SPANDIDOS PUBLICATIONS

Simultaneous confidence intervals to compare gene expression profiles using ABC transporter TaqMan microfluidic cards

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Abstract. The rapid evolution of techniques for measuring gene expression makes available substantial data which require careful analysis. In particular, relative quantification based on microfluidic cards allows performing of rapid large scale analyses. In the present study, we employed ovarian carcinoma cell lines resistant to cisplatin (IGROV-1/Pt1) or to a camptothecin (IGROV-1CPT/L), both characterized by a complex pattern of resistance to multiple agents, to examine the expression of genes of the superfamily of ATP binding-cassette (ABC) transporters by TaqMan microfluidic cards with the aim of developing an analytical tool to process data in this particular framework. The transcript quantification was based on the comparative threshold cycle method, which compares the expression of a target gene normalized to the expression of one or more reference genes (relative quantification). To process expression of ABC transporters, we applied a statistical procedure based on multivariate approaches and re-sampling techniques. The transporters that were significantly modulated included members of the ABCA, ABCB, ABCC and ABCG subgroups. A consistent up-regulation of ABCC2 as compared with the parental IGROV-1 cell line was observed in the IGROV-1/Pt1 cells, whereas down-regulation of ABCC6 and ABCG1 was found in IGROV-1/CPT-L cells. The use of rigorous analytic tools for gene expression data in preclinical models may lead to the identification of signatures to test in ovarian carcinoma clinical samples. Moreover, the developed procedure may be useful in the analysis of relative quantification data obtained with microfluidic cards in different experimental settings.

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Introduction

Ovarian carcinoma is a major cause of death from gynaecological cancers in the Western world. This disease is usually diagnosed at advanced stage when surgery cannot be curative and, after debulking surgery, patients are treated with platinum-taxane combination chemotherapy, which has become a standard treatment for advanced-stage disease (1). A large number of cytotoxic agents including camptothecins, the clinically available DNA topoisomerase I inhibitors, are used in the treatment of recurrence, but most therapies have failed to improve survival (2). Drug resistance represents a major cause of treatment failure, but the cellular bases of resistance to cytotoxic agents remain to be defined. Although extensive cellular studies have underlined that resistance is multifactorial in nature, a major impact of defence mechanisms i.e., factors regulating antitumor drug influx and efflux has been recognized (3,4). Indeed, for all cytotoxic agents, inadequate intra-tumor concentration could explain at least in part the 'pharmacological' resistance to treatment.

Selected components of the super-family of ATP binding-cassette (ABC) transporters have been implicated in conferring resistance to structurally unrelated antitumor drugs including cisplatin and camptothecins (5,6). The recent achievement that ABC transporters are a large family of genes supports the need for novel studies directed at clarifying the relationship between less characterized transporters and resistance. Thus, the availability of techniques allowing the simultaneous analysis of a large number of transcripts with minimal quantity of total RNA may be useful in an attempt to establish procedures to translate to the clinical setting. In this context, it is important to define rigorous analytical procedures to analyze consistently the gene expression data in an attempt to define tools to translate insights from preclinical models into the clinical practise (7). Based on this rationale, we used ovarian carcinoma cell systems including a parental cell line and two drug-resistant sublines exhibiting a variable pattern of response to structurally unrelated antitumor agents to examine the expression of the whole ABC transporters superfamily. The amount of ABC transporter transcripts were quantified by means of TaqMan microfluidic cards, a tool that allows simultaneous real-time PCR analysis of a large number of mRNAs because TaqMan gene expression assays are carried out on a microfluidic card with minimal quantity

of total RNA. The transcript quantification is based on the comparative threshold cycle (Ct) method (8), which compares the expression of a target gene normalized to the expression of one or more reference genes (relative quantification). Although relative quantification based on microfluidic cards enables researchers to perform rapidly large scale analyses, its statistical background needs to be better investigated. Currently, the software programs supplied along with the various qPCR instruments usually do not provide an adequate solution for the processing of the raw data into meaningful results due to their intrinsic restrictions mainly related to a closed software architecture. Consequently, we applied an *ad hoc* statistical procedure based on multivariate approaches and re-sampling techniques specifically developed to process gene expression data obtained using TaqMan microfluidic cards. This procedure, exploiting the comparative Ct method (8), is based on the following steps: data cleaning, outlier detection, data normalization and selection of a small subset of genes confidently inferred to be involved in the multidrug-resistant phenotype.

Materials and methods

Cell culture and drugs. The human ovarian carcinoma IGROV-1 cell line and the cisplatin- and camptothecin-resistant sublines, IGROV-1/Pt1 and IGROV-1/CPT-L, were maintained in RPMI-1640 medium (BioWhittaker Lonza, Lonza Milano S.r.l., Italy) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, San Giuliano M, Italy). The IGROV-1/Pt1 variant was generated as described (9). IGROV-1/CPT-L cells were developed by exposure of the parental cell line to increasing concentrations of the lipophilic camptothecin gimatecan. Resistance was stable for at least 6 months when cells were grown in the absence of selecting agent. Cell cultures were routinely checked for being mycoplasma-free and experiments were carried out using cell lines at similar passages following thawing from a frozen stock. Cisplatin (Teva Italia S.r.l., Milan, Italy) was diluted in saline. Gimatecan (Sigma Tau Spa, Pomezia, Italy) was dissolved in dimethyl sulfoxide and diluted in water. The concentration of the solvent in culture medium of treated cells never exceeded 0.5%.

Growth-inhibition assays. Cell sensitivity was assessed by growth-inhibition assays (10). Exponentially growing cells were seeded in duplicate in 6-well plates at 19000-25500 cells/cm^2. After 24 h, cells were exposed to drugs for 1 h and incubated for 72 h in drug-free medium. When a 72-h drug exposure was used, cells were counted at the end of treatment. Before counting, culture medium was removed and adherent cells were harvested using trypsin and counted with a cell counter (Beckman Coulter, S.p.A., Milan, Italy). As alternative to cell counting, the MTT assay was used to assess growth inhibition (11). IC_{50} is defined as the concentration causing a 50% inhibition of cell growth as compared with control. The resistance index is the ratio between the IC_{50} of resistant and sensitive cells. A cell line is considered resistant when the resistance index is ≥3.0.

RNA extraction and expression analysis with TaqMan microfluidic cards. Harvesting of cells, RNA extraction, and DNase digestion were carried out with the RNAqueous®-4PCR Kit (Ambion Europe LTD, Huntingdon, UK), according to the manufacturer's instructions. RNA purity and integrity were assessed with denaturing gel electrophoresis and the RNA was quantified spectrophotometrically and then stored at -80˚C. cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with a Master Mix containing 2.5 U/μl of MultiScribe Reverse Transcriptase and 1 μg of total RNA. The reaction mixture was incubated at 25˚C for 10 min, followed by 120 min at 37˚C and then by heat inactivation of the enzyme at 85˚C for 5 sec. We then mixed 2 μl of single-stranded cDNA (equivalent to around 100 ng of total RNA) with 48 μl of nuclease-free water and 50 μl of TaqMan Universal PCR Master Mix. After loading 100 μl of the sample-specific PCR mixture into one sample port of the microfluidic cards (Human ABC Transporter Panel; Applied Biosystems), the cards were centrifuged twice for 1 min at 280 g and sealed to prevent well-to-well contamination. The cards were placed in the microfluidic card Sample Block of an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions were 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 30 sec at 97˚C and 1 min at 59.7˚C. The assay for each gene on the microfluidic card was carried out in triplicate, due to the design of this specific panel. The calculation of the threshold cycle (Ct) values was performed using the SDS 2.2 software (Applied Biosystems), after automatically setting the baseline and the threshold.

Methodological background. Microfluidic card experiments allow the detection and quantification of a large number of genes by taking advantage of the real-time PCR method. Usually, each j-th (j=1,2,...J) Ct replicate is processed according to the comparative Ct method to compute the relative quantity (RQ) of the i-th (i=1,2,...I) transcript in the k-th (k=1,2,...K) sample, normalized to an endogenous gene (R) and relative to a calibrator sample (C). With reference to our ovarian carcinoma cell systems, the two target cell lines represent the samples under investigation, which are compared to the parental cell line used as calibrator sample. Following the comparative Ct method, the equation used to compute RQ can be written in terms of base 2 logarithm (log_2) as:

$$\log_2(RQ_{ik}) = -\left[\sum_{j=1}^{J} \Delta\Delta Ct_{jik} \Big/ J\right] \quad [1] \quad \text{where:}$$

$$\Delta\Delta Ct_{jik} = \Delta Ct_{jik} - \overline{\Delta Ct_{iC}} \quad [2] \quad \text{with:}$$

$$\Delta Ct_{jik} = Ct_{jik} - \left(\sum_{j=1}^{J} Ct_{jRk} \Big/ J\right) \quad [3] \quad \text{and:}$$

$$\overline{\Delta Ct_{iC}} = \sum_{j=1}^{J} \Delta Ct_{jiC} \Big/ J \quad [4] \quad \text{with:}$$

$$\Delta Ct_{jiC} = Ct_{jiC} - \left(\sum_{j=1}^{J} Ct_{jRC} \Big/ J\right) \quad [5]$$

Before using raw Ct values to compute $\log_2(RQ_{ik})$ data were pre-processed and only those transporters with at least two

<40, the maximum number of fixed cycles, were ;d in the analysis. Following removal of possible outliers, detected by means of Dixon test (12), data were normalized using as reference the gene that showed the least variation among the 'endogenous genes' available in the card.

Assessment of simultaneous confidence interval (SCI). For each target cell line, relevant ABC transporters were selected using as pivotal statistic the (1-α)% simultaneous confidence intervals (SCI) of $\log_2(RQ_{ik})$ building on the percentile bootstrap approach (13) by taking into account multiplicity adjustment (14). The (1-α)% SCIs for the I parameters of interest within each of the K target cell lines were obtained according to the following algorithm: i) using the pre-processed data, the ΔCt_{jik} and ΔCt_{jiC} values were computed according to equations 3 and 5, respectively; ii) starting from the ΔCt_{jik} and ΔCt_{jiC} distributions, B bootstrap samples were generated; iii) for each bootstrap sample, the $\log_2(RQ_{ik})$ values were computed according to equation 1 to obtain B bootstrap estimates: $\log_2(RQ^*_{ikb})$ (for b=1,2,...B); iv) for each coordinate (ik) the B bootstrap estimates of $\log_2(RQ_{ik})$ were ordered and a rank was defined for each of them; v) for each bootstrap sample the minimum rank [r_{min} (b)] and the maximum rank [r_{max} (b)] were defined as the smallest and the largest rank associated with the b-th bootstrap estimate, respectively; vi) the 100(α/2)-th centile of the r_{min} (b) distributions and the 100(1-α/2)-th centile of the r_{max} (b) distributions were identified and denoted as $r_{\alpha/2}$ and $r_{1-\alpha/2}$, respectively; viii) for each coordinate (ik), the

$$\log_2\left(RQ^{\cdot}_{(ikr_{\alpha/2})}\right) \quad \text{and} \quad \log_2\left(RQ^{\cdot}_{(ikr_{1-\alpha/2})}\right)$$

were the lower and the upper limits of the (1-α)% SCI, respectively. The corresponding limits for RQ_{ik} can be obtained by back-transforming these figures.

According to the conventional 2-fold threshold (RQ ≤0.5 or RQ ≥2), transporters were considered down-regulated if the upper limit of their 95% SCI for RQ_{ik} were ≤0.5; on the other hand transporters were considered up-regulated if the lower limit of their 95% SCI for RQ_{ik} were ≥2. A specific code was developed in SAS® package to carry out data analysis (15).

Results

Phenotypes of ovarian carcinoma drug-resistant cell systems. The drug resistant sublines, IGROV-1/Pt1 and IGROV-1/CPT-L, were generated by chronic exposure to cisplatin and to the lipophilic camptothecin gimatecan, respectively, and were resistant to the selecting agents. The degree of resistance of the cisplatin resistant model was around 10 and that of the camptothecin-resistant subline was around 6. For both cell systems, resistance was associated with reduced sensitivity to drug-induced apoptosis (9, data not shown). The IGROV-1/Pt1 cells also exhibited activation of survival pathways (16). In the IGROV-1/CPT-L cells, resistance was not associated with alterations in the target of campthotecin, i.e., DNA topoisomerase I mutation (data not shown). Both cell lines exhibited a multi-drug resistant phenotype, as reduced sensitivity to structurally unrelated drugs was observed (Table I). In particular, IGROV-1/Pt1 cells were cross-resistant to melphalan and mitoxantrone, and IGROV-1/CPT-L cells were cross-resistant to cisplatin, topotecan and doxorubicin.

Table I. Multidrug resistant phenotype of IGROV-1/Pt1 and IGROV-1/CPT-L cells.[a]

	RI[b]	
Drug	IGROV-1/Pt1	IGROV-1/CPT-L
Cisplatin	12.89	3.69
Gimatecan	0.95	5.75
Topotecan	1.54	17.95
Doxorubicin	2.72	3.76
Etoposide	2.00	1.48
Melphalan	18.06	nd[c]
Taxol	0.24	1.02
Vinblastine	2.29	nd[c]
Mitoxantrone	3.03	2.40

[a]Cell sensitivity to drug was assessed by growth-inhibition assays. Twenty-four h after seeding, cells were exposed to the drug for 1 or 72 h and cell growth was assessed by cell counting or MTT assay 72 h after treatment (for 1-h exposure) or at the end of treatment. IGROV-1/Pt1 data are from Perego *et al* (11). [b]Resistance index, ratio between the IC_{50} value (drug concentration inhibiting cell growth by 50%) of resistant and parental cells. [c]nd, not determined.

Analysis of the expression of ABC transporters. Experiments for the two (K=2) target cell lines (IGROV-1/Pt1 and IGROV-1/CPT-L) and for the calibrator cell line (IGROV-1) were carried out in triplicate (J=3) by considering the fifty (I=50) currently known members of the ABC transporter super-family. Our analysis was performed by taking into consideration only those transporters with at least two determined Ct (i.e., Ct values <40). Fig. 1 reports the frequency distribution of transporters not considered in the analysis according to each ABC subfamily. Overall, a group of 10 transporters (ABCA8, ABCB5, ABCB11, ABCC8, ABCC9, ABCC13/MRP10, ABCD2, ABCG2, ABCG5 and ABCG8) exhibited at least two undetermined Ct values in all the 3 cell lines. Three additional 'undetermined' transporters (ABCB4, ABCC7/CFTR and ABCC12/MRP9) were observed for the IGROV-1/CPT-L cell line. In terms of percentage of undetermined transporters (out of the totality of each ABC subfamily members), we found at the top position the subfamily ABCG (3 of 5; 60%), followed by the subfamily ABCC (5 of 13; 38%), ABCB (3 of 11; 27%), ABCD (1 of 4; 25%) and ABCA (1 of 13; 8%). In the subfamily ABCE and ABCF, all the transporters had at least 2 determined Ct values.

No outlier values were detected by Dixon test. All the Ct values were normalized to the Ct average obtained for the housekeeping gene GAPDH, which showed the least variation

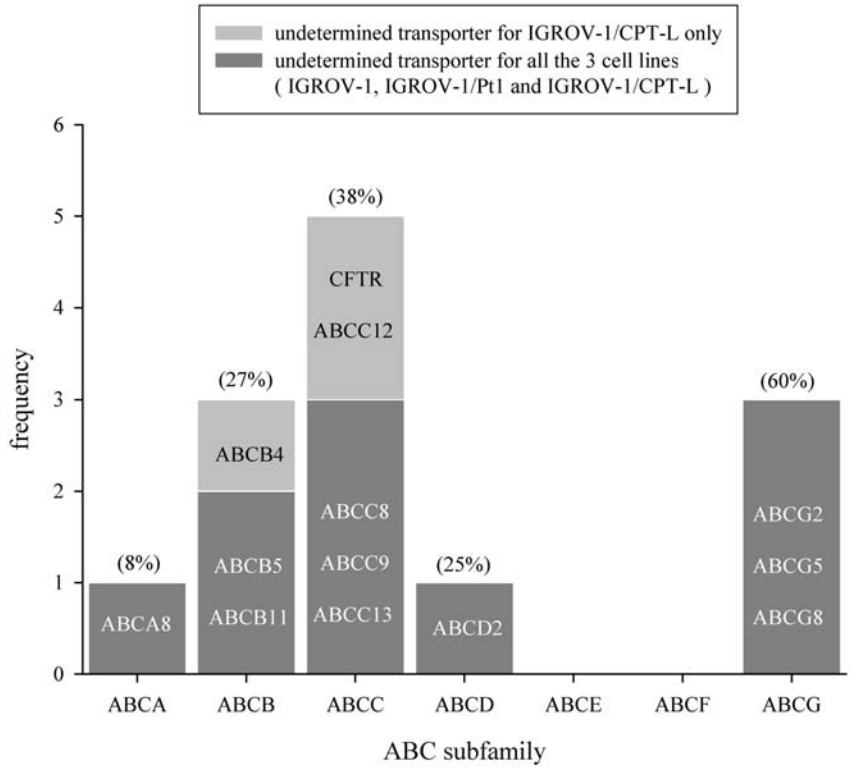

Figure 1. Frequency distribution of the undetermined transporters in the 3 ovarian carcinoma cell lines according to the ABC subfamily. The transporter names are specified inside each bar of the histogram and the percentages of undetermined transporters out of the total number of each ABC subfamily members are reported on the top of the bar.

among other reference genes tested in the studied cell models (data not shown). In panel A and B of Fig. 2, we report the 95% SCIs of RQ obtained from 10000 re-sampling (B=10000), according to the above algorithm for the IGROV-1/CPT-L and IGROV-1/ Pt1 cell lines, respectively. Three and eight transporters appear to be significantly regulated as compared with the parental IGROV-1 cell line for IGROV-1/CPT-L and IGROV-1/Pt1, respectively. Specifically, with reference to the IGROV-1/ CPT-L cell line, a consistent up-regulation of the ABCA1 gene was observed, whereas a down-regulation of ABCC6 and ABCG1 was noted. In the IGROV-1/Pt1 cell line, an increased expression of the ABCA1, ABCC2 and ABCG1 transcripts was found, whereas down-regulation of ABCA11, ABCA12, ABCB1, ABCB4 and ABCC3 was observed.

To confirm these results, a second independent experiment was carried out under the same conditions as the first experiment. The 95% SCIs of RQ for those transporters found to be significantly regulated in at least one experiment for the two target cell lines are reported in Table II.

For the IGROV-1/CPT-L cell line, 2 of 3 transporters (ABCC6 and ABCG1) were confirmed to be significantly regulated; for the IGROV-1/Pt1 cell line, 5 of 8 transporters (ABCA11, ABCA12, ABCB1, ABCC2 and ABCC3) were confirmed to be significantly regulated. Furthermore, in an attempt to investigate the pattern of modulation of ABC transporters in the 2 resistant variants, we examined the differential expression of the above-mentioned transporters (i.e., 2 in camptothecin-resistant cells and 5 in cisplatin-resistant cells) between the two target cell lines by jointly considering both experiments. The expression of 5 of 7 transporters (ABCA12, ABCB1, ABCC2, ABCC6 and ABCG1) was found to be statistically different in the two cell lines. In particular, the modulation of 4 of them was oriented in the same direction in the 2 cell lines, whereas the opposite behaviour was observed for ABCG1, which was up-regulated in IGROV-1/Pt1 cells and down-regulated in IGROV-1/CPT-L cells (Table II).

Discussion

Recent studies underline the importance of developing genomic signatures to be used to define biomarkers to select patients who are more likely to benefit from specific treatments as well as to develop genomic predictors of response to chemotherapy (17). In this context, the combination of gene expression data obtained with different approaches (i.e, microarrays, real-time PCR) and data reflecting tumor cell response to treatment may ultimately be useful to generate models to predict responses (18). In this regard, a lot of effort has already been made in analyses of microarray data (19). Overall, the impact of such strategies in the future clinical practise is expected to be influenced by the development of rigorous analytical tools to evaluate gene expression data. In the present study, we designed an *ad hoc* statistical procedure based on multivariate approaches and re-sampling techniques to analyse relative quantification data obtained using microfluidic cards; these data were generated from 3 cell systems, including a parental cell line and two drug-resistant variants. We provide evidence that development of resistance to cisplatin and to camptothecins in 2 ovarian carcinoma cell lines characterized by defects in the apoptotic

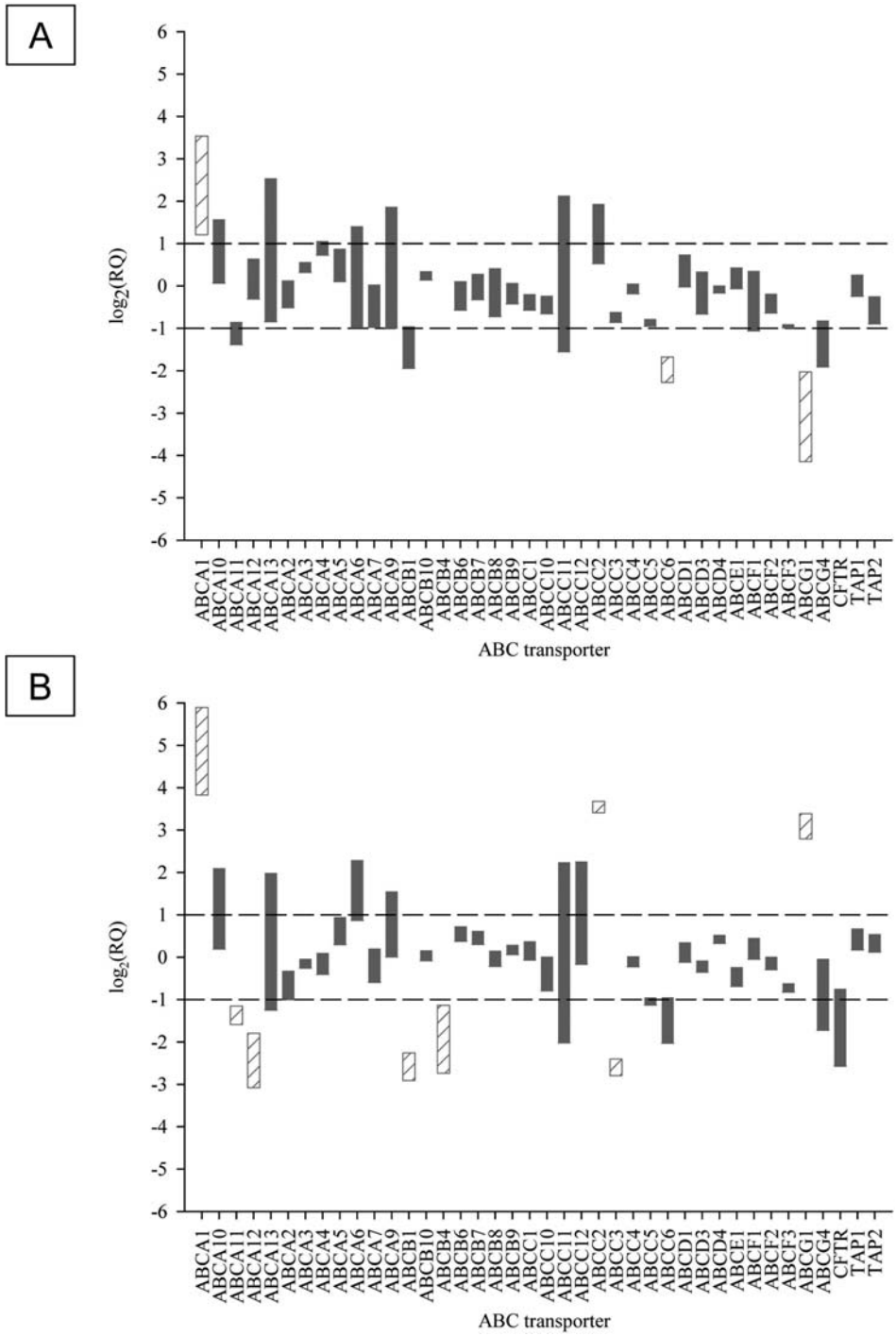

Figure 2. Ninety-five percent of $\log_2$(RQ) for the IGROV-1/CPT-L and IGROV-1/Pt1 cell lines. Each rectangle represents graphically, for transporters with at least two determined Ct values, the 95% SCI of $\log_2$(RQ) for the IGROV-1/CPT-L (A) and IGROV-1/Pt1) (B) cell lines. The upper and lower borders of the rectangles correspond to the upper and lower limits of the 95% SCI, respectively. The two horizontal dashed lines correspond to the thresholds used for considering significantly regulated transporters [$\log_2$ (0.5)= -1 and $\log_2$ (2)= +1]. Dashed rectangles indicate transporters significantly down- or up-regulated.

pathway is associated with the acquisition of resistance to structurally unrelated drugs (Table I). The observed behaviour was associated with modulation of the expression of transporters of the ABC super-family. The transporters that were significantly modulated included members of the ABCA, ABCB, ABCC and ABCG subgroups (Table III, 20-26). In particular, in the IGROV-1/CPT-L cell line a consistent down-regulation of ABCC6 and ABCG1 was found (21,22). A consistent up-regulation of the ABCC2 gene as compared with the parental IGROV-1 cell line was observed in the IGROV-1/Pt1 cell subline in 2 independent biological replicates. Increased levels of ABCC2 were observed only in 1 of 2 experiments in the IGROV-1/CPT-L cell line. In fact, in one case the lower limit of 95% SCI of the RQ value was slightly <2. Based on this evidence, the main cause of the multi-drug reistance phenotype of IGROV-1/Pt1 cells could

Table II. Outline of 95% simultaneous confidence interval of RQs.[a]

	IGROV-1/CPT-L				IGROV-1/Pt1			
	First experiment		Second experiment		First experiment		Second experiment	
	RQ 95% SCI		RQ 95% SCI		RQ 95% SCI		RQ 95% SCI	
Transporter	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
ABCA1	2.314	11.589	1.493	18.779	14.209	59.293	0.087	2.169
ABCA2	0.699	1.093	0.619	0.802	0.500	0.802	0.337	0.456
ABCA4	1.654	2.084	3.363	4.791	0.755	1.073	0.115	0.159
ABCA11	0.384	0.556	0.219	0.398	**0.333**	**0.449**	**0.297**	**0.435**
ABCA12	0.805	1.565	1.239	4.724	**0.119**	**0.288**	**0.052**	**0.320**
ABCB1	0.259	0.517	0.510	0.749	**0.133**	**0.209**	**0.021**	**0.032**
TAP1	0.844	1.203	0.600	0.765	1.128	1.599	0.380	0.412
ABCB4	-	-	0.139	0.639	0.150	0.455	-	-
ABCC2	1.440	3.827	2.210	3.218	**10.611**	**12.802**	**4.471**	**5.744**
ABCC3	0.551	0.652	0.201	0.236	**0.144**	**0.189**	**0.003**	**0.007**
ABCC5	0.519	0.583	0.627	0.834	0.458	0.517	0.314	0.430
ABCC6	**0.207**	**0.313**	**0.068**	**0.166**	0.244	0.519	0.514	0.695
ABCC10	0.634	0.852	0.573	0.826	0.575	1.010	0.332	0.461
ABCC12	-	-	0.304	0.718	0.890	4.788	0.083	0.205
ABCD1	0.983	1.673	1.845	2.293	0.921	1.276	0.368	0.417
ABCD4	0.892	1.007	0.524	0.658	1.254	1.441	0.397	0.485
ABCF2	0.643	0.882	0.483	0.565	0.816	1.008	0.333	0.380
ABCF3	0.504	0.534	0.279	0.318	0.565	0.652	0.267	0.324
ABCG1	**0.057**	**0.246**	**0.102**	**0.188**	6.921	10.461	1.609	2.792
ABCG4	0.266	0.567	0.104	0.491	0.303	0.974	1.957	3.825

[a]The table reports the lower and upper limits of the 95% simultaneous confidence interval (SCI) of the relative quantification (RQ) for those transporters found to be significantly regulated in at least one experiments for the two target cell lines (IGROV-1/CPT-L, IGROV-1/Pt1). In bold are reported the transporters confirmed to be significantly regulated in the second experiment.

Table III. Summary of the role of ABC transporters significantly regulated in the ovarian carcinoma cell lines.[a]

	mRNA expression		
Gene	IGROV-1/Pt1	IGROV-1/CPT-L	Function and associated diseases (references)
ABCC2	↑		Detoxification of glutathione- and glucuronate-conjugated organic anions and bile secretion; mutated in the Dubin-Johnson syndrome (20).
ABCC6		↓	Efflux of anthracyclines and etoposide; mutated in pseudoxanthoma elasticum (21).
ABCG1		↓	Cholesterol distribution on the cell membrane to favor its removal by high-density lipoproteins (22).
ABCA11	↓		Defined as a pseudogene by HGNC[b], proteomic analysis suggests the existence of this protein. (http://www.uniprot.org/uniprot/Q4W5N1 and 23).
ABCA12	↓		Transmembrane lipid transporter, involved in the formation of the *stratum corneum* of the skin; mutated in harlequin ichthyosis (24).
ABCB1	↓		Efflux of multiple molecules (e.g. colchicine, tacrolimus, etoposide, doxorubicin, vinblastine, bilirubin; 25).
ABCC3	↓		Transport of glucuronate-, sulphate- and glutathione-conjugated organic anions and etoposide (26).

[a]↑ Indicates up-regulation; ↓ indicates down-regulation. [b]HGNC, HUGO (Human Genome organization) Gene Nomenclature Committee.

Recognition of widely used anticancer drugs by ABC transporters.[a]

Compound	Targets	ABCB1	ABCC2	ABCC3	ABCC6
Vincristine, vinblastine	Tubulin	+	+		
Taxol	Microtubules	+	+		
Mitoxantrone	Topoisomerase II		+		
Anthracyclines	Topoisomerase II	+	+		+
Etoposide	Topoisomerase II	+	+	+	+
Camptothecins	Topoisomerase I	+	+		
Cisplatin	DNA		+		

[a]Drug recognition may vary depending on the allelic status of genes coding for ABC transporters.

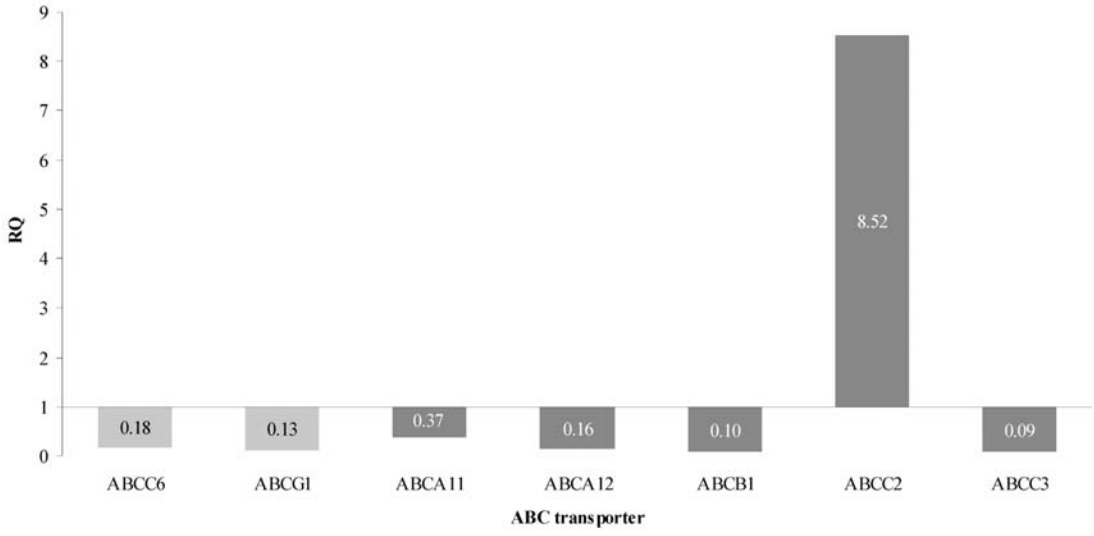

Figure 3. Relative quantity of ABC transporters regulated for the two target cell line. Mean of RQ values obtained in the two experiments for those transporters confirmed to be significantly regulated for IGROV-1/CPT-L (light grey bars) and for IGROV-1/Pt (dark grey bars).

be the up-regulation of ABCC2. This transporter has a broad spectrum of substrates which include DNA topoisomerase I and II inhibitors (Table IV). However, lipophilic camptothecins are poorly recognized by MRP2/ABCC2 (25,26), in keeping with the lack of resistance to gimatecan in IGROV-1/Pt1 cells (Table I). In IGROV-1/ CPT-L cells, a down-regulation of the ABCG1 transcript may suggest an altered content of cholesterol in cell membrane, possibly affecting drug permeability. Indeed, the features of the plasma membrane may change during the development of drug resistance, thus leading to reduced membrane fluidity (29).

Among the transcripts that were found not to be expressed in the studied ovarian carcinoma cells lines (Fig. 1), we found members of almost all the subgroups including ABCA8, ABCB5 and ABCB4, ABCB11, components of the ABCC subgroup (i.e., the cystic fibrosis trans-membrane conductance regulator ABCC7/CFTR, ABCC8, ABCC9, MRP9/ABCC12, MRP10/ABCC13) and ABCD2, ABCG2, ABCG5 and ABCG8. This data are in keeping with the pattern of expression documented in public databases for ABC transporters in ovarian carcinoma cells, as the expression of some of those genes appears to be specific for other tissues (http://biogps.gnf.org).

In conclusion, it appears that the final pattern of cross-resistance of the studied cell lines is the result of multiple alterations also involving the ABC super-family of transporters. According to our analysis, a group of seven ABC transporters may be implicated in conferring multidrug resistance to the IGROV-1/Pt1 and IGROV-1CPT/L cell variants (Fig. 3). The multidrug resistance gene signatures identified in our preclinical model, throughout the *ad-hoc* statistical procedure, may be tested in clinical samples from ovarian cancer patients in an attempt to identify genes implicated in clinical resistance to chemotherapy. The rational design of inhibitors of those transporters that are up-regulated in resistant cells may be useful to optimize antitumor therapy (30). Moreover, the developed procedure may be useful in the analysis of relative quantification data obtained with microfluidic cards in different experimental settings.

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