (16). Interestingly, tumors with more than 1 WGD event had fewer regions of copy number amplification, suggesting a specific association with $\text{CCNE1}$ (Fig. 6C). Moreover, tumors with increased $\text{CCNE1}$ copy number did not show a higher proportion of total amplification events (Fig. 6D). Tumors that showed no evidence of either WGD (Supplementary Fig. S9B) or $\text{CCNE1}$ amplification (Supplementary Fig. S9C) had a higher number of deletions, consistent with previous reports (22).

Taken together these findings suggest that high ploidy genomes are a common property of tumors with $\text{CCNE1}$ amplification. Genome-doubled HGSC samples have previously been reported to have a greater increase of cancer recurrence (22). Consistent with these findings, we found that samples without $\text{CCNE1}$ amplification and no WGD had improved overall survival over patients with at least one WGD event (Fig. 6E). In contrast, $\text{CCNE1}$-amplified tumors, irrespective of WGD status, showed the shortest overall survival. Using immunohistochemistry to interrogate primary tumor samples known to have $\text{CCNE1}$ gene amplification, we observed intense nuclear staining of cyclin E1, and identified some positively stained cells that had giant nuclei consistent with increased ploidy (Fig. 6F).

**Discussion**

Tumors with amplification of the $\text{CCNE1}$ gene are associated with poor clinical outcome in HGSC, and we...
(7) and others (8) have previously shown the essentiality of maintained CCNE1 overexpression in these tumors. Here, we show a dependency on CDK2, the partner protein of cyclin E1, in ovarian and other tumor types with amplification of the 19q12 locus. The essentiality of CCNE1 and CDK2 in ovarian tumors with 19q12
amplification is consistent with a recent report in breast cancer (23).

Expression of other 19q12 genes may also contribute to the oncogenic effect of amplification. Suggested targets include the prosurvival protein URI1 (7, 24), and CCNE1, POP4, PLEKHF1, and TSHZ3 in breast cancer (23). Furthermore, genes elsewhere in the genome such as TPX2, that we have shown to be frequently coamplified with CCNE1 (7), may function in essential cooperative networks.

To date, no clinical trials have used CCNE1 copy number status to inform treatment decisions, and our data suggest that CDK inhibitors may be an effective treatment strategy in HGSC. The use of CCNE1 copy number status as a predictive tool should be explored in a clinical setting. As with other molecularly targeted therapeutics, we also showed that resistance to CDK inhibition can emerge despite initial sensitivity. Resistance to molecularly targeted therapies can arise through mutation or amplification of target proteins or deregulation of other signaling pathway components (19). The level of resistance we observed in cell lines after prolonged drug exposure was modest and may relate to both inhibitors targeting multiple CDKs or compensation by other CDKs. We were, however, able to identify two possible mechanisms of resistance to CDK2 inhibitors; one involving upregulation of CDK2 protein, consistent with previous studies with other CDK inhibitors (1), and a second novel mechanism of resistance to CDK inhibitors associated with selection of polyploid cells.

Resistance to CDK inhibitors through naturally occurring mutations in target genes has not been described to date. We found no evidence of CCNE1 copy number change or CDK2 mutation in resistant cell lines derived after extended exposure to PHA-533533. Recent studies in Xenopus show that engineering of compound mutations in the kinase domain of Cdk2 can achieve resistance to CDK inhibitors (25). However, the requirement for multiple residue changes may limit the likelihood of emergence of resistance by mutation in vivo.

Previous studies have shown an association between tetraploidization of tumor cells to DNA-damaging agents
(26) and targeted agents (27). In our study, rather than inducing an increase in DNA ploidy leading to drug resistance, treatment with CDK2 inhibition seemed to select for preexisting polyploid cells. Defective apoptotic pathways, facilitating the survival of polyploid cells, may also influence their sensitivity to cytotoxic and targeted agents. Recently, tetraploid cells have been shown to have an increased sensitivity to Aurora B inhibition (28), presenting a potential therapeutic approach for these tumors. However, we were unable to show increased sensitivity of the 533533-R1 line or FACS-sorted hyperpentaploid cells to an Aurora B-specific inhibitor (data not shown).

Although flow cytometry-sorted hyperpentaploid cells had increased resistance to CDK2 inhibition, this was not to the extent of cells selected in the presence of drug, suggesting that high DNA ploidy does not fully account for the resistance observed in drug-exposed cell lines. Indeed, decreased genomic stability in polyploid cells may facilitate the accumulation of further genomic changes. SNP-based copy number analysis and cell karyotyping revealed structural and copy number changes that may contribute to increased resistance, including increased copy number of AKT1. Activation of the AKT pathway may promote DNA repair and cell survival and has been associated with resistance to chemo- and radiotherapy previously (29). The lack of resistance to cisplatin suggests that the reduced sensitivity of sorted hyperpentaploid cells to PHA-533533 is not due to the selection of cells with a generalized attenuation of apoptotic responses to cytotoxic agents.

Consistent with our in vitro data, we found a clear association between CCNE1 copy number increase and high DNA ploidy in primary tumors. The association was specific to CCNE1 gene amplification and not an increased number of amplification events overall. Our analysis of primary tumor data is consistent with previous in vitro studies showing that constitutive overexpression of CCNE1 does not increase the overall number of gene amplification events, but does increase the frequency of polyploid tumor cells (30). Expression of the hyperactive low-molecular weight isoform of CCNE1 has also been shown to lead to failed cytokinesis and polyploidy in breast tumor cells (31).

Our findings show that although therapeutic strategies designed to inhibit CDK2 function may prove useful in the treatment of CCNE1-amplified tumors, resistance related to a propensity for increased ploidy in these tumors is likely to emerge.

Disclosure of Potential Conflicts of Interest

P.M. Waring has commercial research grant and a honoraria from speakers’ bureau from Ventana Medical Systems. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: D. Etemadmoghadam, D.D. Bowtell
Development of methodology: D. Etemadmoghadam
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Etemadmoghadam, G. Au-Yeung, M. Wall, C. Mitchell, M. Kansara, E. Loehrer, C. Batzios, S. Founi, I. Gresshoff, W.C. Hahn, P.M. Waring
Writing, review, and/or revision of the manuscript: D. Etemadmoghadam, M. Wall, E. Loehrer, B.A. Weir, L. Mileskin, D. Ruchin, W.C. Hahn, P.M. Waring, C. Cullinane, L.J. Campbell, D.D. Bowtell
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.D. Bowtell
Study supervision: D. Etemadmoghadam, W.C. Hahn, D.D. Bowtell

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References


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Dariush Etemadmoghadam, George Au-Yeung, Meaghan Wall, et al.


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