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Chemosensitivity and radiosensitivity profiles of four new human epithelial ovarian cancer cell lines exhibiting genetic alterations in *BRCA2*, *TGFβ-RII*, *KRAS2*, *TP53* and/or *CDNK2A*

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Abstract To address the cellular basis for the response to ovarian cancer treatment, we characterized the chemosensitivity and radiosensitivity of four human epithelial ovarian cancer cell lines that harbor different genetic alterations. The TOV-21G, TOV-81D, OV-90, and TOV-112D cell lines were derived from ovarian tumors (TOV) or ascites (OV) from chemotherapy- and radiotherapy-naive patients and were characterized by their mutation spectrum of *BRCA2*, *TGFβ*-RII, *KRAS2*, *TP53*, and *CDKN2A*. Cells were monitored for survival following exposure at various concentrations to different cytotoxic agents including cisplatin, camptothecin or paclitaxel or to different doses of γ-irradiation. At the lowest doses, the *TGFβ*-RII-mutated and *KRAS2*-mutated cell line, TOV-21G, and the *BRCA2*-mutated cell line, TOV-81D, demonstrated a significantly higher sensitivity to cisplatin and γ-irradiation than the *TP53*-mutated cell lines, TOV-112D and OV-90. At higher doses, differences between the *TP53*-mutated lines were observed with TOV-112D being less sensitive to cisplatin than OV-90 that also harbors a *CDNK2A* mutation. All cell lines were similarly sensitive to high doses of γ-irradiation. In contrast, sensitivity to camptothecin or paclitaxel was not significantly different between all cell lines, irrespective of the mutation status of *BRCA1*, *BRCA2*, *TGFβ*-RII, *KRAS2*, *TP53*, and *CDKN2A*. The observed responses to treatment are consistent with the current knowledge concerning *BRCA2*, *TGFβ*-RII, *KRAS2*, *TP53*, and/or *CDKN2A* aberrant function.

Keywords Chemosensitivity · Radiosensitivity · Ovarian epithelial cell line · Genetic alterations in *BRCA2*, *TGFβ*-RII, *KRAS2*, *TP53*, and/or *CDNK2A*

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Introduction

Epithelial ovarian cancer (EOC) remains the most lethal gynecological cancer mainly due to a late diagnosis when symptoms are related to the invasion of pelvic organs and/or abdominal metastasis. The biological mechanisms leading to the malignant transformation of the

ovarian surface epithelium (OSE) are still unclear. Accumulation of a number of genetic abnormalities is frequently observed in ovarian cancers, implicating known pathways leading to transformation, such as *TP53*, *CDKN2A*, and *KRAS2*, as well as unknown genes that map to chromosomal regions frequently associated with loss or gain [9, 13, 19–21, 23, 26, 28, 38, 46, 57].

Four human ovarian cancer cell lines (TOV-21G, TOV-81D, OV-90, and TOV-112D) derived from ovarian tumors or ascites from chemotherapy- and radiation-therapy naive patients were established in our laboratory and characterized using biological, morphological, histological, cytogenetic, and genetic criteria [34]. The cell lines differ in their morphological and growth characteristics, which correlate with the histological subtype and clinical aggressiveness of the original tumor. In particular, we observed that TOV-81D, which is derived from a clinically indolent ovarian cancer (> 10 years survival), is unable to form tumors in the nude mouse model in contrast to the rapid tumor formation seen with TOV-21G, OV-90, and TOV-112D [34]. These phenotypic differences were also revealed in the analysis of global patterns of gene expression using oligonucleotide expression microarrays. Comparing patterns of expression in the four cell lines to that in a primary culture of normal surface epithelial cells, we have noted strong similarities between the normal and indolent TOV-81D tumor cell line, while the TOV-21G, OV-90, and TOV-112D exhibit characteristics compatible with an aggressive phenotype [53].

The EOC cell lines harbor mutations in genes previously shown to occur in the ovarian cancer context. The TOV-81D cell line carries a mutation in *BRCA2* [34]. Germline mutations in *BRCA2* significantly increase the risk for developing both breast and ovarian cancers and usually occur in the context of a strong family history of these cancers [11]. The TOV-21G cell line harbors a frameshift mutation in *TGFβ*-RII (transforming growth factor beta receptor II) resulting from the deletion of one adenine base within the 10-bp polyadenine repeat present in the RII coding region [23]. This type of acquired sequence variation is often observed in the context of microsatellite instability [23]. While a mutation in a mismatch excision repair (MMR) gene has not been identified in TOV-21G, microsatellite analysis suggests that TOV-21G displays a RER^+ phenotype consistent with harboring a defect in MMR [23]. Both The TOV-112D and OV-90 cell lines harbor a mutation in the DNA binding domain of *TP53* [34]. The OV-90 cell line also carries a mutation in *CDNK2A* [34]. Mutations in *TP53* and *CDNK2A* inactivation are frequent occurrences in EOC [27]. Hence the molecular genetic signature of these established EOC cell lines reflect those observed in the analysis of ovarian cancers.

Since these genes are involved in the control of the integrity of the genome and the maintenance of genomic stability, mainly during cell division and/or in response to DNA damage, the genetic alterations described in our cell lines could be of importance in the therapeutic and carcinogenetic process. The association of Brca1 and Brca2 proteins with recombination/DNA repair proteins suggests that they are both involved in the preservation of genome integrity [32, 40, 41, 61]. *BRCA1* is involved at multiple levels in the DNA damage response and there is now evidence that *BRCA2* is essential for double strand break repair by homologous recombination [59]. Cells that contain truncated Brca2 proteins progressively accumulate aberrations in chromosome structure [55]. *p53* is involved in a DNA damage-induced G_1/S and G_2/M cell cycle checkpoint and contributes together to DNA repair mechanisms [39]. *CDKN2A* is also involved in the G_1/S checkpoint control [27, 43, 56]. Mutations in codons 12 and 13 of Ras are activating mutations that occur frequently in carcinomas [1, 3, 5, 9, 13, 17, 44–46, 48, 51]. These point mutations result in a constitutive activation of the abnormal Ras protein, which leads to continuous activation of the MAP kinases cascade, triggering uncontrolled cell proliferation and tumorigenesis [16, 51]. To complete the analysis of our cell lines, we also determined the Ras mutation status of our cell lines in the present study.

A large number of cytotoxic agents including platinum compounds, alkylants, and more recently paclitaxel and camptothecin derivatives, have been used as treatment for advanced stage EOC [31]. In the recent past, irradiation was also offered as an option [2, 50]. Despite an improved efficiency resulting from the use of these drugs in combination, such as paclitaxel and platinum combined therapy, the prognosis of this disease remains poor [31]. The cellular basis for response to these chemotherapeutic agents remains unclear in ovarian cancers, although recent work has focused on linking response to apoptosis and AKT pathways [7, 12], as well as molecular [6] and genetic markers [22] with the development of chemoresistance. Treatment sensitivity studies of ovarian cancer cell lines mutated for genes involved in ovarian carcinogenesis could be useful to further dissect cellular response to therapies. In this study, we characterized the response to paclitaxel, cisplatin, camptothecin, and γ-irradiation of four recently described human ovarian cancer cell lines harboring specific molecular genetic signatures.

Material and methods

Cell lines

The TOV-21G, TOV-81D, OV-90, and TOV-112D cell lines were established either from ovarian carcinoma (TOV) of different histologies or ascites (OV) from previously untreated patients. These four cell lines were previously characterized according to their morphology, behavior in culture, and tumorigenicity [34] and global gene expression profiles [53]. Briefly, they have been shown to harbor mutations in *BRCA1*, *BRCA2*, *TGFβ*-RII, *TP53*, and/or *CDKN2A* [34]. Genomic DNA from the EOC cell lines was investigated for codons 12 and 13 *KRAS2* mutations. DNA fragments for sequencing were

generated by PCR reactions in a total volume of 25 µl:2 µl DNA (200–400 ng), 1× PCR buffer, 5 pmol of each dNTP, 100 ng each of forward and reverse primers [35], and 2.5 U Taq DNA polymerase (Amersham Biosciences). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR system 9600 under the following conditions: 3 min at 95°C, then 35 cycles of 15 s at 94°C, 15 s at 55°C and 30 s at 72°C. The PCR products were sequenced using a Sequenase PCR sequencing kit (Amersham Biosciences) according to the manufacturer's instructions. Heat-denatured products were electrophoresed at 70 W in 5% polyacrylamide under denaturing conditions. Autoradiography was performed with Kodak Biomax MR film for 2–5 days.

Cell growth experiments, cytotoxic drugs, and irradiation treatments

Cells were grown in OSE medium (1:1 mixture of medium 199 adjusted to pH 7.3 and medium 105 adjusted to pH 7.6) [18]. The medium (Sigma, St Louis, Mo.) was supplemented with 10% fetal bovine serum (FBS) (Wisent, Rocklin, Calif.), 0.5 µg/ml amphotericin B (Cellgro, Herdon, Va.), and 50 µg/ml gentamicin (Wisent) as described previously [21]. The TOV-81D cells were grown in 15% FBS.

Paclitaxel, *cis*-diamminedichloroplatinum(II), and 20(S)-camptothecin were purchased from Sigma. All drugs were diluted in dimethyl sulfoxide and used as ×100 concentrated stock. Cells were plated at 10^5 cells/dish (60-mm dishes for cytotoxic drug treatments and 25-cm^2 T-flasks for irradiations) 18–20 h before treatments.

Cytotoxic drug exposure Initial experiments were performed to identify the concentrations of cisplatin (0.1 µ*M* and 1.0 µ*M*), camptothecin (0.001 µ*M* and 0.01 µ*M*), and paclitaxel (0.001 µ*M* and 0.01 µ*M*) that represented high and low dose responses in the EOC model system. After 24 h of drug treatment, cells were washed with phosphate-buffered saline and grown in fresh complete medium without drug.

Irradiation Gamma-ray irradiation was performed at 4°C with a ^{60}Co-source (1.60 Gy/min). Following initial experiments to determine EOC cell response to this treatment, escalating doses of 2, 4 and 6 Gy were tested.

Survival curves Cell counts were determined using a trypan blue exclusion assay in duplicate from two to four independent experiments. For cytotoxic drug treatment, cells were counted prior to drug treatment (day 0) and on days 2, 4 and 6. For irradiation experiments, cells were counted before irradiation (day 0) and on days 4 and 7. Survival was scored by comparing treated cells to untreated cells (control) and expressed as a percentage of cell survival. The standard error of the means of each different independent experiment was calculated when at least three independent experiments were performed.

Results

Mutation characterization

Our cell lines were previously characterized for mutations in *BRCA2*, *TGFβ*-RII, *TP53*, and/or *CDNK2A* (Table 1). Mutations in codons 12 and 13 of *KRAS2*

Table 1 Relationship between the mutational status of the four different ovarian cell lines and the percentage cell survival 6 days after exposure to drugs and 7 days after irradiation. Numbers in this table are the mean survival of treated cells in two to four independent experiments expressed as a percentage of the survival of untreated control cells (*wt* wild-type)

	Cell line			
	TOV 112D	OV90	TOV 81D	TOV 21G
Mutation				
BRCA1[a]	Negative[b]	Negative[b]	Negative[b]	Negative[b]
BRCA2[a]	Negative[b]	Negative[b]	8765delAG	Negative[b]
TP53[a]	Arg175His	Ser215Arg	wt	wt
CDNK2A[a]	wt	211delTC Ala86Asn	wt	wt
TGFβ-RII[c]	wt	wt	wt	709delA
KRAS2	Negative[d]	Negative[d]	Negative[d]	Gly13Cys
Treatment				
Cisplatin 0.1 µ*M*	113 ± 13%	88 ± 7%	53%[f]	33 ± 6%
Cisplatin 1.0 µ*M*	76 ± 12%	9 ± 1%	32%[f]	25 ± 16%
γ-irradiation 2 Gy	109 ± 32%	86 ± 6%	52 ± 3%	59 ± 3%
γ-irradiation 4 Gy	49 ± 16%	59 ± 10%	42 ± 8%	31%[f]
Camptothecin 0.001 µ*M*	89 ± 5%	111 ± 7%	75 ± 6%	108 ± 3%
Camptothecin 0.01 µ*M*	14 ± 2%	22 ± 2%	58 ± 7%	17 ± 2%
Paclitaxel 0.001 µ*M*	110 ± 5%	104 ± 8%	77 ± 7%	78 ± 10%
Paclitaxel 0.01 µ*M*	4 ± 0%	11 ± 2%	29 ± 1%	13 ± 2%

[a]Mutations reported in reference 34
[b]Negative for the founder *BRCA1* or *BRCA2* French Canadian mutations described in reference 52
[c]Mutations reported in reference 23
[d]Negative for mutations in codons 12 and 13 of *KRAS2*
[f]Standard error of the mean was only calculated when at least three independent experiments were done

were investigated to further elucidate the molecular genetic characteristics of the EOC cell lines. Mutation analysis of TOV-21G revealed that it acquired a nucleotide substitution, G → T, in codon 13 (GGC → TGC) resulting in an amino acid substitution of glycine to cysteine (Gly13Cys). This amino acid substitution results in constitutive activation of ras activity. The sample was heterozygous for this sequence variation.

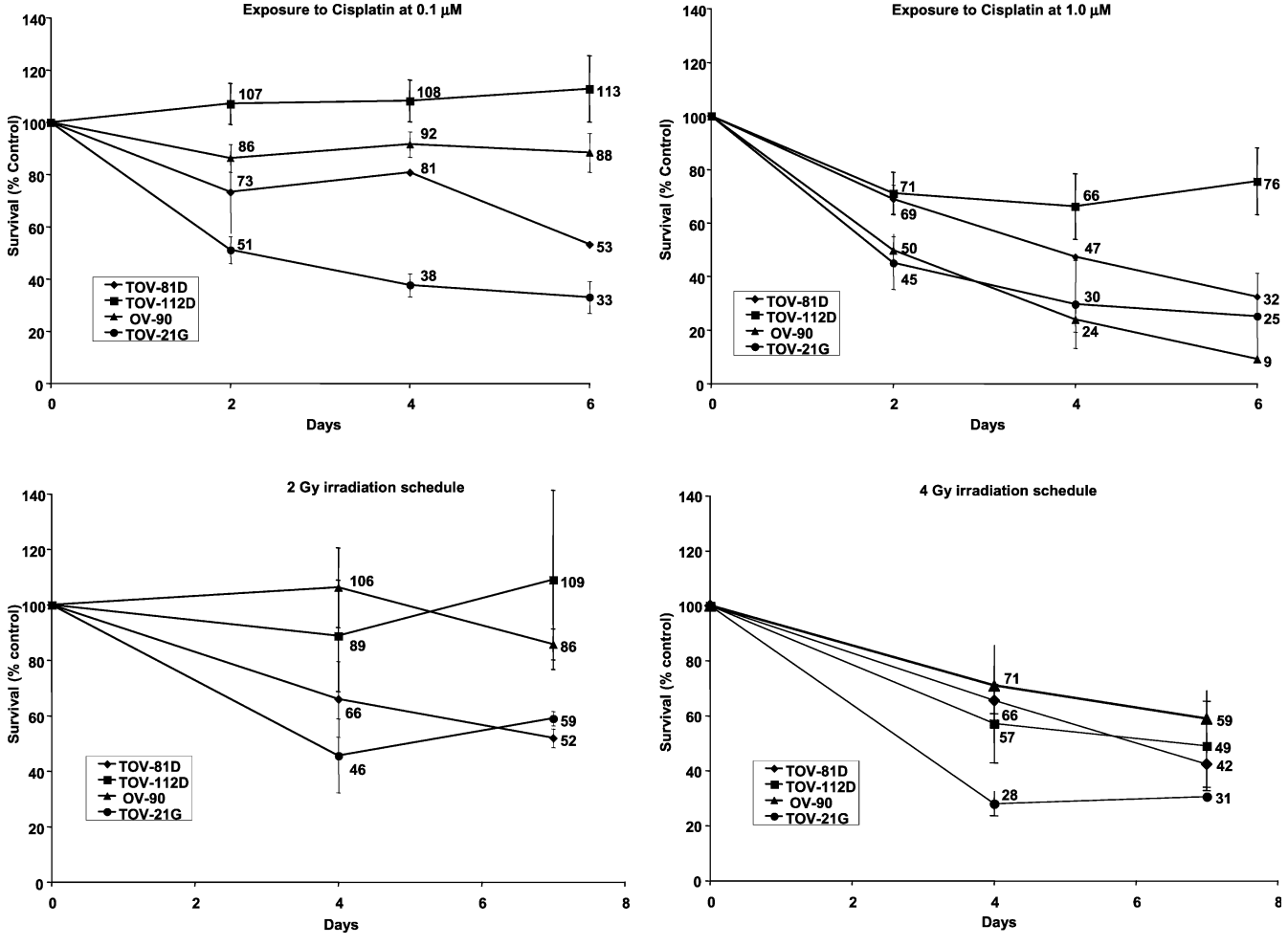
Sensitivity to the DNA-damaging agents cisplatin and γ-irradiation

The effect of cisplatin and γ-irradiation, both DNA-damaging agents, was characterized for each EOC cell line according to the percentage survival of treated compared to untreated cells (controls) (Table 1; Fig. 1). The *TP53*-mutated cell lines (OV-90 and TOV-112D) appeared insensitive to both the lowest dosage of cisplatin (0.1 μ*M*) and γ-irradiation (2 Gy). The *TGFβ*-RII and *KRAS2*-mutated cell line, TOV-21G, and the *BRCA2*-mutated cell line, TOV-81D, both demonstrated significant sensitivity to cisplatin and γ-irradiation at low doses. While all cell lines were sensitive to higher doses of cisplatin (1.0 μ*M*), OV-90 showed the highest sensitivity (9 ± 1% survival at day 7) while TOV-112D was the most resistant (76 ± 12% survival at day 7). With high doses of γ-irradiation (4 and 6 Gy) all cell lines displayed similar sensitivity profiles, although the effect was noted earlier in the TOV-21G cell line. The results following irradiation at 6 Gy were similar to those following irradiation at 4 Gy (data not shown).

Chemosensitivity to camptothecin and paclitaxel

Sensitivities to camptothecin, a DNA-topoisomerase I inhibitor, and paclitaxel, an inhibitor of microtubule depolymerization, are shown in Table 1 and Fig. 2. Two days after exposure to camptothecin at the lowest dosage (0.001 μ*M*), OV-90 and TOV-21G demonstrated only a modest decrease in growth and appeared to rapidly escape the effect of the drug at later time points. TOV-112D and TOV-81D cell survival was slightly

Fig. 1 Survival curves after exposure to cisplatin or γ-irradiation. TOV-112D (*TP53*-mutated), OV-90 (*TP53*-mutated and *CDNK2A*-mutated), TOV-21G (*TGFβ*-RII-mutated and *KRAS2*-mutated), and TOV-81D (*BRCA2*-mutated) were exposed to different concentrations of cisplatin (*top panels*) and to different doses of γ-irradiation (*bottom panels*). Cell survival after treatment was determined as a percentage of that of non-treated cells



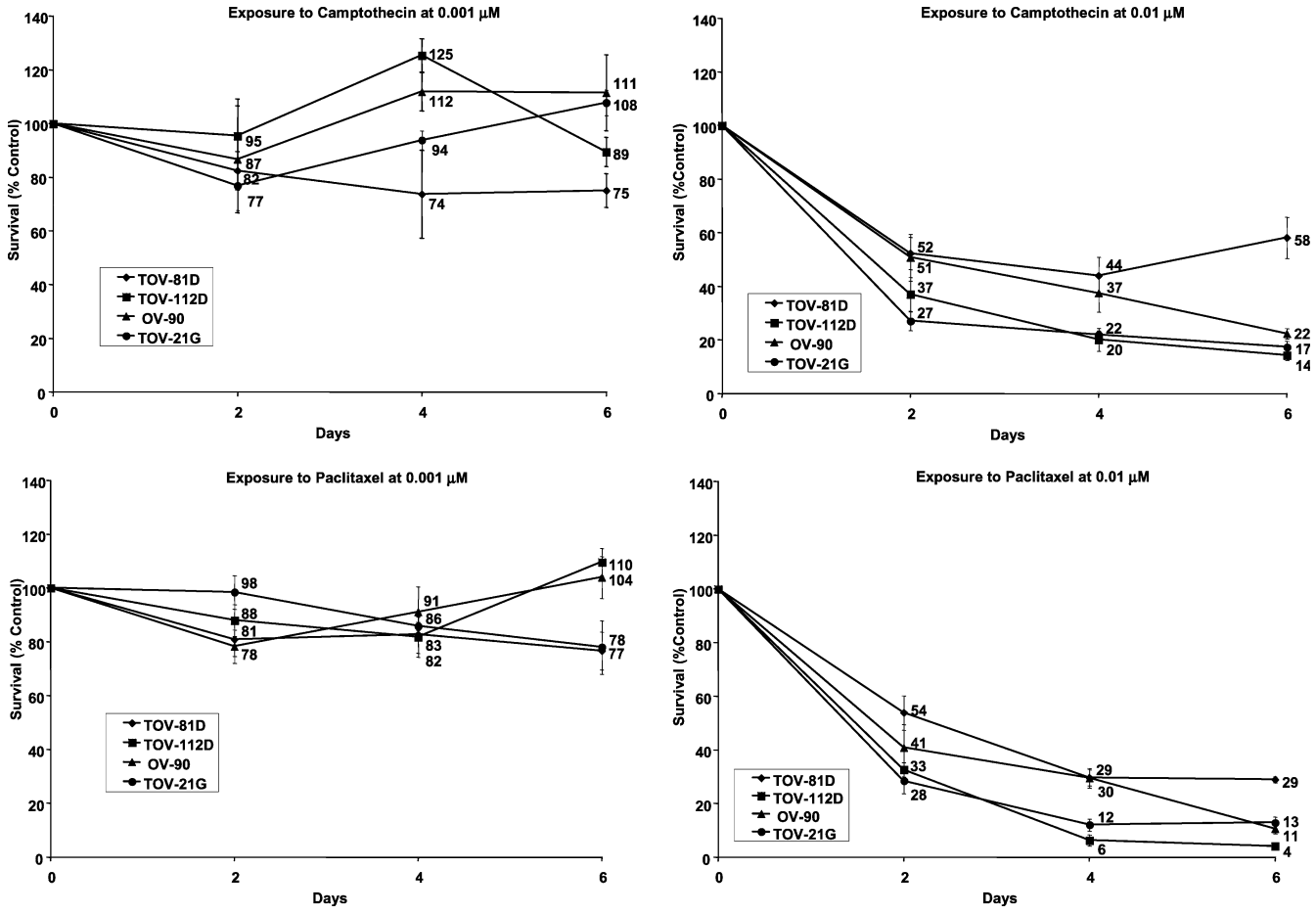


Fig. 2 Survival curves after exposure to camptothecin and paclitaxel. Cells were exposed to different concentrations of camptothecin (*top panel*) and paclitaxel (*bottom panel*). Cell survival after treatment was determined as a percentage of that of non-treated cells

affected at these dosages with a $89 \pm 5\%$ and $75 \pm 6\%$ cell survival, respectively, 6 days after exposure to this drug. Although all cell lines were sensitive to 0.01 μ*M* camptothecin, the effect on the TOV-81D was less pronounced than the effects on the other cell lines.

Exposure to 0.001 μ*M* paclitaxel did not affect the survival of TOV-112D and OV-90 cells, whereas a slight effect was observed on TOV-21G and TOV-81D cells. In contrast, all cell lines were highly sensitive to a dose of 0.01 μ*M* where the relative sensitivity appeared as TOV-112D > TOV-21G/OV-90 > TOV-81D from most to least sensitive.

Discussion

We evaluated the survival following treatment with different anticancer agents and γ-irradiation of newly described EOC cell lines harboring mutations in the key genes *BRCA2*, *TGFβ*-RII, *K-ras2*, *TP53*, and/or *CDNK2A*, previously shown to be important in ovarian cancers [34]. These EOC cell lines displayed various sensitivities to chemotherapeutic agents and radiation. Recent studies have shown somatic *TP53* mutations to be a predictor of response to treatment in ovarian cells resistant to cisplatin [14]. *TP53* mutations are observed in more than 50% of advanced ovarian carcinomas [24]. Wild-type *p53* is required for the induction of a G_1 and/or G_2 irreversible arrest after γ-irradiation [58]. While loss of *p53* function has been associated with the lack of response to high-dose cisplatin in ovarian cancer patients [36], it does not appear to affect sensitivity to paclitaxel [25]. The introduction of a dominant negative mutated copy of *TP53* in ovarian cancer cells reduces their sensitivity to cisplatin and γ-irradiation, but the transfected cells retain their sensitivity to paclitaxel and camptothecin [54]. At the lower doses tested in our study, the results with the TOV-112D and OV-90 cell lines, which both harbor *TP53* mutations, are consistent with those found in the cited studies, suggesting that mutations in this gene confers resistance to cisplatin and γ-irradiation. For cisplatin at the higher doses tested, the responses of TOV-112D and OV-90 cells differed, with OV-90 cells displaying an increased sensitivity while both cell lines showed similar profiles for γ-irradiation at the higher doses. Whether this is related to the specific *TP53* mutation, the presence of an additional mutation in the *CDNK2A* in OV-90, or to other genetic lesions has not yet been determined.

Cisplatin resistance can be circumvented by the use of either topotecan (a camptothecin analog) or paclitaxel [33, 37]. TOV-112D, which displays resistance to cisplatin, is sensitive to paclitaxel and camptothecin at the highest doses. While *TP53* is not mutated in the TOV-21G cell line, results suggest that this cell line has an RER^+ phenotype [23] as well as an activating mutation in *KRAS2*. The TOV-81D cell line also retains a wild-type *TP53* although this cell line harbors a *BRCA2* mutation. The TOV-21G and TOV-81D cell lines were the most sensitive to paclitaxel, cisplatin, and γ-irradiation. Significant differences were observed with camptothecin. At a low dose camptothecin, TOV-81D was the most sensitive of the cell lines tested, while the TOV-21G line, initially sensitive, escaped rapidly the effect of the drug based on recovery of cell growth with prolonged exposure to the drug. In contrast, at the higher dose of camptothecin, TOV-81D demonstrated a higher level of cell survival. How these results relate to *TP53*, *TGFβ*-RII, and *BRCA2* mutational status remains to be determined.

As *BRCA2* interacts in the DNA damage repair process [4], the mutated status of the *BRCA2* gene might play a role in the sensitivity of TOV-81D cells to γ-irradiation. It has previously been shown that human cell lines carrying a heterozygous mutation of *BRCA2* display a greater sensitivity to γ-irradiation and an impaired proliferative capacity when compared to non-mutated cells [10]. The observation that *BRCA2* heterozygous knockout mice are more sensitive to γ-irradiation-induced DNA damage [42] also supports this hypothesis. A recent study has demonstrated that inactivation of *BRCA2* incurs gross chromosomal rearrangement between non-homologous chromosomes during division [60]. Moreover, *BRCA2* has been shown to control *Rad51* recombination and DNA repair by regulating both the intracellular localization and DNA binding ability of *Rad51* [8]. Consequently, cells harboring a *BRCA2* mutation could be hypersensitive to DNA-damaging agents such as cisplatin or γ-irradiation. While an association between *TP53* mutations and EOC sensitivity to drugs or γ-irradiation has already been observed, our data also suggest that *BRCA2* mutations could influence EOC response to treatment.

To our knowledge, the relationship between mutations affecting the *TGFβ*-RII gene and response to treatment in ovarian cancer has not been well documented as most studies have focused on the potential implication of the *TGFβ* signaling pathway in carcinogenesis. In the studies reported here, the TOV-21G cell line, which originates from a clear-cell carcinoma, displayed sensitivity to all the treatments tested, even at the lowest dosages. This is in contrast to the response rate to platinum-based chemotherapy in patients with a stage III clear-cell carcinoma, which is significantly lower than in patients with serous adenocarcinoma [47]. However, the overall response may be influenced by the fact that the TOV-21G cell line carries a mutation in the *TGFβ*-RII gene, which is not a frequent event in EOC [23].

Here we report a codon 13 *KRAS2* mutation in the TOV-21G cell line. Acquired somatic point mutations in codons 12 and 13 are activating mutations that occur frequently in ovarian carcinomas. They have been encountered in serous borderline tumors, and in non-invasive and invasive micropapillary serous carcinomas, but not in high-grade (conventional) serous carcinomas, suggesting that at least two distinct pathways exist in serous ovarian carcinogenesis [9, 13, 46]. *KRAS2* mutations have also been observed in mucinous, clear-cell, and endometrioid ovarian carcinoma [13, 29]. No relationship has been found between Kirsten-Ras protein levels and cisplatin sensitivity in human ovarian cancer carcinoma cell lines, both comprising cisplatin-naive and those with acquired platinum resistance [15]. A recent independent study has confirmed the cisplatin sensitivity of the TOV-21G cell line and points out the apparent loss of FANCD2-L expression in this cell line in an attempt to correlate the sensitivity to cisplatin of this cell line with the Fanconi anemia-BRCA (FANC-BRCA) pathway [49]. BRCA and Fanconi's anemia proteins have recently been associated with the FANC-BRCA pathway required for the response to DNA damage induced by cisplatin or other DNA crosslinking agents and the disruption of the FANC-BRCA pathway has been observed in 8% of ovarian tumor cell lines [30, 49].

Together, these findings suggest that the model system described here provides a starting point to further elucidate the role of different genetic backgrounds in determining the response of ovarian cancers to chemotherapy and radiation therapy. The appearance of multiple genetic lesions within this well-defined model system points to the need for further investigations to elucidate how complex gene interactions influence response to therapy. To obtain an extensive picture of ovarian cancer mutations in key genes could be useful to address the question of the individual sensitivity to drugs and to tailor the treatment of each ovarian cancer patients.

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