Expression of ABC transporters in multidrug resistant ovarian carcinoma cell lines using TaqMan Micro Fluidic Cards

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Introduction
The optimization of treatment with anti tumor drugs remains a major effort of preclinical and clinical research. The over-expression of ATP binding cassette (ABC) transporters by tumor cells has been recognized as a mechanism contributing to the poor response of tumor cells to structurally and mechanistically unrelated antitumor drugs in experimental models. Whole genome approaches have documented the existence of a wide family of ABC transporters in human cells (1). Quantitative analysis of the expression of ABC transporters in cell lines characterized with respect to the pattern of response to clinically useful antitumor agents may be helpful to define those genes that can be associated with the multidrug resistant (MDR) phenotype. In the present study, we used TaqMan Micro Fluidic Cards to identify the ABC transporters transcripts that were associated with the drug-resistant phenotypes of ovarian carcinoma cell lines developed in vitro. A statistical procedure based on multivariate approaches and re-sampling techniques was implemented to process expression values of ABC transporters.

Material and methods

Cell lines
The drug-resistant cell lines used in the present study were IGROV-1/Pt1 and IGROV-1/CPT-L, generated by chronic exposure of the human ovarian carcinoma IGROV-1 cell line to cisplatin and to a liposomal carboplatin, respectively. The cell variants are characterized by a stable drug-resistant phenotype when grown in the absence of the selecting agent for more than 6 months (2,3).

TaqMan Micro Fluidic Cards
Harvesting of cells, RNA extraction, and DNase digestion were carried out with the RNeasyàMicro 48PCR Kit (Ambion Europe LTD, Hampshire, 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 stained at -60°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 μl of total RNA. The reaction mixture was incubated at 25°C for 10 min, followed by 10 min at 37°C and then by heat inactivation of the enzyme at 95°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 we loaded 100 μl of the sample-specific PCR mixture into one sample port of the Micro Fluidic 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 loaded in the Micro Fluidic 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 95°C and 1 min at 58.7°C. Threshold cycle (Ct) values were determined in triplicate by setting the baseline and the threshold automatically.

Methodological background

Each j-th (j=1,2,…,J) Ct replicate was processed according to an ad-hoc statistical procedure developed exploiting the comparative Ct method (4). According to this method, the percentile bootstrap approach (6) according to the algorithm reported in the references was implemented to process expression values of ABC transporters.

Statistical Analysis

The (1-α)% SCIs for the I parameters of interest within each of the K target cell lines [log2(RQ)] were the pivotal statistics adopted to analyze expression values of ABC transporters.

Simultaneous confidence interval (SCI)

The percentiles of interest within each of the K target cell lines [log2(RQ)] were the pivotal statistics adopted to analyze expression values of ABC transporters. The lower and upper limits of these intervals were computed on the bases of the percentile bootstrap approach (6) according to the algorithm reported in the Box.

For each target cell line transporters were considered, according to the conventional 2-fold threshold (RQ ≤ 0.5 or RQ ≥ 2), as follows:
- **down-regulated**: upper limit of the (1-α)% SCI of log2(RQ) ≤ -1; log2(RQ) = -1
- **up-regulated**: lower limit of the (1-α)% SCI of log2(RQ) > +1; log2(RQ) = +1

**Results**

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 transporters superfamily. A group of 10 (ABCA8, ABCB5, ABCB11, ABCC8, ABCC9, ABCC13, ABCD2, ABCG2, ABCG5 and ABCG8) transporters presented at least two undetermined Ct values in all the cell lines. Three (ABCB4, CFTR and ABCB12) additional “undetermined” transporters were observed for IGROV-1/CPT-L. No outlier values were detected by Dixon test (significance level, α = 0.001). All the Ct values were normalized to the Ct average obtained for the housekeeping gene GAPDH (equation [2] and [3]) which showed the least variation among other reference genes previously tested in our cell models (data not shown).

In the figures are reported the 95% SCIs obtained from B=10,000 resampling. Three and 8 transporters appear to be significantly regulated as compared with the parental IGROV-1 cell line for IGROV-1/CPT-L. Two of the transporters are the previously characterized up-regulation of the ABCA1 gene was observed, whereas a down-regulation of ABCB6, ABCG1 was noticed. In the IGROV-1/Pt1 cell line an increased expression of the ABCA1 ABCB2 and ABCG1 transcripts was found, whereas down-regulation of ABCA11, ABCA12, ABCB1, ABCB4 and ABCB3 was observed. To confirm these results, a second independent experiment was carried out under the same conditions of the first experiment. In the Table are reported the 95% SCI in terms of RQ for those transporters found to be significantly regulated in at least one experiments for the two target cell lines.

**Conclusions**

According to our analysis, a group of seven ABC transporters may be implicated in conferring multidrug resistance to the IGROV-1/Pt1 and IGROV-1/CPT-L cell variants. The MDR gene signatures identified in our preclinical model, throughout an ad-hoc statistical procedure, may be tested in clinical samples from ovarian cancer patients in an attempt to identify gene expressions linked to chemotherapy.

**References**


**Box: Simultaneous bootstrap percentile confidence interval - algorithm**

1. generate B bootstrap samples from the \( \Delta Ct_{j} \) and \( \Delta Ct_{k} \) distributions;
2. for each b-th (b=1,2,…,B) bootstrap sample, calculate the \( \log_{2}(RQ_{ik}) \) (equation [1]) to obtain B bootstrap estimates: \( \log_{2}(RQ_{ik,b}) \);
3. for each coordinate (ik), order the B bootstrap estimates and denote them by \( \log_{2}(RQ_{ik,b}) < \log_{2}(RQ_{ik,b+1}) < ... < \log_{2}(RQ_{ik,b'}) \). For each estimate define the rank: \( r_{ik,b} \);
4. for each bootstrap sample, define the minimum and the maximum rank as:
   - \( r_{min} = \min(r_{ik,b}) \) to be the smallest rank associate with the b-th bootstrap estimate;
   - \( r_{max} = \max(r_{ik,b}) \) to be the largest rank associate with the b-th bootstrap estimate;
5. identify the \( r_{min} \) and the \( r_{max} \) Percentile of the \( r_{min} \) and \( r_{max} \) distributions, respectively;
6. take the lower and the upper limits of the (1- α)% SCI for each coordinate (ik) to be \( \log_{2}(RQ_{ik,min}) \) and \( \log_{2}(RQ_{ik,max}) \), respectively. The corresponding limits for RQ can be obtained by back-transforming these figures.

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\log_{2}(RQ_{ik}) = \log_{2}(\frac{Ct_{ik}}{Ct_{R}}) \]

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\Delta Ct_{ik} = \log_{2}(\frac{Ct_{ik}}{Ct_{R}}) - \log_{2}(\frac{Ct_{j}}{Ct_{R}}) = \log_{2}(\frac{Ct_{ik}}{Ct_{ik}}) - \log_{2}(\frac{Ct_{j}}{Ct_{j}}) \]

where: \( i \) is the gene of interest, \( j \) is the reference gene, \( k \) is the cell line, \( R \) is the reference gene and \( Ct_{R} \) is the Ct average of the reference gene.