Polo-like kinase PLK 2 is an epigenetic determinant of chemosensitivity and clinical outcomes in ovarian cancer

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Abstract:

Resistance to platinum and taxane based chemotherapy remains a major clinical impediment to effective management of epithelial ovarian cancer (EOC). To gain insights into resistance mechanisms, we compared gene and confirmed expression patterns of novel EOC cell lines selected for paclitaxel and carboplatin resistance. Here we report that resistance can be conferred by downregulation of the polo-like kinase Plk2. Mechanistic investigations revealed that downregulation occurred at the level of transcription via associated DNA methylation of the CpG island in the Plk2 gene promoter in cell lines, primary tumors, and patient sera. RNAi-mediated knockdown and ectopic overexpression established a critical functional role for Plk2 in determining apoptotic sensitivity to paclitaxel and carboplatin. In drug resistant human EOC cell lines, Plk2 promoter methylation varied with the degree of drug resistance and transcriptional silencing of the promoter. RNAi-dependent knockdown of Plk2 abrogated G2-M cell cycle blockade by paclitaxel, conferring resistance to both paclitaxel and platinum. Conversely, ectopic expression of Plk2 restored sensitivity to G2-M cell cycle blockade and cytotoxicity triggered by paclitaxel. In clinical cases, DNA methylation of the Plk2 CpG island in tumor tissue was associated with a higher risk of relapse in patients treated post-operatively with carboplatin and paclitaxel ($p= 0.003$). This trend was also reflected in analysis of matched serum samples. Taken together, our results implicate Plk2 as a clinically important determinant of chemosensitivity, in support of the candidacy of Plk2 as a theranostic marker to inform EOC management.

Introduction

The Polo-like kinases (Plks) are a family of serine-threonine kinases invovled in cell cycle regulation and cellular response to stresses e.g. DNA damage. Plk1 is the best characterised and positively regulates progression through G2 (1). Polo-like kinase 2/Snk (Plk2) and Polo-like kinase 3 Plk3/Fnk/Prk (Plk3) were identified as immediate-early transcripts in mouse fibroblasts (2 - 4), whilst Plk4 (SAK) functions in centriole biogenesis (5 - 9). Plk1 is over-expressed in various cancers supporting treatment strategies designed to target it (10, 11). Plk2 has a role in development and is required for centriole duplication (12, 13), although Plk2 -/- mice are viable (14). Plk2 induces apoptosis in Burkitt's lymphoma (BL) cells (15) and expression is directly induced by wild-type p53 (16). Under normal physiological conditions, Plk2 protein is subject to proteosomal degradation via the ubiquitin ligase hVPS18 (17). Methylation-dependent transcriptional silencing of Plk2 is common in BL (15). Transcriptional silencing of many genes is described for EOC (18). EOC is often a chronic disease, characterized by long-term responsiveness to chemotherapy. Cisplatin and carboplatin are key agents in EOC management and cancers may retain sensitivity to these drugs for prolonged periods and sometimes, even after clinical relapse. Paclitaxel (Taxol®) and docetaxel (Taxotere®) are frequently used in combination with platinum for treating EOC. There is emerging evidence that epigenetic changes contribute significantly to acquired chemotherapy resistance (19 -21). We show herein that Plk2 is a determinant of sensitivity to paclitaxel and platinum.

Materials and Methods

Drugs used:

Carboplatin, cisplatin, paclitaxel, 5'-Aza-cytidine and Trichostatin A were obtained from Sigma Aldrich, Poole, UK).

Cell lines and plasmids

Primary normal ovarian surface epithelial cells (OSE) were prepared as described (22). A2780 cells were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). SKOV-3 cells were obtained from ATCC (LGC Promochem, Teddington, UK). Cell lines were authenticated at source by STR profiling, morphology (ATCC) and DNA profiling (ECACC). Sub-lines of A2780 and SKOV-3 ovarian carcinoma cell lines with acquired resistance to paclitaxel and carboplatin (SKOV-3TaxR and SKOV-3CR, respectively) were derived by sequential, pulsed exposure to increasing concentrations of drug. Maintenance doses for the drug resistant cell lines were generally sub-IC_{50} doses and representative of clinically achievable levels.

Transfection (Knock-in) and Silencing (Knock-down) of *Plk2*

Stable knock-down was achieved using plasmid-based RNAi. Sequences (from Ambion) were ligated into pSilencer. A2780 cells were transfected with pSilencer plasmids using Lipofectamine (Invitrogen, Paisley, UK) and selected in hygromycin B. Cells were then challenged with paclitaxel and subjected to flow cytometry. The plasmid for ectopic expression of *Plk2* was described previously (15). For transient, RNAi-mediated knockdown of *Plk2*, we used reagents provided by Smartpool (Dharmacon;

ThermoScientific, UK) according to the manufacturer's protocol. Following 72h of silencing, SKOV-3 cells were sub-cultured into $T25^2$ tissue culture flasks at approximately 30-50% confluence and exposed to paclitaxel (10nM) or carboplatin (100μM) (both used at approximately twice the IC_{50} dose) for a period of 48h, then harvested and subjected to the annexin assay.

Clinical Samples

Samples of EOC were obtained from the Tissue Bank of the Charité Hospital, Berlin, Germany. Tissue obtained at initial debulking surgery and at first relapse (obtained with informed consent and local ethical committee approval) were collected as snap-frozen biopsies at surgery. Serum was collected at the time of diagnosis and relapse and stored at -80 ^{o} C. Genomic DNA was obtained from tissues using proteinase K/ phenol and from serum using the Qiagen system. Cases were predominantly serous adenocarcinomas (n= 43), but also included endometrioid carcinomas (n= 5), mucinous (n=1), clear cell (n=1), mixed histology (n=2) and 2 unclassified cases (Table 2). Post-operative chemotherapy comprised carboplatin and paclitaxel for all patients, given on either a 21 day (q21) or 7 day (q7) schedule. In some cases Gemcitabine was added. Response was assessed by standard criteria including clinical examination, serum CA125 and computerized tomographic (CT) imaging.

mRNA analysis

Analysis of Plk2 expression by RT-PCR and qPCR was as described previously (15).

Methylation analysis

Genomic DNA was purified from cell pellets by proteinase K digestion and from serum using a commercially available system (Qiagen). For methylation analysis, genomic DNA (1μg) was modified by sodium bisulphite as described previously (15). The location of primers for bisulphite sequencing and MSP is shown in Supplementary Material, Figure 1. qMSP of the Plk2 CpG island was carried out using primer pair 4. PCR was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SyBRGreen (Qiagen).

Western blotting

Cell pellets were prepared from attached and floating cell populations, then subjected to osmotic rupture in hypotonic detergent based buffer (1mM PMSF, NaVO4, aprotinin and leupeptin as protease inhibitors, 150mM NaCl, in 50mM Tris buffer, 0.2% SDS, 1% NP-40, pH 7.5) and 40-50μg of protein/sample electrophoresed on SDS-PAGE gels. Membranes were incubated overnight at 4^0C with primary antibody- Plk2 antibody (sc-25421, H-90 rabbit polyclonal antibody, Insight Biotechnology, London, UK) or Plk1,3 and 4 (AbCam, Cambridge, UK). In some experiments, the anti-Plk2 antibody used was as described previously (13, 15). Following the probing of each membrane with the primary antibody of choice, the membrane was stripped and re-probed using a GAPDH, PCNA or actin antibody as loading control. Antibodies against Plk1, Plk3 and Plk4 were from AbCam.

Cytotoxicity assays

A2780 and SKOV-3 parental and resistant cells for cytotoxicity testing were seeded into 96-well plates at a density of 3-4 x 10^4 cells/ml to allow approximately 3-doublings (approximately 10-20-fold increase in control cell number) for the duration of the drug exposure. Cells were allowed to attach and equilibrate for 24h, then treated with various concentrations of drug over 2-3 log orders of concentration. Results were not materially affected by initial plating densities. Drugs were diluted in tissue culture medium containing 10% FCS. Control cell wells were treated with drug-free tissue culture medium. Drug-treated cells were then returned to the incubator for a period of 72h. Cell viability was determined by addition of 0.1mg MTT (thiazoyl blue tetrazolium bromide) to each well for 4h at 37° C. Wells were aspirated to remove the medium, the resulting formazan crystals solubilized in 200µl DMSO and the absorbance read at 570nM. The absorbance of the formazan product obtained for drug-treated cells was calculated as a fraction of that for the untreated control wells. Growth curves were constructed using PRISM software and results expressed as IC_{50} values i.e. dose of drug causing a 50% reduction in cell viability.

Annexin V assay for assessment of apoptosis:

The effect of drugs on cell cycle distribution was assessed by flow cytometry as described previously (15). Cell death was assessed by measurement of apoptosis using Annexin V. Briefly, cells were seeded into tissue culture flasks to give a density approximately 30-50% confluence (for SKOV-3 and A2780 cell lines this corresponded to 4 x 10^5 cells / T25flask), allowed to attach for 2-3 hours and then treated for 48h with the appropriate compound at various concentrations. In our hands, initial plating densities between 20 and 50% did not significantly affect apoptosis induction in this assay. An Annexin V-FITC conjugated

apoptosis detection kit incorporating PI was used according to the manufacturer's protocol (Oncogene; supplied by CN Biosciences, Beeston, UK). Harvesting of cells included collection of attached cells (following trypsinization) and floating cells. Samples were analysed by flow cytometry, using the FL1 (FITC) and FL3 (PI) laser lines and each sample was assessed using a collection of 10,000 events. All analyses were carried out in triplicate.

Statistical analysis

Statistical analysis was carried out using SPSS (version 14) and MiniTab programs. Tissue and serum methylation results were assessed in the groups of responders and non-responders in terms of the utility of DNA methylation as a potential marker of chemo-responsiveness.

Results

Ovarian carcinoma cell lines with primary resistance to paclitaxel show cross-resistance to platinum and *vice versa*

We derived, *de novo*, a series of novel A2780 EOC cell lines with increasing degrees of acquired resistance to paclitaxel (supplementary Table1). Calculating the relative resistance (as a Resistance Factor), using the ratio of IC_{50} resistant variant / IC_{50} of parental cell line (1.5 nM) we derived the nomenclature for the A2780TaxR panel of cell lines: hence

A2780TaxR$_{354}$ is 354-fold resistant to paclitaxel, etc. We also developed independent SKOV-3 cell lines with primary resistance to paclitaxel and carboplatin SKOV-3TaxR was 52 -fold resistant, compared to the parental SKOV-3 cell line (IC_{50} = 5.7 nM) and SKOV-3CR cell lines 3-fold resistant to carboplatin (IC_{50}= 298.0 μM) and 2-fold resistant to cisplatin (IC_{50}= 21.7μM) (supplementary Table1). We tested whether cell lines with acquired resistance to paclitaxel were cross-resistant to platinum compounds and *vice versa*. Paclitaxel-resistant cell lines were challenged with carboplatin or cisplatin and cytotoxicity evaluated. In each cell line in our series, paclitaxel-resistant cell lines showed decreased sensitivity to cisplatin and carboplatin (Figure 1, supplementary Table1). Conversely, SKOV-3CR cells showed decreased sensitivity to paclitaxel (Figure1, supplementary Table1).

Drug resistance is associated with loss of G2M checkpoint

Using annexin V staining and flow cytometry, we asked whether resistance to paclitaxel was associated with changes in drug-induced apoptosis and/or cell cycle distribution. In A2780TaxR$_{354}$ paclitaxel-resistant cell lines, there was greatly reduced apoptosis on exposure to paclitaxel relative to parental cells (Figure 2A). The apoptotic effects of drug treatment could be seen by the emergence of annexin V positive (quadrants top right and bottom right) and PI positive populations (emergence towards top left/right quadrants). Summation of the apoptotic quadrants revealed approximately 49% and 25% cell death for A2780 parent and A2780TaxR$_{354}$ cells, respectively following treatment with paclitaxel. Likewise, following treatment with cisplatin a clear reduction in the extent of cell death is also seen comparing A2780 parent and A2780TaxR$_{354}$ resistant cells. Next, we analyzed

perturbations in cell cycle following drug exposure. Following 24h paclitaxel exposure A2780 parent cells, sub-G1 (apoptotic) and G2M arrested populations were clearly increased (Figure 2B), but in paclitaxel-resistant A2780TaxR$_{354}$ cells, there was no increase in the sub-G1 population. Furthermore, the G2M population was greatly reduced in A2780TaxR$_{354}$ cells relative to A2780 parental cells (Figure 2B). Together, these results imply that acquired resistance to paclitaxel involves loss of G2M cell cycle checkpoint function and abrogation of apoptosis. In SKOV-3 parent cells, paclitaxel exposure resulted in clear increase in the apoptotic cell population which was completely absent in SKOV-3TaxR cells (Figure 2C). In carboplatin resistant SKOV-3CR cells, there was reduced apoptosis on exposure to paclitaxel (Figure 2C).

Transcriptional silencing of Plk2 in cell lines with acquired drug resistance

Next, we analysed the expression and regulation of Plk2. *Plk2* mRNA was abundantly expressed in normal ovarian surface epithelium (OSE) and in the parent A2780 and SKOV-3 lines (Figure 3A and 3B). There was, however, down-regulation of Plk2 mRNA in A2780TaxR$_{354}$, SKOV-3TaxR (paclitaxel-resistant) and SKOV-3CR (carboplatin resistant) cells relative to the respective parent lines (Figure 3A and 3B). Western blot analysis confirmed reduced levels of Plk2 protein in paclitaxel resistant A2780 and SKOV-3 cell lines (Figure 3C). In contrast, there was no change in expression of Plk1 or Plk3 between parent and paclitaxel-resistant A2780 and SKOV-3 cell lines (Figure 3D). Plk4 levels were higher in A2780 TaxR$_{354}$ cells but not in SKOV-3TaxR. To investigate whether down-regulation of *Plk2* mRNA is associated with methylation changes, we performed bisulphite sequencing of the *Plk2* CpG island, The CpG island was unmethylated in OSE, in the parent

A2780 and SKOV-3 EOC cell lines and in other EOC cell lines. However, in A2780TaxR$_{354}$ and SKOV-3TaxR cells, there was acquisition of methylation within a defined region of the CpG island (Figure 4A). We tested whether demethylating agents reactivate expression of Plk2 mRNA and observed an increase in *Plk2* mRNA after exposure to 5'-Aza-cytidine (Aza-C) (Figure 4B), consistent with methylation-dependent silencing. Using both conventional and quantitative MSP (qMSP), we confirmed that methylation was detectable in the paclitaxel-resistant derivatives A2780TaxR$_{354}$ and SKOV-3TaxR, but not in the respective parent cell lines (Figure 4C and 4D).

Methylation and expression of Plk2 vary quantitatively with drug resistance

We analysed expression and methylation of *Plk2*. in our panel of paclitaxel resistant variants of A2780. Bisulphite sequencing showed a progressive, quantitative increase in methylation with increasing drug resistance (Figure 5A). qMSP analysis confirmed that methylation increased with increasing paclitaxel resistance (Figure 5B). Expression of *Plk2* mRNA (Figure 5C) and protein (Figure 5D) decreased with increasing paclitaxel resistance, consistent with changes in CpG methylation.

Paclitaxel resistance correlates with epigenetic status of the Plk2 CpG island

We next tested the stability of the drug resistant phenotype. Clones of the A2780 paclitaxel-resistant cell line panel with increasing levels of primary resistance to paclitaxel, were transferred to drug-free medium and grown continuously in the absence of paclitaxel. After 6 weeks growth in drug-free medium, A2780TaxR$_{354}$ cells grown in the absence of paclitaxel (-) had substantially reverted to a paclitaxel sensitive phenotype (Figure 6A). In

A2780TaxR$_{615}$ (-) cells, there was a clear, but less complete, restoration of the paclitaxel-induced G2M block (Figure 6A). Plk2 expression in A2780TaxR$_{354}$ (-) had returned to almost 75% of the level of parental cells and that of A2780TaxR$_{615}$ (-) to approximately 33% of parent A2780 cells (Figure 6B, light grey panels) but remained suppressed in cells exposed to maintenance paclitaxel (dark grey panels). MSP analysis of the Plk2 CpG island showed a decrease in methylation with recovery of Plk2 expression (Figure 6C) and this was confirmed by bisulphite sequencing of the *Plk2* CpG island (Supplementary Material, Figure 2).

Plk2 expression modulates cytotoxicity of carboplatin and paclitaxel

We used plasmid-mediated inhibitory RNA (RNAi) to stably down-regulate Plk2 in the drug sensitive A2780 parent cell line. Knock-down was confirmed by RT-PCR ("KD" in Figure 7A). Clonal cell lines with "knocked-down" expression of Plk2 (A2780KD) were challenged with a cytotoxic concentration of paclitaxel (Figure 7B). G2M block and increase in the sub-G1 population were greatly reduced in the knocked-down cells (Figure 7B). To exclude so-called "off-target" effects, A2780 cell lines with knocked-down Plk2 (A2780KD) were re-transfected with a *Plk2* expression plasmid (A2780KD + KI). The effect of Plk2 re-expression was increased paclitaxel-induced G2M blocked and sub-G1 populations, relative to the A2780KD cells (Figure 7B). Next, we ectopically expressed *Plk2* in A2780TaxR$_{354}$ cells which have undetectable levels of endogenous Plk2. Flow cytometry revealed that in A2780TaxR$_{354}$ KI cell lines engineered to ectopically express *Plk2*, there was restoration of the paclitaxel-dependent G2M checkpoint which was lost in the A2780TaxR$_{354}$ cells (Figure 7C). Finally, we used transient transfection of *Plk2* siRNA to silence expression in SKOV-3

parental cells. Western blotting confirmed down-regulation of Plk2 expression (Figure 7D). The silenced cells were exposed to paclitaxel and carboplatin for 48h. There was a reproducible reduction in apoptosis induced by both paclitaxel and carboplatin in cells silenced for *Plk2* expression compared to control cells (Figure 7E).

Plk2 is a candidate predictor of chemotherapy resistance in EOC

We used quantitative MSP (qMSP) to analyse methylation in the *Plk2* CpG island in a test series of 54 cases of EOC, treated in a single centre with post-operative carboplatin/ paclitaxel chemotherapy. 34 cases had relapsed clinically at the time of censor (as evidenced by rising serum CA125 levels and/or CT evidence of progressive disease), whereas 20 cases remained disease-free at the time of censor. Full clinical details of the cases are given in supplementary Table 2. Methylation in tumour tissues in the Plk2 CpG island was detected, using qMSP, in 19/54 (31%) cases at diagnosis. Cases with methylation in tumour tissue at diagnosis were more likely to have relapsed at the time of censor than those with undetectable methylation in tumour tissue at diagnosis (p=0.0032, Fisher's exact test). We also performed qMSP for *Plk2* CpG methylation in serum (where available). 39/50 sera were concordant with the methylation status of their matched tumour tissue (Fisher's exact test, p=0.00216). We asked whether the presence of methylated *Plk2* DNA in serum was more common at relapse than at diagnosis. Paired sera at diagnosis and relapse were available for 32 cases. 23/32 sera were concordant with the matched tissue (p=0.011, Fisher's exact test). The proportion of sera with detectable methylation was higher at relapse than at diagnosis but because of small sample numbers this did not reach statistical significance (0.47 vs 0.62, p=0.204). Although there was a clear

trend for shorter relapse-free survival in cases with detectable methylated DNA from the *Plk2* CpG island, both in tumour tissue and serum, this did not reach statistical significance with the small numbers of cases available for study (p= 0.142 for tissue and 0.054 for serum). See Supplementary Material, Figure 3 for additional data and Kaplan-Meier survival curves. These data suggest that detection of methylated Plk2 DNA may have utility in prediction of clinical outcome in EOC.

Discussion

We show that Plk2 is a determinant of cellular sensitivity to paclitaxel and carboplatin and via a mechanism of methylation-dependent transcriptional silencing, *Plk2* is associated with drug resistance. Paclitaxel resistance in A2780 cell lines was associated with reduction or complete loss of the drug-induced G2M checkpoint and reduced apoptosis. Similarly, in paclitaxel or carboplatin resistant SKOV-3 cells, there was reduced apoptosis relative to drug-sensitive parental cells. Importantly, in A2780 and SKOV-3 cell lines with acquired resistance to paclitaxel, there was significantly reduced apoptosis when challenged with platinum compounds. Moreover, carboplatin-resistant SKOV-3 cells exhibited reduced apoptosis when exposed to paclitaxel.

Plk2 has both G2M checkpoint (16) and pro-apoptotic (15) function(s), prompting us to ask whether changes in expression of Plk2 occur with acquisition of drug resistance. Plk2 expression was reduced relative to parent cell lines in A2780 and SKOV-3 cell lines with acquired resistance to paclitaxel and also in a SKOV-3 cell line resistant to carboplatin. The specificity of these effects was emphasised by analysis of the related genes *Plk1* and *Plk3/Fnk/Prk* in which there were no changes in expression with acquisition of resistance to paclitaxel. Three other lines of experimental evidence support an important role for Plk2 as a determinant of drug sensitivity. First, ectopic expression of *Plk2* in cells in which endogenous *Plk2* was epigenetically down-regulated, substantially restored sensitivity to paclitaxel. Secondly, RNAi-mediated "knock-down" of Plk2 in drug sensitive A2780 and SKOV-3 parental cell lines conferred reduced sensitivity to paclitaxel and platinum compounds, accompanied by reduced G2M block and apoptosis, an effect reversible by re-expression of Plk2. Thirdly, low to moderate degrees of paclitaxel resistance were

reversible when cells were allowed a drug-free "holiday". In such cell lines, with reacquired drug sensitivity, expression of Plk2 increased with increasing drug sensitivity. Our results are compatible with studies showing that decreased sensitivity to paclitaxel is associated with compromised checkpoint function or spindle assembly (24-26). Our data are also consistent with micro-array analyses of platinum-resistant cell lines in which *Plk2* is down-regulated (21; 27). Together, these results afford further support for the candidacy of Plk2 as a determinant of chemotherapy sensitivity and for a predominantly epigenetic basis for acquired drug resistance (28). Our data also imply that Plk2 expression and Plk2-associated kinase activity may be required, at least in some cell types, for sensitivity to chemotherapy. This is supported by a recent study in chronic lymphocytic leukaemia in which failure of Plk2 upregulation was associated with chemotherapy resistance (29).

Our hypothesis, that dynamic changes in the epigenetic status of *Plk2* influence the drug sensitivity of cancer cells, is supported by various lines of evidence from other authors. For example, changes in DNA methylation within the CpG islands of specific genes are detectable very rapidly after exposure to cytotoxic agents (30). Also, increasing cisplatin resistance *in vivo* is associated with elevated expression of methyltransferases and quantitative changes in CpG island methylation (21). In vivo supportive evidence comes from studies of the DNA mismatch repair gene *hMLH1*. Inactivation of *hMLH1* is associated with increased resistance to cisplatin *in vitro*. Analysis of methylated DNA in serum of individuals receiving platinum-based adjuvant chemotherapy for EOC revealed that methylation levels changed during chemotherapy and had utility in predicting clinical outcome (23). A profiling study of global changes in the methylome of drug sensitive and

resistant MCF-7 cells, supports a general model of acquired drug resistance in which opposing and ongoing hypo- and hypermethylation in multiple genes is a major mechanism driving the process of drug resistance (31). In initial clinical studies we show that cases of EOC with *Plk2* methylation at diagnosis were at significantly increased risk of early clinical relapse with a less favourable response to post-operative chemotherapy than cases lacking methylation. Also, methylated *Plk2* DNA in serum was more common at clinical relapse than at initial diagnosis. Our results imply that analysis of *Plk2* methylation may have utility as a biomarker of both disease activity and of chemo-sensitivity in EOC. In the context of our results, Plk2 has been identified as a significantly down-regulated gene in chemotherapy-resistant primary ovarian carcinomas, affording independent validation of our hypothesis for it being a determinant of chemotherapy sensitivity and candidate biomarker in EOC (32). An important feature of the taxane resistant cell line models we use in the current study is their cross-resistance with platinum agents. In support of this, previous reports in the literature cite decreased platinum sensitivity with cross resistance to chemotherapeutic agents such as taxanes in EOC cell lines (33). Clinically, a proportion of EOC patients show platinum-taxane non-cross resistance (34). Both our cell line panel data and our EOC patient cohort used in the present study are representative of platinum and taxane cross-resistance.

We previously reported that *Plk2* is subject to transcriptional down-regulation in B lymphomas and expression profiling has shown that Plk2 down-regulation is a predictor of poor clinical outcome in B lymphomas (35). Our data are in support of clinical trials of epigenetic therapies in EOC patients, and other cancer patients (36) and suggest that detection of methylated *Plk2* DNA warrants independent evaluation in larger clinical series

as a candidate predictor of response of EOC patients to chemotherapy and as a serum biomarker of drug-resistant clinical relapse.

Conflict of Interest

The authors declare they have no competing financial interests in relation to the work in this study.

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Figure legends

Figure 1: A: Dose-response curves for A2780 cells with increasing levels of acquired paclitaxel resistance. B: Dose-response curves of A2780 cells with varying levels of paclitaxel resistance challenged with carboplatin. Cells were treated with varying concentrations of each drug as indicated and survival determined (see Methods). Data are mean +/- 1SD from multiple independent experiments (at least four). C: Dose-response curves for SKOV-3 cells with acquired resistance to paclitaxel or carboplatin, challenged with paclitaxel. D: Dose-response curves for SKOV-3 cells with acquired resistance to paclitaxel or carboplatin, challenged with carboplatin. Cell lines were treated with varying concentrations of each drug as indicated and survival determined as described in Methods. Data shown are mean +/- 1SD from multiple independent experiments (at least four).

Figure 2:

Paclitaxel resistance and cross-resistance to platinum is associated with reduced apoptosis and changes in cell cycle distribution on exposure to drugs. **A:** Annexin staining of parent A2780 and A2780 paclitaxel-resistant cells grown in the presence of the indicated concentrations of paclitaxel or cisplatin and then subjected to the annexin V (AV) assay with propidium iodide (PI) staining. The proportions of cells in each compartment are indicated. A representative analysis (mean and (SD) from 3 independent experiments) is shown. Bottom left = viable cells; bottom right = early apoptotic (annexin- FITC +ve); top right = PI and AV +ve cells mid/late phase apoptosis; top left = end stage apoptosis/necrotic PI +ve. A representative analysis (typical of three independent experiments) is shown, with mean and (SD) indicated. **B**: Parent A2780 cells and

A2780TaxR$_{354}$ were exposed to paclitaxel then subjected to flow cytometry. **C**: SKOV-3 parent cells, SKOV-3TaxR and SKOV-3CR were exposed to paclitaxel, then subjected to flow cytometry (data shown are representative of repeat experiments).

Figure 3.

A and B: RT-PCR and qPCR analysis of expression of Plk2 in EOC cell lines and primary, normal ovarian surface epithelial (OSE) cells. Upper panel (A) shows RT-PCR analysis of Plk2 and the control gene GAPDH. Mr is molecular weight markers. The lower panel (B) shows qPCR analysis of expression of Plk2 in the same samples. **C**: Western blot analysis of Plk2 expression in parental A2780 and SKOV-3 EOC cell lines and the paclitaxel-resistant derivatives A2780TaxR$_{354}$ and SKOV-3TaxR. **D**: Western blot analysis of Plk1, Plk3 and Plk4 expression in parent A2780 and SKOV3 cells and in A2780TaxR$_{354}$ and SKOV-3TaxR. The anti-Plk2 antibody used for western blotting detects a doublet, the upper band of which is Plk2 (arrowed). PCNA is shown as a loading control.

Figure 4

A: Bisulphite sequence traces showing acquired CpG methylation in A2780TaxR$_{354}$ cells. **B**: Cell lines were treated with 5-aza-cytidine (+A) or 5-aza-cytidine and Trichostatin A (+AT) and expression of *Plk2* analysed by RT-PCR. **C:** MSP analysis of methylation in parent and paclitaxel-resistant EOC cell lines. Controls are C_U (unmethylated DNA) and C_M (methylated DNA). MSP reactions for unmethylated (U) and methylated (M) alleles are shown. **D**: qMSP analysis of the *Plk2* CpG island in parent and paclitaxel-resistant EOC cell lines.

Figure 5

A: Bisulphite sequence analysis of the *Plk2* CpG island in A2780 parent cells and sub-lines with increasing resistance to paclitaxel. Methylation is quantified by the number of black squares: 0 - no black blocks; 1-25% - 1 black block; 25-50% - 2 black blocks; 50-75% - 3 black blocks; 75-100% - 4 black blocks. **B**: qMSP analysis of the *Plk2* CpG island in parent A2780 cells and sub-lines with increasing levels of paclitaxel resistance. **C:** qPCR analysis of *Plk2* mRNA in A2780 cell lines with increasing paclitaxel resistance. Levels of *Plk2* mRNA are indicated relative to A2780 parent cells. **D:** Western blot analysis of Plk2 expression in A2780 cell lines with increasing paclitaxel resistance. As in Figure 3, the anti-Plk2 antibody used for western blotting detects a doublet, the upper band of which is Plk2 (arrowed). PCNA is shown as a loading control.

Figure 6

Reversal of paclitaxel resistance by growth in the absence of drug. **A**: Flow cytometry analysis of A2780 parent cells and paclitaxel-resistant A2780TaxR$_{354}$ and A2780TaxR$_{615}$ sub-lines. Parent A2780 cells and derivative cell lines allowed a six week period of growth in drug-free medium (-) were exposed to paclitaxel then subjected to flow cytometry. **B**: Expression of Plk2 mRNA in A2780 cell lines grown with (dark grey boxes) or without maintenance paclitaxel (light grey boxes). Expression is relative to parent A2780 cells. **C:** MSP analysis of the *Plk2* CpG island in A2780 cell lines grown with (+) or without (-) maintenance paclitaxel. Controls are: C_U (control unmethylated DNA) and C_M (control methylated DNA). MSP reactions for unmethylated (U) and methylated (M) alleles are shown.

Figure 7

Expression of Plk2 modulates response to paclitaxel and carboplatin . **A**: Expression of *Plk2* mRNA in A2780 parent cells was "knocked-down" using inhibitory RNA (RNAi) and stable **K**nock-**D**own confirmed by RT-PCR. Expression of *Plk2* was then restored to the knocked-down cells by ectopic expression (**K**nock-**I**n: "KD+ KI"). **B**: Flow cytometric analysis of the effect of paclitaxel in A2780 cells. Cells were treated with paclitaxel and subjected to flow cytometry 48 hours later as indicated. **C:** Knock-in of *Plk2* in A2780 cells with acquired paclitaxel resistance. A2780TaxR_{354} cells were transfected with a plasmid expressing *Plk2* (A2780TaxR_{354} KI), then challenged with paclitaxel. **D:** Western blot analysis of expression of Plk2 in SKOV-3 cells following knock-down. **E:** Parent SKOV-3 cells were treated with subcytotoxic doses of paclitaxel or carboplatin following *Plk2* knock-down or treatment with irrelevant SiRNA (control). Cells were harvested 48 hours after addition of cytotoxic agent. Similar results were obtained after 72 hours (n = 3).

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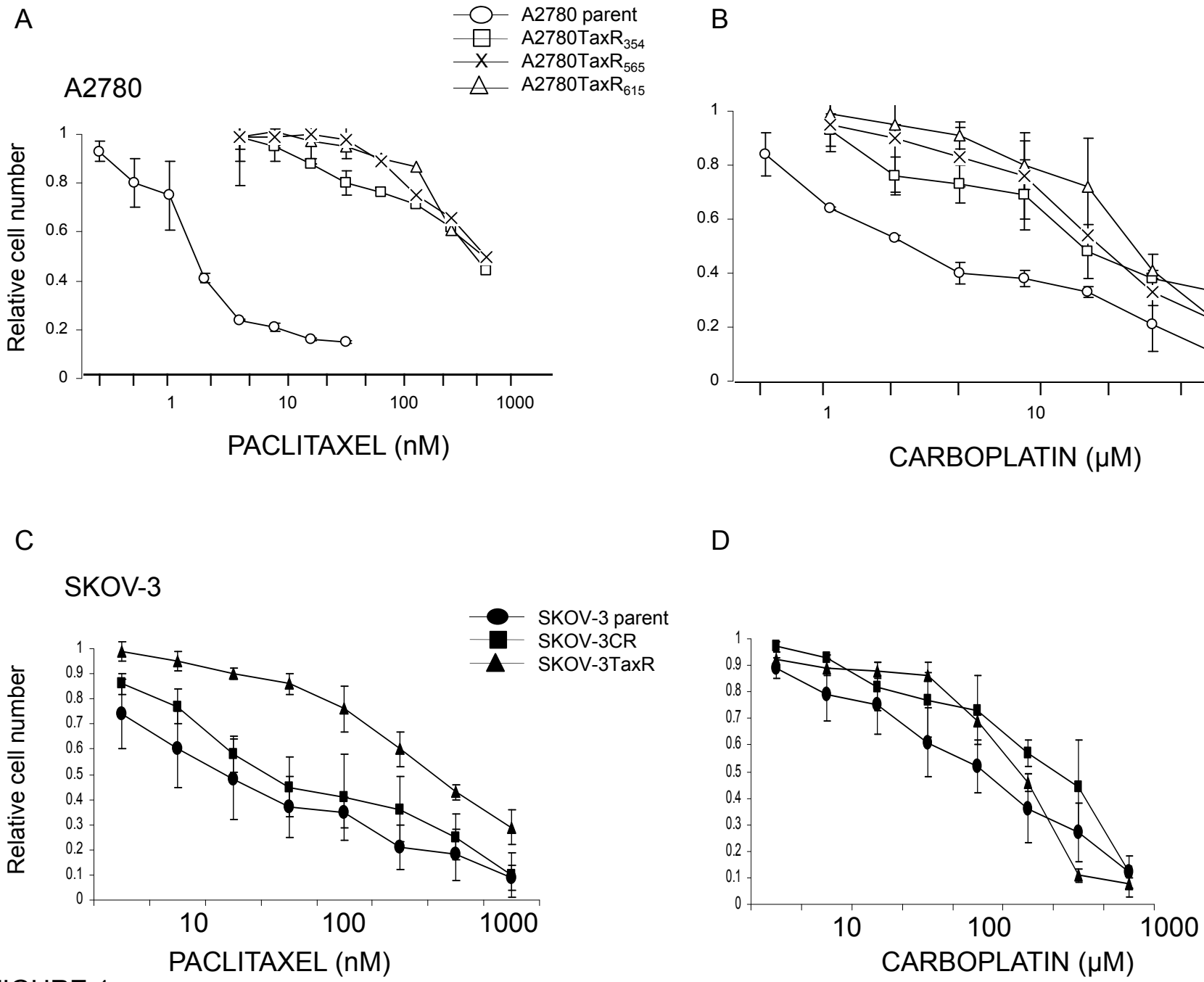

A
A2780
A2780 parent
A2780TaxR354
A2780TaxR565
A2780TaxR615
Relative cell number
PACLITAXEL (nM)
B
CARBOPLATIN (μM)
C
SKOV-3
SKOV-3 parent
SKOV-3CR
SKOV-3TaxR
Relative cell number
PACLITAXEL (nM)
D
CARBOPLATIN (μM)

FIGURE 1

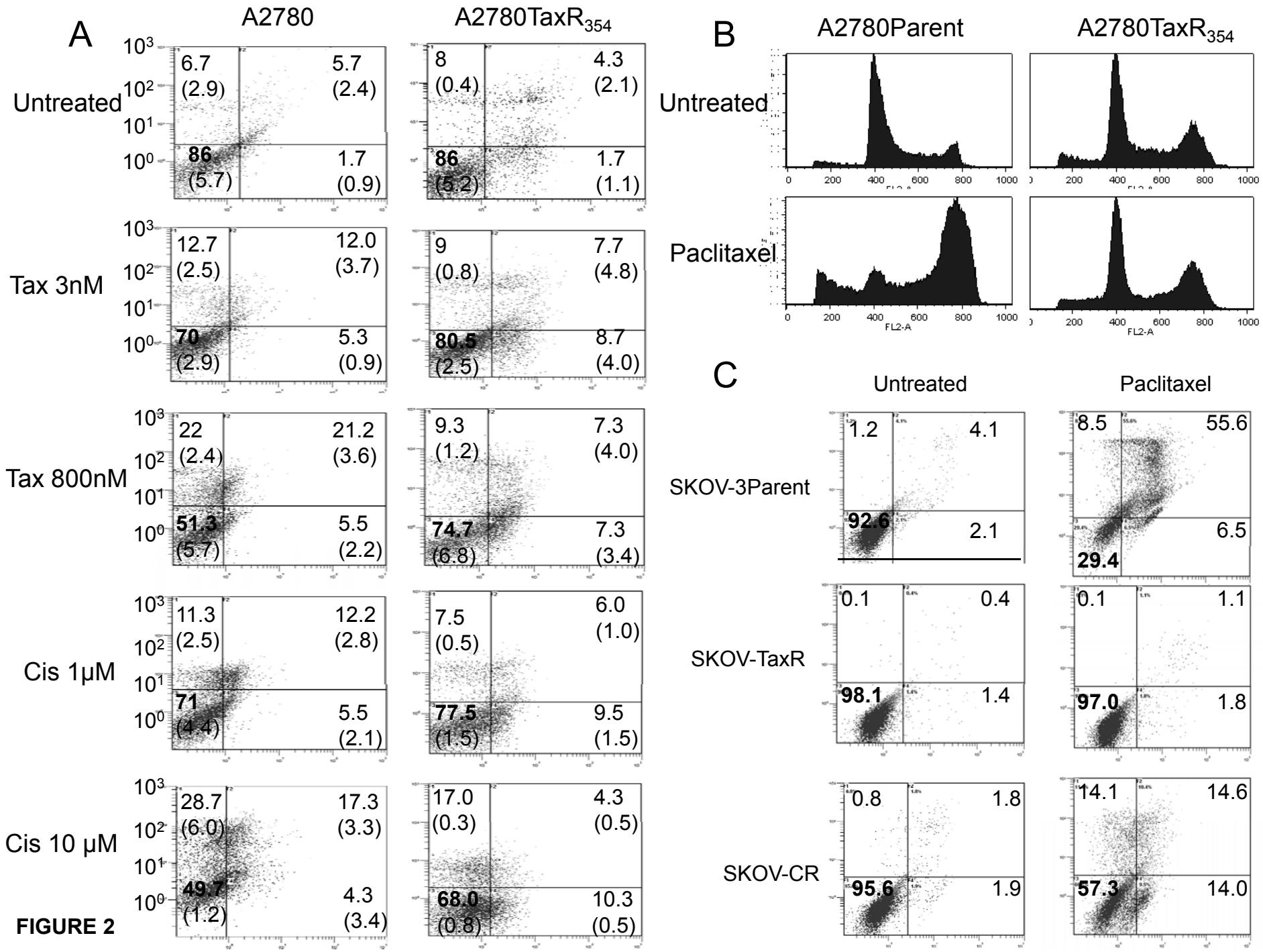

A
A2780
A2780TaxR354
Untreated
6.7 (2.9)
5.7 (2.4)
86 (5.7)
1.7 (0.9)
8 (0.4)
4.3 (2.1)
86 (5.2)
1.7 (1.1)
Tax 3nM
12.7 (2.5)
12.0 (3.7)
70 (2.9)
5.3 (0.9)
9 (0.8)
7.7 (4.8)
80.5 (2.5)
8.7 (4.0)
Tax 800nM
22 (2.4)
21.2 (3.6)
51.3 (5.7)
5.5 (2.2)
9.3 (1.2)
7.3 (4.0)
74.7 (6.8)
7.3 (3.4)
Cis 1μM
11.3 (2.5)
12.2 (2.8)
71 (4.4)
5.5 (2.1)
7.5 (0.5)
6.0 (1.0)
77.5 (1.5)
9.5 (1.5)
Cis 10 μM
28.7 (6.0)
17.3 (3.3)
49.7 (1.2)
4.3 (3.4)
17.0 (0.3)
4.3 (0.5)
68.0 (0.8)
10.3 (0.5)
B
A2780Parent
A2780TaxR354
Untreated
Paclitaxel
FL2-A
C
Untreated
Paclitaxel
SKOV-3Parent
1.2
4.1
92.6
2.1
8.5
55.6
29.4
6.5
SKOV-TaxR
0.1
0.4
98.1
1.4
0.1
1.1
97.0
1.8
SKOV-CR
0.8
1.8
95.6
1.9
14.1
14.6
57.3
14.0

FIGURE 2

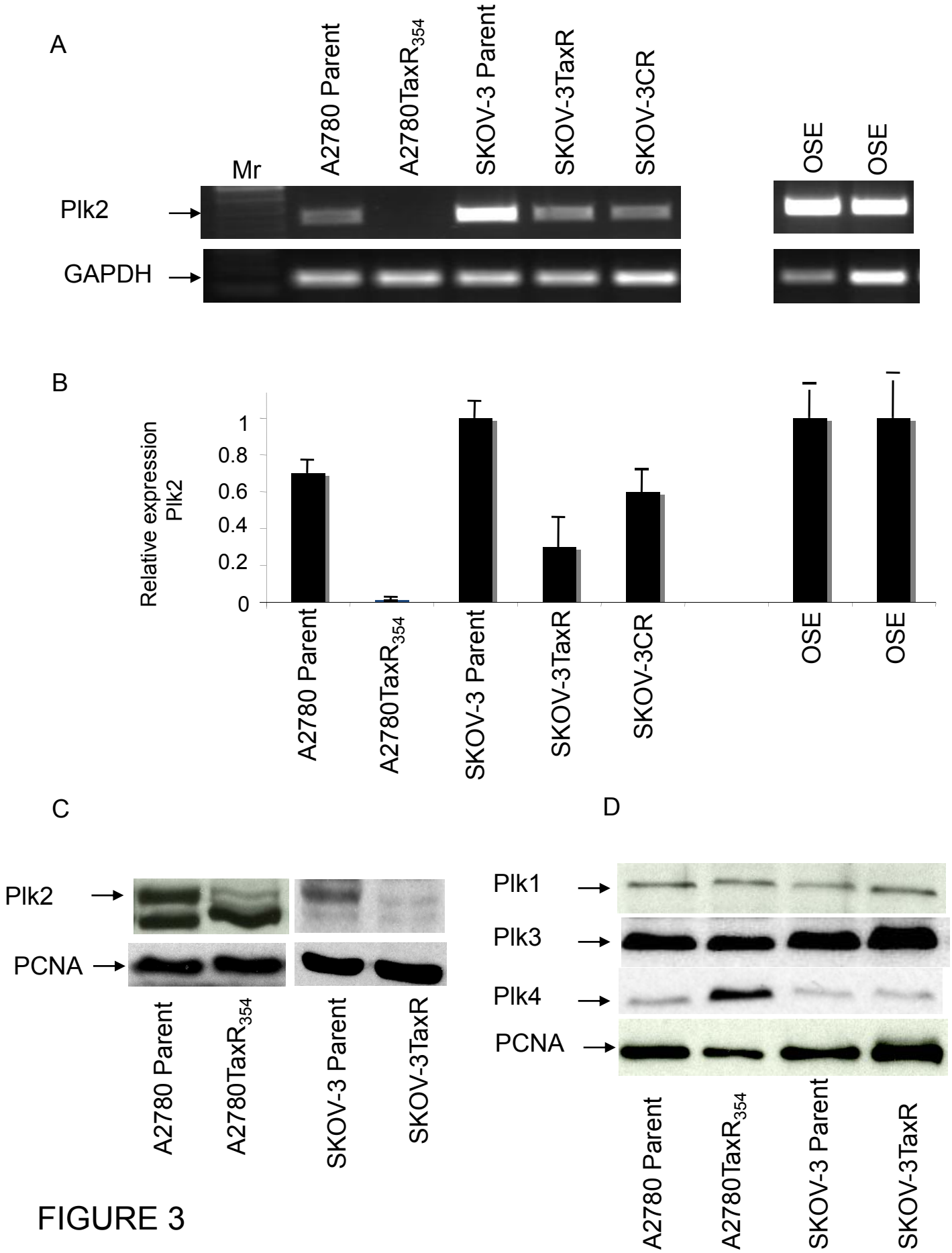

A
Mr
A2780 Parent
A2780TaxR354
SKOV-3 Parent
SKOV-3TaxR
SKOV-3CR
OSE
OSE
Plk2
GAPDH
B
Relative expression Plk2
1
0.8
0.6
0.4
0.2
0
A2780 Parent
A2780TaxR354
SKOV-3 Parent
SKOV-3TaxR
SKOV-3CR
OSE
OSE
C
Plk2
PCNA
A2780 Parent
A2780TaxR354
SKOV-3 Parent
SKOV-3TaxR
D
Plk1
Plk3
Plk4
PCNA
A2780 Parent
A2780TaxR354
SKOV-3 Parent
SKOV-3TaxR

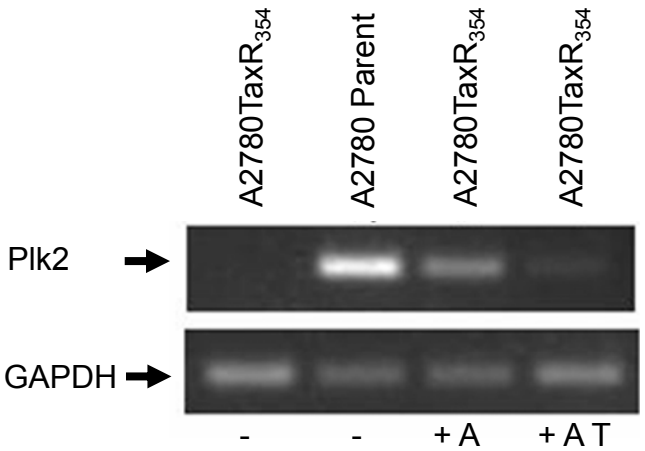
FIGURE 3

A

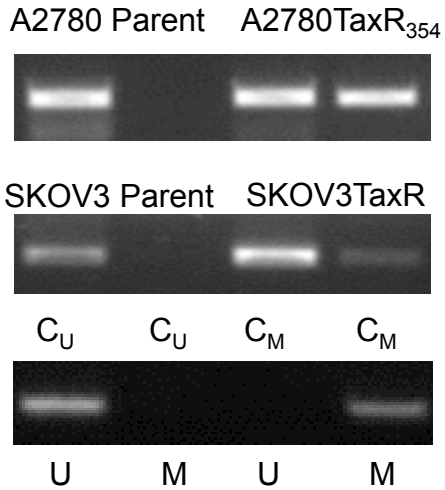
A2780 Parent

A2780TaxR$_{354}$

B



C



D

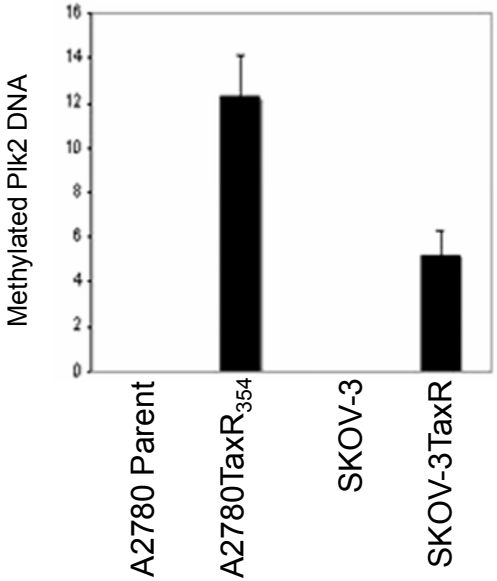
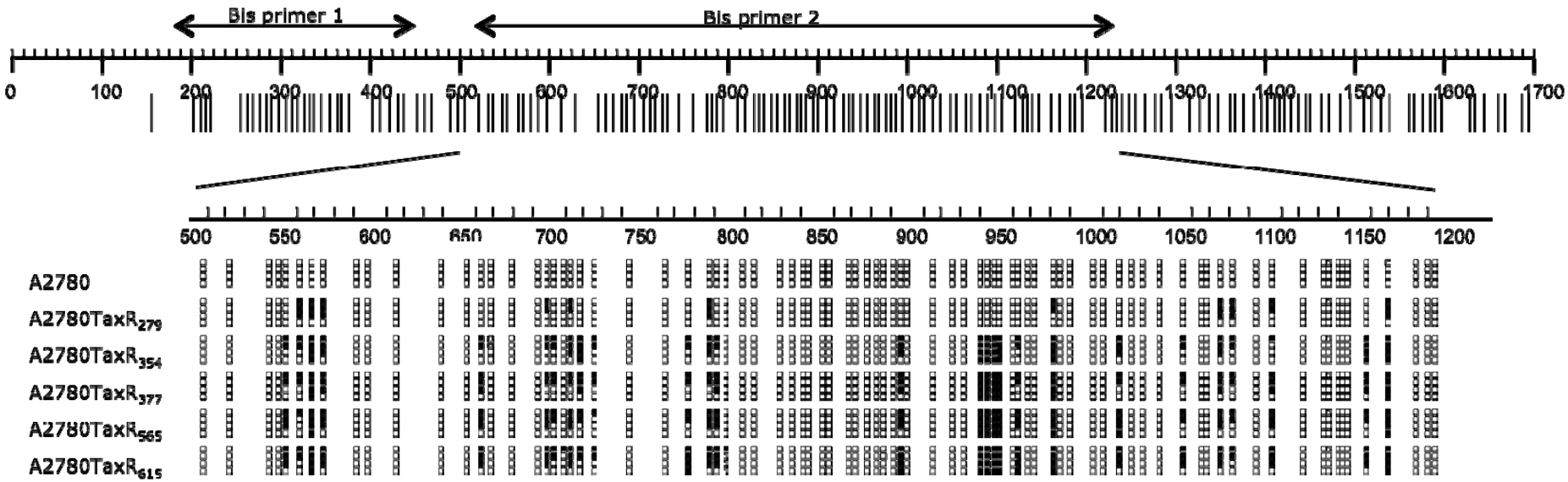
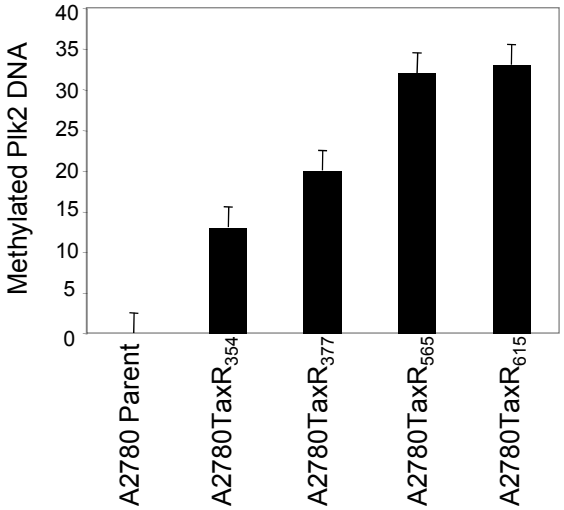


FIGURE 4

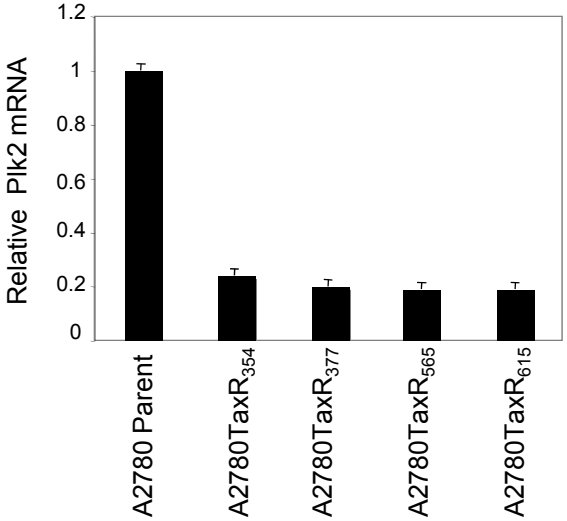
A



B



C



D

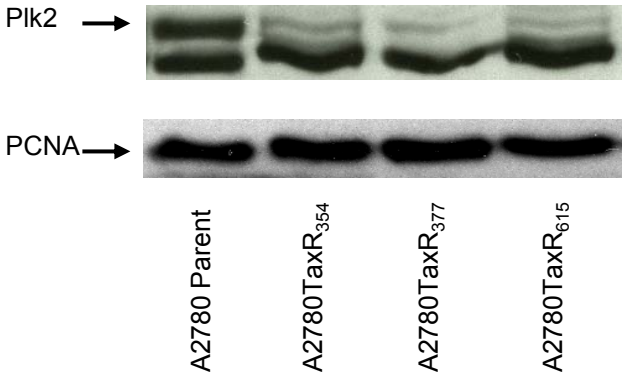
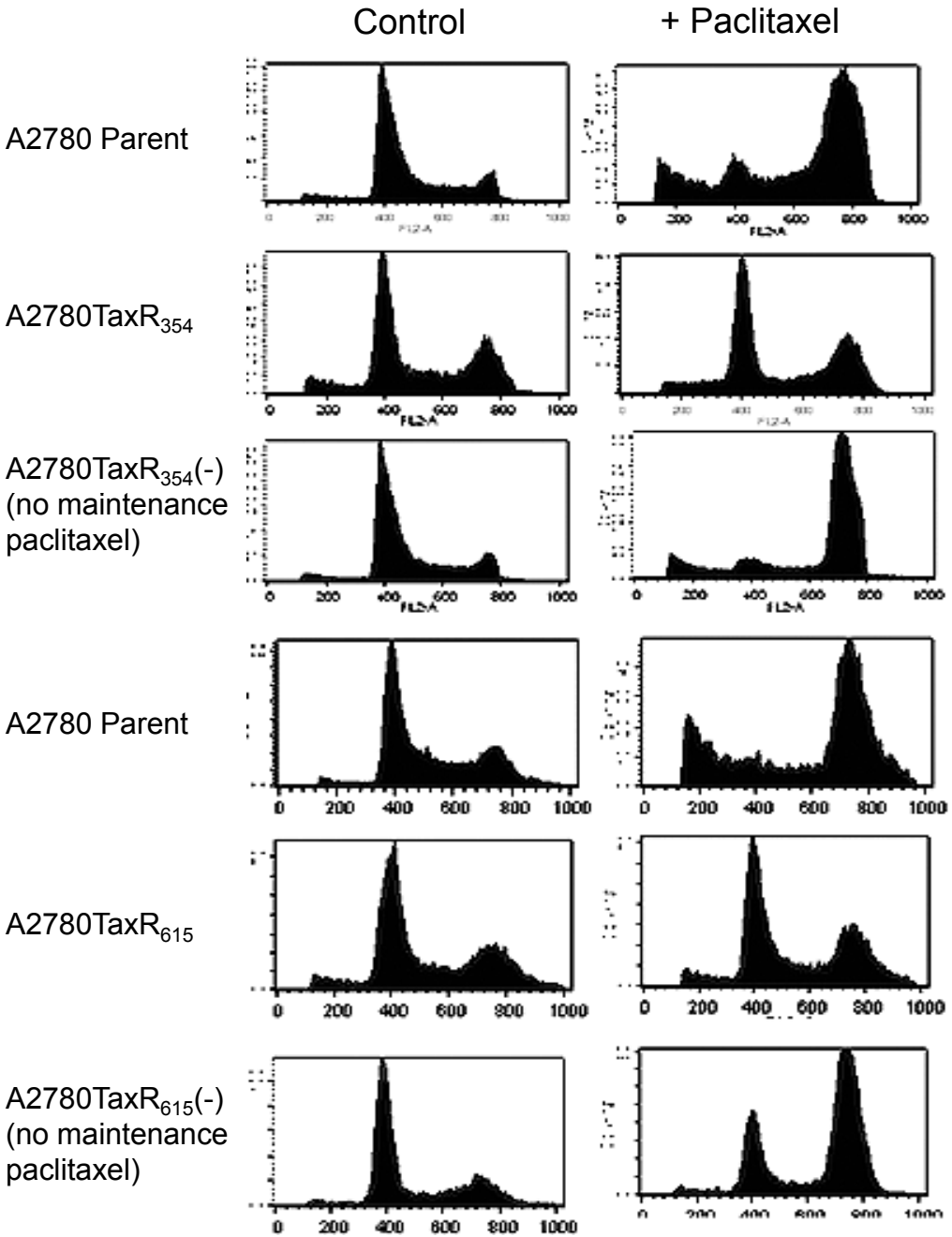
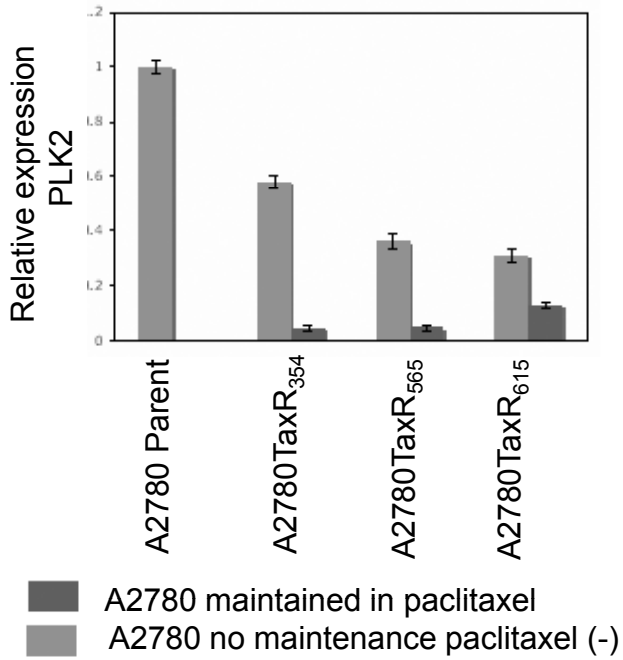


FIGURE 5

A


Control
+ Paclitaxel
A2780 Parent
A2780TaxR354
A2780TaxR354(-)
(no maintenance
paclitaxel)
A2780 Parent
A2780TaxR615
A2780TaxR615(-)
(no maintenance
paclitaxel)
0
200
400
600
800
1000

B


Relative expression
PLK2
A2780 Parent
A2780TaxR354
A2780TaxR565
A2780TaxR615
A2780 maintained in paclitaxel
A2780 no maintenance paclitaxel (-)

C

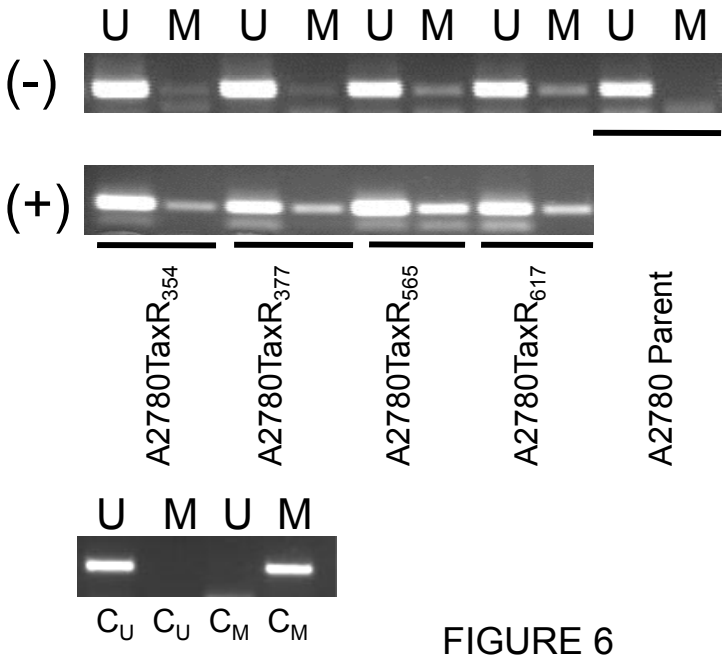

U M U M U M U M U M
(-)
(+)
A2780TaxR354
A2780TaxR377
A2780TaxR565
A2780TaxR617
A2780 Parent
U M U M
CU CU CM CM

FIGURE 6

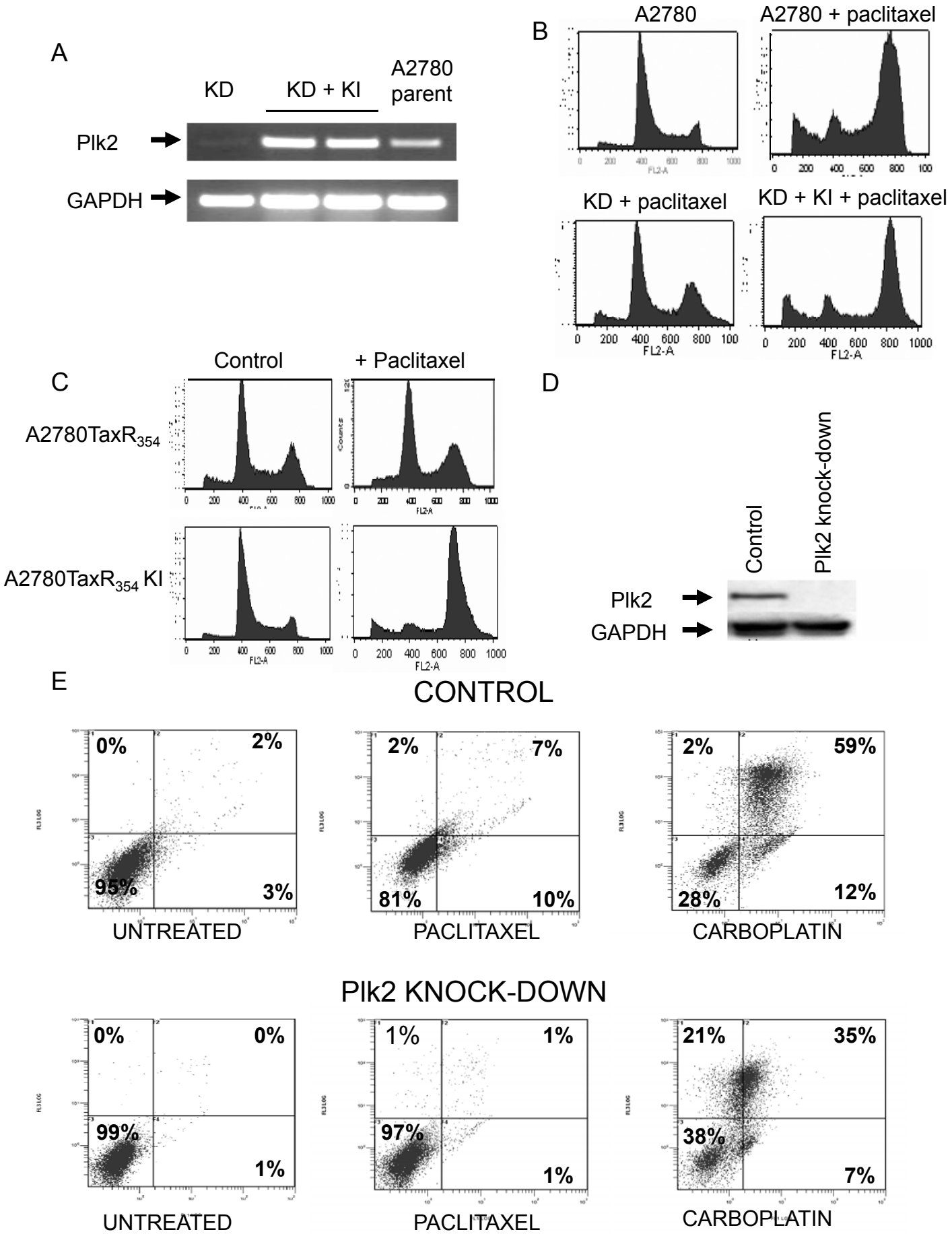

A
KD
KD + KI
A2780 parent
Plk2
GAPDH
B
A2780
A2780 + paclitaxel
KD + paclitaxel
KD + KI + paclitaxel
FL2-A
C
Control
+ Paclitaxel
A2780TaxR354
A2780TaxR354 KI
D
Control
Plk2 knock-down
Plk2
GAPDH
E
CONTROL
0%
2%
95%
3%
UNTREATED
2%
7%
81%
10%
PACLITAXEL
2%
59%
28%
12%
CARBOPLATIN
Plk2 KNOCK-DOWN
0%
0%
99%
1%
UNTREATED
1%
1%
97%
1%
PACLITAXEL
21%
35%
38%
7%
CARBOPLATIN

FIGURE 7