

Original Research

Establishment and Genetic Profiling of Platinum/Taxane Doublet-Resistant Cells Generated by Hybridizing Single Resistant Cells

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Abstract

Background: Current standard chemotherapy for gynecologic malignancies consists of platinum agent and taxane though, many patients experience the relapse of disease with drug resistance making the following therapy unsuccessful. It's a compelling question whether the mechanisms of doubly resistance is a simple combination of single agent resistance or whether the core novel mechanism common to platinum and taxane resistance stands out as a result of combination therapy. The purpose of this study is to establish the doublet drug resistant cell line and to find its genetic characteristics. **Methods:** Platinum/taxane doublet resistant cell lines (F3 and F4) were generated by hybridizing two independent, platinum or taxane resistant subline (C13 or PX24) stemmed from grand parental ME180 cells. The resistant cells were selected through repeated exposure to cisplatin and paclitaxel. For the assessment of drug sensitivity, colony forming assay was used. For the gene expression analysis, genome-wide expression profiling was done using the Human Genome U133A Array. Protein-protein interaction network (PPI) scaffold networks were retrieved from the Search Tool for the Retrieval of Interacting Genes database and, for the enrichment of pathway analysis, WebGestalt was used. **Results:** Colony forming assay showed C13 was 5.8-fold cisplatin resistant while PX24 was 5.3-fold paclitaxel resistant compared with parental ME180 cells. F3 and F4 acquired resistance to cisplatin and paclitaxel by 8.3/4.9- and 3.7/3.3-fold (F3/4) respectively. Microarray analysis demonstrated, out of 22284 genes, 103 genes were >4-fold up-regulated in F3/4 and 33 (32%) were identified as simultaneously upregulated genes (SUG) in C13, PX24 and F3/4. The Protein-protein interaction analysis of 33 SUG displayed a scaffold network pivoting aldo-keto reductase 1C1 (*AKR1C1*), aldo-keto reductase1C2 (*AKR1C2*) and aldo-keto reductase1C3 (*AKR1C3*). The enrichment pathway analysis demonstrated *AKR1C* gene family anchored to molecular function of oxidoreductase and aldo-keto reductase activity and biological process of daunorubicin and doxorubicin metabolism. **Conclusions:** We report here the establishment of doubly drug-resistant hybridoma to platinum and taxane. Analysis of SUG indicated the *AKR1C* gene family plays a key role for doubly resistant mechanism that would be possible targets for therapeutic strategies.

Keywords: gynecologic malignancy; chemotherapy; drug sensitivity; resistance; taxane resistance; hybridoma

1. Introduction

The majority of gynecologic malignancies are treated with surgery followed by adjuvant chemotherapy comprising a platinum agent combined with a taxane. The difference in mode of action and mechanisms of acquiring resistance between platinating agents and taxane is the big advantage of the combination chemotherapy to increased efficacy. Treatment has advanced remarkably to develop much better efficacy in the field of breast cancer [1], ovarian cancer [2,3], uterine endometrial cancer [4], and cervical cancer [5]. However, any numbers of these patients will eventually relapse around 2 years after the start of chemotherapy with persistent drug resistance, either intrinsic or acquired. The optimal management of these patients is currently not established and chemoresistance arising from combined platinum and taxane therapy is usually more challenging than single agent resistance [6]. It's always a compelling question whether the mechanisms of doubly resis-

tance is a simple combination of single agent resistance or whether the core novel mechanism common to platinum and taxane resistance stands out as a result of combination therapy. The concept of doubly resistance to cisplatin and paclitaxel, rather than mutual cross-resistant between these drugs, has ever been discussed only in few papers [7,8] preparing doubly resistant cells. However, generating the drug resistant cells upon exposure to single agent often accompanies inverse resistance between platinum and taxane [9]. Whereas, when the cells are concurrently exposed to both drugs, the doubly resistance could be acquired but the maximally tolerated dose and the degree of resistance is usually small [8]. To avoid the backflow in this conventional way to establish the drug resistant cells, we have introduced cell fusion technique where the resistant-coding genes for each drug should be incorporated to implement the high degree of drug resistance. The upregulated genes shared among the cell lines contained the strictly-selected



unique gene cluster displaying one scaffold protein-protein interaction (PPI) network centered on *AKRIC* gene family associated with signaling for molecular function of oxidoreductase and aldo-keto reductase activity and biological process of daunorubicin and doxorubicin metabolism. Other PPI non-committed genes are independently playing an integral part of dual resistance, suggesting the feature of multifactorial resistant mechanism. Our established cell line enabled us to explore the core gene cluster and signaling pathways necessary to acquire resistance to platinum and taxane and they could be another molecular target for developing future therapeutic strategy.

2. Materials and Methods

2.1 Tumor Cell Lines

The human cancer cell line 2008 was originally established from a patient with a serous cystadenocarcinoma of the ovary [10]. For many years, it was thought to be an ovarian carcinoma cell line, but genetic testing subsequently disclosed that it was identical to the ME180 cervical carcinoma cell line that had been isolated by the same investigator at the same institution in the same year [11–13]. A resistant subline, designated C13*5.25 (C13), was obtained by 13 monthly selections with cisplatin followed by chronic exposure to stepwise increased cisplatin concentrations [14]. A paclitaxel resistant subline (PX24) was developed over an 8-month period by growing ME180 cells in the presence of progressively higher concentrations of paclitaxel [15]. The cells were grown on tissue culture dishes in a humidified incubator at 37 °C and 5% carbon dioxide atmosphere. Antibiotic was added in each cell culture experiments to keep the cells negative for contamination.

2.2 Cell Fusion

C13 cells (1×10^6 cells/mL) were fused with PX24 cells (1×10^6 cells/mL) at a 1:1 ratio using 40% polyethylene glycol (PEG; BDH Daiichi, Japan) in RPMI-1640 medium. These cells were centrifuged at 1200 rpm for 5 min at 25 °C and 1 mL of 50% PEG was added to the cell pellets. An additional 2.5 mL of warm serum-free medium was added to dilute PEG twice every 2 min. PEG-treated cells were centrifuged at 1200 rpm for 5 min at 25 °C, re-suspended with RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin, and cultured overnight. The next day the hybridoma cells were sequentially exposed to 20 nM paclitaxel for 24 h and then 8 μ M cisplatin for 1 h. Once the cells began growing again the same selection was repeated twice more for a total of three rounds of selection. The drug concentrations used were IC90 inhibitory concentration of paclitaxel for C13 and of cisplatin for PX24 and were sufficient to kill all of the parental non-drug-resistant cells and any non-hybridized drug resistant cells.

2.3 Colony Assays

Colony forming assay was used to assess drug sensitivity. Five milliliters of cell suspension, containing 1000 cells, was plated on 60-mm polystyrene tissue culture dishes. After 24 hours, drug-containing solution was added to triplicate plates at each drug concentration. Cells were exposed to drugs for 1 hour except for paclitaxel to which the cells were exposed for 24 hours because the paclitaxel-resistant PX24 cells were established by repeated 24-hour drug exposure [15]. The drug-containing medium was then aspirated and replaced with drug free medium. After colonies formed the plates were washed, fixed and stained. Colonies of over 100 cells were counted macroscopically.

2.4 Flow Cytometry

Cell monolayers were incubated for appropriate time and were washed with PBS twice. Trypsin was quenched with complete medium and the cell suspensions were centrifuged at $500 \times g$ for 5 min. The pellet was resuspended in PBS at a density of 1×10^6 cells/mL and fixed with 2 mL of 100% ethanol while vortexing. The cells were then placed at -20 °C until used. For cell cycle analysis, ethanol was removed, and the cells were washed once with PBS. The cells were stained with propidium iodide, and flow cytometry was performed on a FACS Calibur (Becton Dickinson). Argon laser was used for excitation at 488 nm and fluorescence emission was collected through a 620 nm Long pass filter. At least 6000 cells were analyzed per sample. The DNA-diploid cell population of ME180 is used as a reference standard for the identification of DNA-aneuploid clones. The ratio of aneuploid G0-G1 peak values to diploid G0-G1 peak values was expressed as a DNA index (DI). The cases with DI between 0.95 and 1.05 were considered as DNA diploids.

2.5 Quantitative Real Time PCR (Q-PCR)

We used the manual method using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Extracted RNAs for BRCA1 associated protein and BRCA1 is Breast Cancer gene 1 (BRAP), Protein kinase C theta type (PRKCC), Regulating synaptic membrane exocytosis protein 2 (RIMS2), O-linked N-acetylglucosamine (GlcNAc) Transferase (OGT), and Carboxylesterase (CES) mRNA assessment were subjected to reverse transcription using qScript cDNA SuperMix™ (Quantabio, Beverly, MA, USA). Complementary DNAs (cDNAs) were subjected to quantitative real-time PCR using PerfeCTa SYBR™ Green FastMix (Quantabio, Beverly, MA, USA). All PCR reactions were performed in 96-well plates using the tepOnePlus™ real-time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control during mRNA PCR.

2.6 Immunoblotting

Cell extracts were boiled for 5 min and fractionated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) minigels (7.5% separating gel) followed by electro transfer to nitrocellulose paper. The blots were incubated with antibodies specific for proteins of BRAP, PRKCQ, RIMS2, OGT, and CES followed by horseradish peroxidase conjugated anti-mouse Ig. Beta-actin was used as internal control.

2.7 Expression Profiling

Genome-wide expression profiling was done using the Human Genome U133A Array (HG-U133A: Gene Chip, Affymetrix, Santa Clara, CA, USA). RNA was isolated then double-stranded cDNA was synthesized, and the cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleotide triphosphates. The biotinylated cRNA was hybridized with a probe array, and the hybridized biotinylated cRNA was washed and stained with streptavidin-phycoerythrin and then scanned with a Gene Array Scanner. The fluorescence intensity of each probe was quantified using Gene Chip Analysis Suite 5.0 software (Affymetrix, Santa Clara, CA, USA). The expression level of a single RNA was determined as the average fluorescence intensity among the intensities obtained by 11-paired (perfect-matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the Microarray Analysis Suite 5.0 program. The data were processed with Affymetrix's default variables, except for scaling, without normalization procedures to calculate the level of gene expression as the signal.

2.8 Pathway Analysis

WebGestalt [16] was used (<http://bioinfo.vanderbilt.edu/webgestalt/>) to test for the enrichment of any pathways that might relate to the doublet drug resistance phenotype. Gene Ontology (GO) [17], one of the integrated multiple centrally curated functional database, was analyzed using this system.

2.9 Protein-Protein Interaction Network (PPI)

Protein-protein interaction network (PPI) scaffold networks were retrieved from the STRING (Search Tool for the Retrieval of Interacting Genes) database [18,19] on the basis of simultaneously upregulated 33 genes.

2.10 Statistical Analysis

Differences between samples or groups of samples were determined by two-sample *t*-test using two-sided *p*-values for significance at $p < 0.05$. For the gene expression analysis, data files were imported from the Affymetrix Expression Console where the background was corrected

and quantile was normalized. The data were then analyzed using a two-sample *t*-test for significance at $p < 0.05$ with a fold change cutoff of 4.0. To assess the possible functional connections between the differentially expressed genes, a pathway analysis that assesses statistically represented functional terms within a list was conducted using Ingenuity Pathways Analysis for all comparisons. To determine the correlation between gene expression intensity and anchored-frequency, lineal regression analysis was performed.

3. Results

3.1 Drug Sensitivity Profiles of Hybridoma Cells

Two hybridomas (F3 and F4) were selected for >3-fold resistant to cisplatin and paclitaxel that is far more clinically relevant drug resistance and thus these cell lines could be reasonably selected for further studies. Fig. 1 illustrates dose-response curves for parental C13 and PX24, and two hybridomas of F3 and F4 that are compared to grand-parent ME180 cells. Based on those lines, IC50s and the degree of resistance for four resistant cells were calculated (Table 1). C13 cells were 5.75 ± 0.42 (mean \pm SE)-fold resistant to cisplatin while PX24 cells were 5.30 ± 0.58 -fold resistant to paclitaxel compared with the parental ME180 cells and neither the C13 nor PX24 cells demonstrated cross-resistance to either cisplatin or paclitaxel. The F3 and F4 hybrids had similar levels of statistically significant resistance to cisplatin 8.27 ± 0.46 - and 4.88 ± 0.44 -fold and to paclitaxel 3.66 ± 0.14 - and 3.29 ± 0.03 -fold [$N = 3$; $p < 0.05$], respectively. The level of cisplatin resistance of the hybrids was similar to that of the C13 cells while paclitaxel resistance was slightly inferior to that of the PX24 cells. C13 and hybridoma cells were approximately 2-fold hypersensitive to doxorubicin and vincristine. These results indicate that F3 and F4 cells exhibited similar drug sensitivity profiles and the sensitivity pattern is more likely to C13.

3.2 Measurement of Cellular DNA Content

DNA histograms for five cell lines were shown in Fig. 2. All those five cells showed the narrow and high peak associated with diploid G0/G1 with a small peak identified as G2/M phase. DNA index (DI) for F3 and F4 was 0.86 and 0.81 compared to C13 and PX24 of 0.86 and 0.98. The similarity of diploid DNA-frequency histogram pattern showed no significant difference could be seen in DNA content among those cells indicating DNA diploid clones of F3 and F4.

3.3 Gene Expression Patterns in C13, PX24, and F3/4 Cells

To identify genes important to the acquisition of resistance to both drugs, gene expression profiles were determined for the C13, PX24, and F3/4 cells. Fig. 3 presents Venn diagrams depicting the number of genes more than or less than four-fold differentially expressed relative to

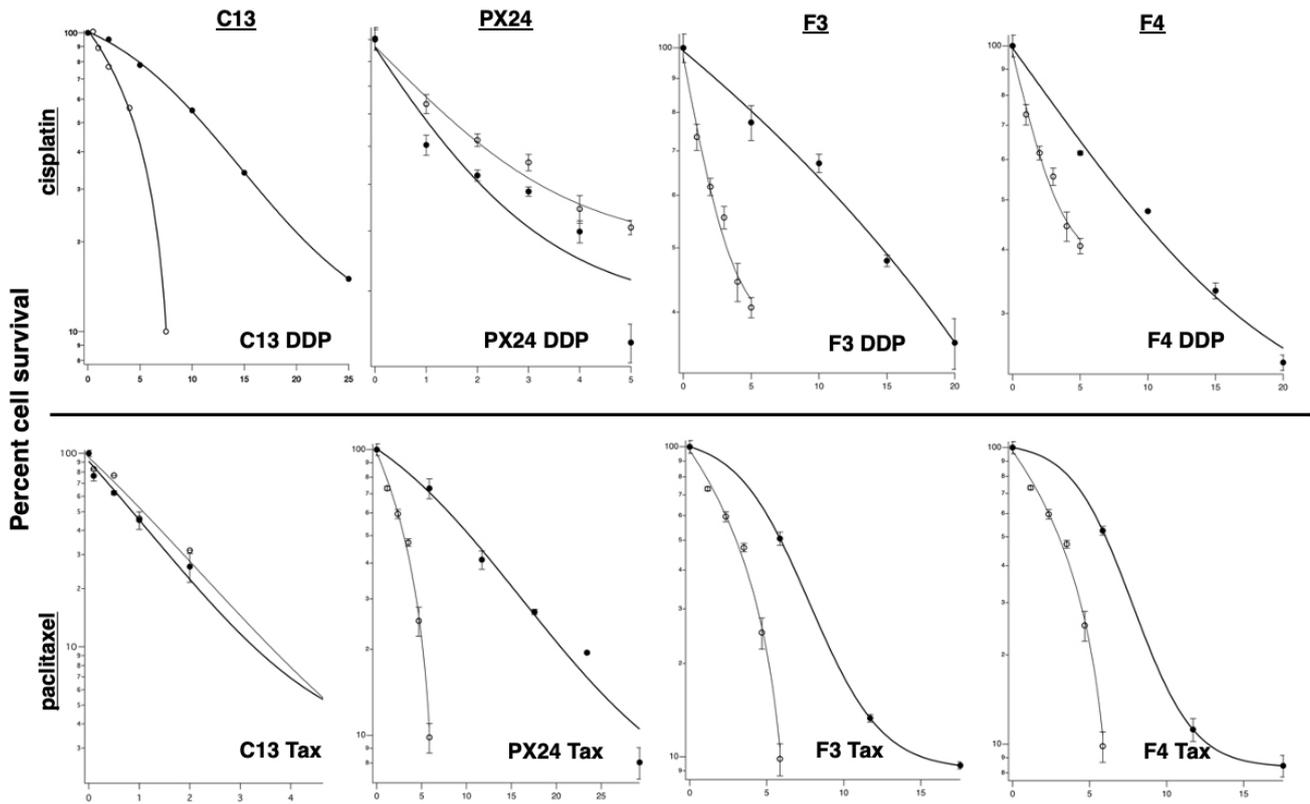


Fig. 1. Dose-response curves for parental C13 and PX24, and two hybridomas of F3 and F4 (Closed circle) that are compared to grand-parent ME180 cells (open circle). DDP, cisplatin; Tax, paclitaxel.

Table 1. Drug sensitivity profiles¹.

		ME180	C13	PX24	F3	F4
Cisplatin ²	IC 50 ⁴ (μ M)	1.76 \pm 0.17	10.12 \pm 0.26	2.14 \pm 0.35	14.55 \pm 0.81	8.58 \pm 0.08
	-fold resistance	–	5.75 \pm 0.42 ⁵	1.21 \pm 0.14	8.27 \pm 0.46 ⁵	4.88 \pm 0.04 ⁵
Paclitaxel ³	IC 50 (nM)	1.88 \pm 0.49	1.04 \pm 0.03	9.97 \pm 1.09	6.88 \pm 0.26	6.18 \pm 0.07
	-fold resistance	–	0.56 \pm 0.05	5.30 \pm 0.58 ⁵	3.66 \pm 0.14 ⁵	3.29 \pm 0.03 ⁵
Doxorubicin ²	IC 50 (nM)	2.60 \pm 0.38	1.27 \pm 0.20	2.26 \pm 0.23	1.01 \pm 0.16	1.27 \pm 0.23
	-fold resistance	–	0.49 \pm 0.08 ⁵	0.87 \pm 0.09	0.39 \pm 0.06 ⁵	0.48 \pm 0.08 ⁵
Vincristine ²	IC 50 (μ M)	1.29 \pm 0.08	0.77 \pm 0.06	1.14 \pm 0.06	0.93 \pm 0.07	0.79 \pm 0.06
	-fold resistance	–	0.60 \pm 0.05 ⁵	0.89 \pm 0.05	0.72 \pm 0.06 ⁵	0.61 \pm 0.05 ⁵
5-FU ²	IC 50 (μ M)	15.07 \pm 1.53	13.53 \pm 0.97	17.28 \pm 2.19	14.84 \pm 1.55	12.65 \pm 0.82
	-fold resistance	–	0.90 \pm 0.06	1.15 \pm 0.15	0.99 \pm 0.10	0.84 \pm 0.05

1; Mean \pm SE.

2; One hour exposure.

3; Twenty-four hours exposure.

4; Fifty percent inhibitory drug concentration.

5; $p < 0.05$ (compared to ME180).

ME180 cells either uniquely or in common among the C13, PX24 and F3/4 hybridoma cells. Out of 22,284 genes examined, when assorted by each resistant phenotype (C13, PX24 and F3/4), 163, 415, and 103 genes were significantly up-regulated ($p < 0.01$) and 535, 986 and 321 were down-regulated in the development of resistance to cisplatin, paclitaxel and both. Thirty-three genes were elevated in common to all three resistant phenotype and were considered

as core gene cluster to acquire drug resistance, while 109 genes were simultaneously down-regulated.

3.4 Hierarchical Clustering of Genes Expressed in Common in C13, PX24 and F3/4 Cells

To further visualize the differences in gene expression patterns among four types of resistant cells, we performed hierarchical clustering of the 33 up-regulated genes and the

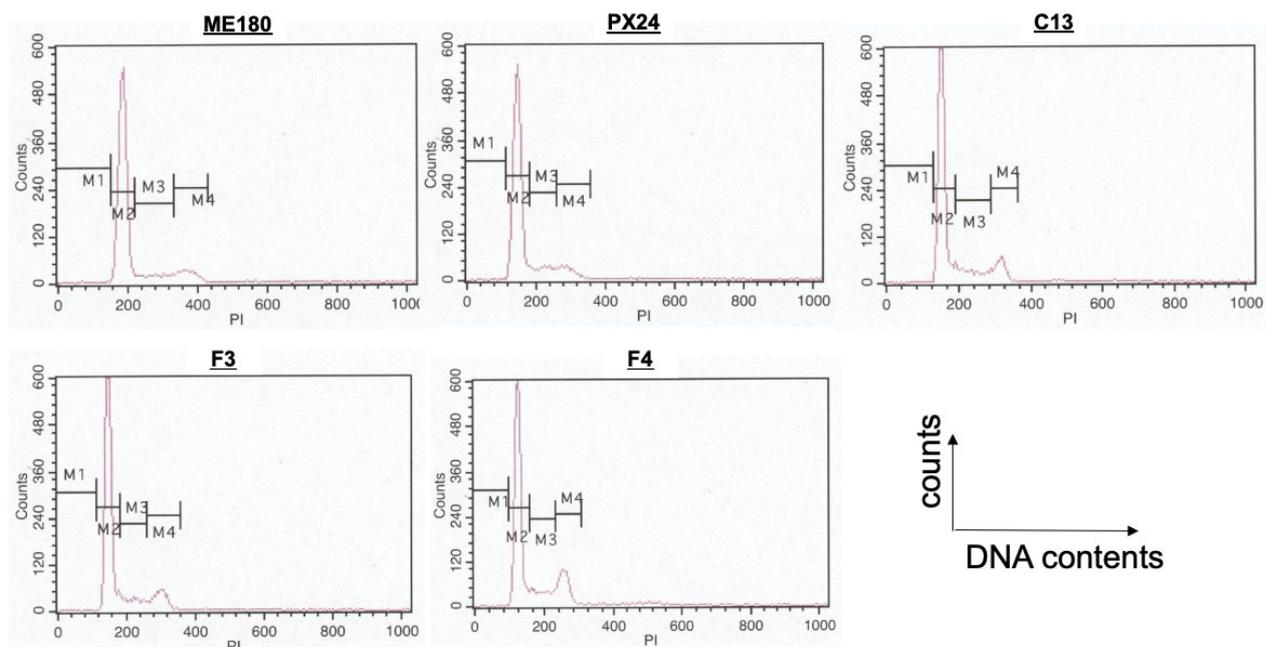


Fig. 2. DNA histograms for five cell lines. M1: Cell death, M2: G0-G1 phase, M3: S phase, M4: G2 phase.

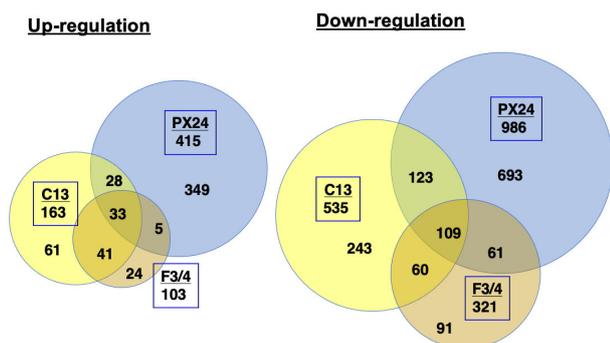


Fig. 3. Venn diagrams presenting the number of genes differentially or commonly expressed in each C13, PX24 and F3/4 hybridoma cells. A total of 33 genes were up-regulated and 109 were down-regulated in common to all three types of resistant cells.

109 down-regulated genes. The dendrogram pictured in Fig. 4 shows the segregation of the gene expression patterns and demonstrates greater similarity between C13 and F3/4 than between PX24 and the F3/4 cells. This suggests that the cisplatin-resistant C13 cells contributed to a greater degree than the PX24 cells to the phenotype of the F3/4 cells that is consistent with the results of drug sensitivity pattern; F3/4 cells acquired cisplatin resistance while they demonstrated collateral sensitivity to doxorubicin and vincristine at the same level of C13.

3.5 Validation of Cell-Specific Gene Expression

For validation by Q-PCR, five genes out of simultaneously up-regulated 33 genes were selected based on the

five-ranks of up-regulation level and these were shown in red letters in Fig. 4. It is worth nothing to dig up further in down-regulated genes for resistant-relevant gene profiling. In general, significant up-regulation in transcript levels from the microarray results were confirmed by Q-PCR and, regarding to the fold change, the degree of transcription was mostly compatible with microarray results except for BRAP and OGT in F4 and RIMS2 in F3 and F4 cells showing the negative direction of change (Fig. 5). In hybridoma, transcription might not always correspond to microarray results.

3.6 Protein Expression Determined by Immunoblotting

Changes in gene expression was further confirmed at protein level. Immunoblot signals of five proteins (CES1, PRKCQ, BRAP, OGT and RIMS2) were normalized to loading control of β -actin density and then band density ratio was calculated between each resistant cells and ME180 parental cells (Fig. 6). The protein over-expression ratio was better consistent with microarray results than mRNA and the density ratios were well allied in the order of up-regulation demonstrated by microarray (CES1, PRKCQ, BRAP, OGT and RIMS2). However, compared to the exceptional results of Q-PCR in BRAP, OGT and RIMS2 in F3 or F4, those genes amplifications and mRNA levels might not always correspond to protein levels.

3.7 Protein-Protein Interaction Network

Fig. 7 depicted protein-protein interaction network (PPI) built on the basis of simultaneously up-regulated 33 genes in common to 4 resistant cells. This showed one tightly constituted scaffold comprising four group of genes

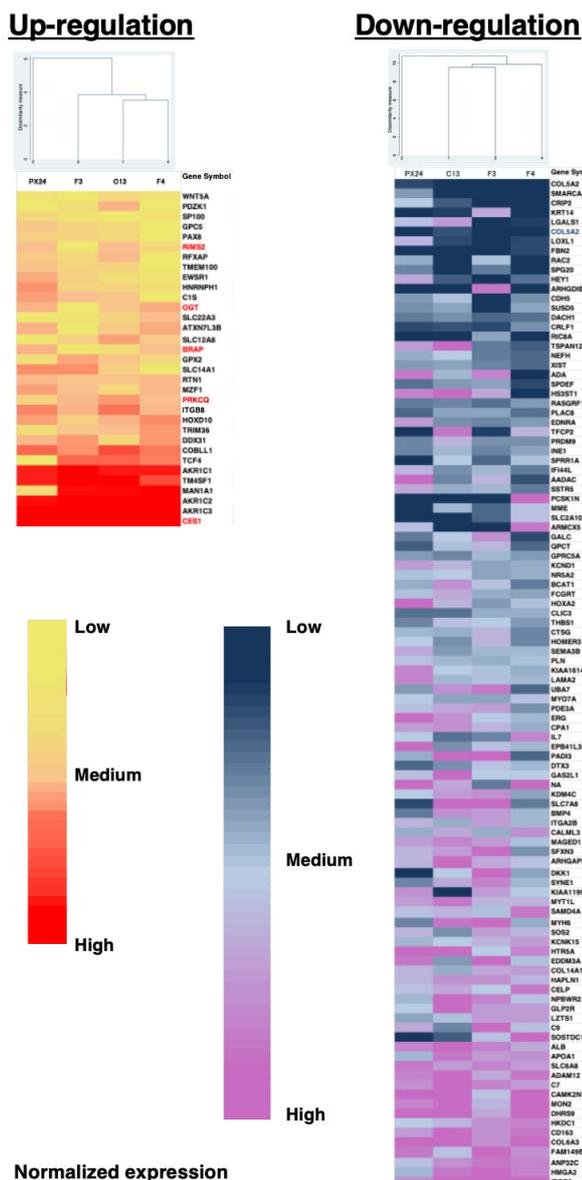


Fig. 4. Hierarchical clustering of commonly up-regulated 33 genes and down-regulated 109 genes. Columns represent individual samples and rows represent genes. A visual doublet color code is utilized with red and yellow in up-regulated genes and violet and dark-blue in down-regulated genes indicating relatively high and low expression levels. Dendrogram illustrate the separation of samples based on their degree of similarity.

(*AKRIC1*, *AKRIC2*, *AKRIC3* and *GPX2*) and two small scaffolds. Other genes were likely to be enrolled as individual roles for rendering resistance, suggesting multifactorial mechanism of drug-resistance.

3.8 Enrichment Analysis for Signaling Pathway

Enrichment analysis for pathways with the Gene Ontology (GO) database revealed that proteins incorporated

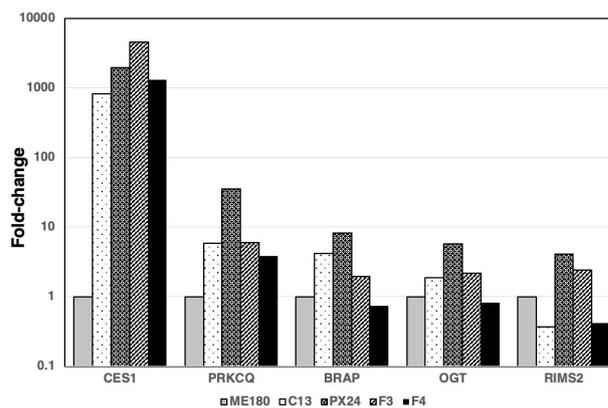


Fig. 5. Q-PCR analysis of genes elevated in four drug resistant cells and parental ME180. The y-axis represents fold up-regulation in the different drug resistant cell lines over the parental ME180 cell line.

as main scaffold in above networks, particularly *AKRIC* gene family, are enriched for molecular function and biological processes as listed in Table 2. The molecular function contains ketosteroid- and phenanthrene monooxygenase, androsterone-, alcohol- and steroid dehydrogenase activity, aldo-keto reductase (NADP) activity and oxidoreductase activity. The biological process contains cellular response to jasmonic acid, daunorubicin-, doxorubicin-, aminoglycoside and glycoside metabolic process, steroid hormone metabolic. The results indicated that those pathways are functioning in common among all four types of resistance. Studies for pathway enrichment on 33 simultaneously up-regulated genes in these resistance phenotypes showed 16 significantly overrepresented pathways but they involved three genes of *AKRIC* gene family.

4. Discussion

Just about every patients with advanced-stage gynecologic malignancies receive platinum with taxane as a primary chemotherapy. Intrinsic resistance or acquired resistance to this combination is associated to resistance to both drugs individually. One possibility is that such tumors contain clones that are separately resistant to one or the other drug in such proportions that treatment with either as a single agent fails to produce a clinically detectable response. Another possibility is that it contains individual cells that are resistant to both drugs. It has not been possible to characterize such doubly resistant cells because combined exposure of naïve cells to both drugs produces such a high level of cell kill. There are some reports of cross-resistance [7,20], but these doubly resistant cells were not genomically characterized. We utilized hybridoma technology as an alternative way of generating doubly resistant cells that could be characterized by expression profile analysis.

The F3/F4 hybrids exhibited levels of resistance similar to those of the parental C13 and PX24 cells (Table 1).

Table 2. Pathways enriched in up-regulated 33 gene sets.

Sub-root	Category name	p value	Reference genes
Molecular function	Ketosteroid monooxygenase	4.74×10^{-7}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Phenanthrene 9,10-monooxygenase	4.74×10^{-7}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	4.74×10^{-7}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Androsterone dehydrogenase activity	1.42×10^{-6}	AKR1C1, AKR1C2, AKR1C3
Molecular function	alditol:NADP+ 1-oxidoreductase activity	5.64×10^{-6}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Aldo-keto reductase (NADP) activity	5.64×10^{-6}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Alcohol dehydrogenase (NADP+) activity	2.80×10^{-5}	AKR1C1, AKR1C2, AKR1C3
Molecular function	Oxidoreductase activity	0.0002	AKR1C1, AKR1C2, AKR1C3
Molecular function	Steroid dehydrogenase activity	0.0002	AKR1C1, AKR1C2, AKR1C3
Biological process	Cellular response to jasmonic acid	1.11×10^{-5}	AKR1C1, AKR1C2, AKR1C3
Biological process	Daunorubicin metabolic process	6.18×10^{-5}	AKR1C1, AKR1C2, AKR1C3
Biological process	Doxorubicin metabolic process	6.18×10^{-5}	AKR1C1, AKR1C2, AKR1C3
Biological process	Aminoglycoside antibiotic metabolic process	6.18×10^{-5}	AKR1C1, AKR1C2, AKR1C3
Biological process	Progesterone metabolic	0.0003	AKR1C1, AKR1C2, AKR1C3
Biological process	Glycoside metabolic process	0.0004	AKR1C1, AKR1C2, AKR1C3
Biological process	C21-steroid hormone metabolic	0.0024	AKR1C1, AKR1C2, AKR1C3

Table 3. Genes enrolled in drug resistance.

Gene symbol	Summary	Reference
TM4SF1	B7-H3 suppresses doxorubicin-induced senescence-like growth arrest in colorectal cancer through the AKT/TM4SF1/SIRT1 pathway	Wang <i>et al.</i> Cell Death and Disease 2021; 12: 453 [26]
PRKCQ	PRKCQ inhibition enhances chemosensitivity of triple-negative breast cancer by regulating Bim	Byerly <i>et al.</i> Breast Cancer Research 2020; 22: 72 [27]
OGT	O-GlcNAc Transferase (OGT) Inhibitor Synergistically Enhances Doxorubicin-Induced Apoptosis in HepG2 Cells	Cancers 2020; 12: 3154 [28]
TCF4	Tumor xenograft modeling identifies an association between TCF4 loss and breast cancer chemoresistance	Disease Models & Mechanisms 2018; 11 [29]
ITGB8	Overexpression of ITGB8 restored CDDP resistance inhibited by miR-199a-3p	Oncol Rep 2018; 39: 1649 [30]
EWSR1	Relationship of sensitivity of Ewing's sarcoma cells harboring EWSR1 gene translocation to poly(ADP-ribose) polymerase (PARP) inhibitors	Nature. 2012; 483: 570 [31]

Similar doubly resistant cell line was reported by Armstrong *et al.* [8]. They established the cells acquiring combined resistance to carboplatin and docetaxel by exposing parent chemotherapy-naïve A2780 cells to compare IC50s on concurrent exposure [8]. However dual drug treatment produce efficacy at far lower doses probably because of the synergistic effect. Their cells showed more than 10-fold decrease in IC50s of carboplatin and docetaxel when exposed to both drugs simultaneously compared to when exposed to each drug alone. On this score, our hybridoma technology is superior to conventional method of exposing cells to step-wise higher concentration of drugs when producing doubly resistant cells. When individual cisplatin and taxol resistant cell nucleus fused with each other, the substantially important drug-resistant coding genes should be incorporated to survive the following selection for resistance. The cells get gene cluster necessary to acquire resistance first, resulting in generation of cells implemented with similar level of drug resistance.

Genomic analysis demonstrated that the large number of unique changes in gene expression were detected in C13

and PX24. It is interesting in knowing fraction of genes differentially expressed between the ME180 and C13 or PX24 cells are still differentially expressed between the ME180 and F3/F4 cells (74 or 38 genes). Probably not all the genes that are differentially expressed between the ME180 and C13 or PX24 cells (163 or 415 genes), particularly up-regulated genes uniquely to the drug used (61 out of 163 in C13 or 349 out of 415 in PX24). However, the subsets of these that are still differentially expressed between the ME180 and F3/F4 cells (74 or 38 genes) are further enriched for more curated genes that are constitutively important to the cisplatin or paclitaxel resistant phenotype. Since F3 and F4 were generated by cell fusion technology, the more sophisticated genes were selected in hybridoma and genes that are not actually contributing doubly resistance were whittled down in the process of nuclear fusion.

Hierarchical cluster analysis on 33 genes over-expressed in common demonstrated greater similarity between the C13 and F3 or F4 cells than with the PX24 cells (Fig. 4) and Venn graph showed that more genes were shared with C13 cells (74 genes) than with PX24 cells (38

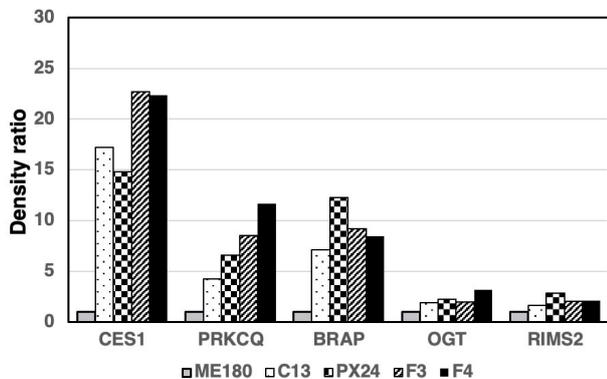
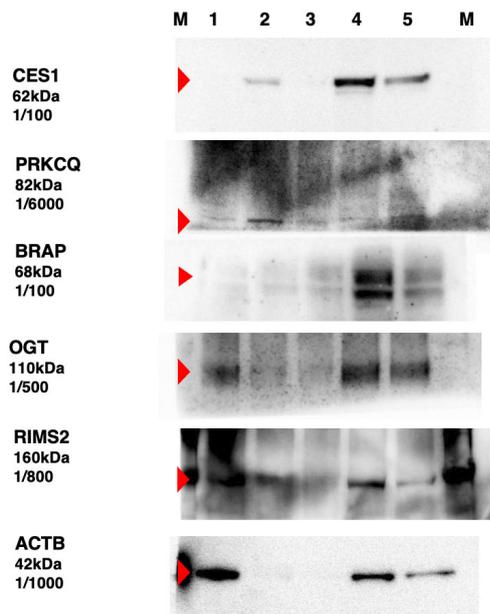


Fig. 6. Representative immunoblot analysis of selected gene products identified by microarray and Q-PCR as altered in four drug resistant cells and parental ME180. The y-axis represents density ratio in the different drug resistant cell lines based on parental ME180 cell.

genes) (Fig. 3). It is interesting that the similar results were reported in carboplatin/docetaxel dual resistant cells showing that the gene expression changes were more likely to carboplatin resistant cells than docetaxel resistant cells [8,11].

Protein-protein interaction network showed one tightly constituted scaffold comprising four group of genes (*AKR1C1*, *AKR1C2*, *AKR1C3* and *GPX2*) and these *AKR1C* gene superfamily were enriched for molecular function and biological process as listed in Table 2. Aldo-keto reductases (AKRs) catalyze the NADPH-dependent reduction of carbonyl groups and mediate resistance to cancer chemotherapeutics including cisplatin and anti-tubulin agents. As shown in subcategory on Table 2, enrichment for molecular function included alcohol dehydrogenase activity, aldo-keto reductase (NADP) activity and oxidoreductase activity and these pathways are playing integral

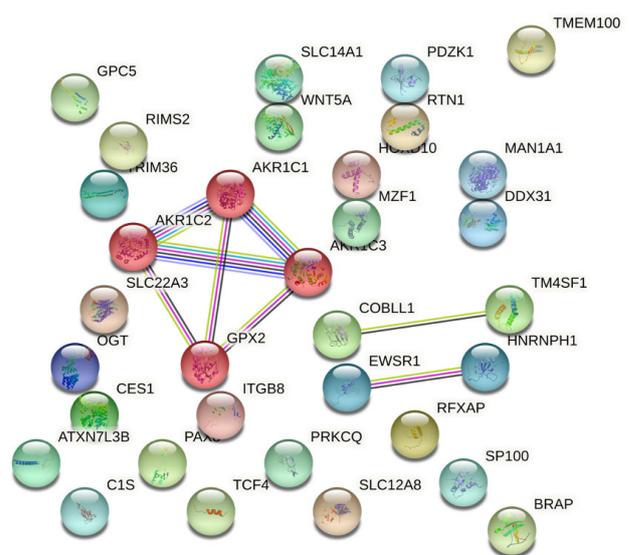


Fig. 7. Protein-protein interaction network scaffold, built on the basis of 33 simultaneously up-regulated genes among C13, PX24 and F3/4 cells, identifying one tight scaffold network framed by three hub genes of *AKR1C1-3*.

part of drug resistance [21–25]. Significant up-regulation of *AKR1C3* was reported in carboplatin and docetaxel dual resistant cells [8], supporting the important role of aldo-keto reductase in drug resistance. Enrichment analysis also documents the *AKR1C*-family associated metabolic process on daunorubicin and doxorubicin that might link to the collateral sensitivity in F3 and F4 cells (Table 1 and Fig. 1). Other up-regulated genes than *AKR1C* family were excluded from the PPI network constitution (Fig. 7), however, some of them were enrolled in drug resistance individually. Reports of drug sensitivity related genes are listed in Table 3 (Ref. [26–31]) showing that each gene are uniquely contributing to drug resistance, suggesting multiple mechanisms can mediate the development of drug resistance. Among them, EWS (Ewing Sarcoma) RNA Binding Protein 1 (EWSR1) could be the candidate for powerful biomarker to guide the use of Poly(ADP-Ribose) Polymerase (PARP)-inhibitor now world-widely used to circumvent drug resistant gynecologic tumor.

Significant up-regulation in transcript levels were confirmed by Q-PCR and the degree of transcription was mostly compatible with microarray results except for part of BRAP, OGT and RIMS2 in hybridoma showing the negative direction of change. The dissociation may be due to design of the Q-PCR primers being based on transcript specific sequences while the oligonucleotides used in microarray are designed to detect possible transcript genes containing non-coding transcript. Whereas, in the protein level, over-expression ratio was well consistent with microarray results and the density ratios were well corresponding to the order on the data demonstrated by microarray. The discordance among microarray, Q-PCR and protein expression

results is not quite rare and this was observed in other experiments [32–34]. They documented some discrepancy eventually happen in transcription and translation of gene products, suggesting the requirement of protein-expression assessment for the precise knowledge. Finally, the limitation of this work is the lack of functional analysis of those selected genes. Whether the identified genes and pathways are causally related to doubly resistance remains to be determined and it will be important to follow up these findings with mechanistic studies.

5. Conclusions

We report here the establishment of novel cell line showing resistant to cisplatin and paclitaxel. Genomic analysis suggested that *AKRIC* gene family is playing core part of doubly resistance by framing protein-protein-interaction network while some other genes should be committed individually. Our data allowed for the identification of new candidate druggable genes and pathways linked to dual drug resistance and targets future development.

Author Contributions

SI worked out the conception of the whole study, oversaw the selection of the cell lines, generated the graphs and figures, contributed to the final data analysis and drafted the manuscript. RS performed the drug sensitivity assay, the cell fusion experiment, microarray analysis, flowcytometry. NY performed the qPCR validation experiments and immunoblotting for the selected genes. SS supervised and conducted the cell fusion technique, microarray experiments. All authors read and approved the final manuscript and are accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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