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ABC transporter gene expression in benign and malignant ovarian tissue

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ABSTRACT

Objective. ATP-binding Cassette (ABC) transporters are thought to cause multiple drug resistance (MDR) in various carcinomas. Gene expression data from individual transporters in ovarian cancer tissue is contradictory and also scarce for some of them. RNA levels of a panel of ABC transporters were collected and analyzed to get a more detailed overview which transporters are of importance in resistance to chemotherapeutic agents in ovarian carcinoma.

Methods. Real-time PCR was used to determine RNA expression levels of 9 ABC transporters in 50 benign tissue samples and 50 recurrent ovarian cancer samples. Genes exhibiting a significant difference between those two groups were further evaluated in 50 primary cancer samples. Data were analyzed with receiver operating characteristic (ROC) curves and multiple Wilcoxon–Mann–Whitney *U*-tests with Shaffer correction.

Results. Gene expression of four transporters (ABCC1, ABCC2, ABCC3, and ABCB3) was significantly elevated in recurrent cancer lesions compared to benign tissue. Expression levels of these 4 ABC transporters were further analyzed in primary ovarian cancer lesions. A significant difference between primary and recurrent tumor tissue was found in all four genes. Changes in gene expression between benign samples and primary lesions were minor and not relevant.

Conclusions. Four of the examined ABC transporters are likely to play a role in the MDR of ovarian carcinoma. Gene expression of these transporters seems only up regulated through chemotherapy. The thesis that MDR in ovarian cancer is acquired through therapy itself and not present *ab initio* is supported by these findings.

Introduction

Ovarian cancer is a rare disease with an incidence of 42,700 in the European Union 2004 [1] and 21,650 new cases in the US 2008 [2]. Nevertheless it is ranked 6th in cancer-related deaths in the western world [3]. An explanation for this high death rate is that ovarian cancer is usually diagnosed at late stage. 5-year survival for patients diagnosed at stage III and IV is 20–30% [4] and 50–75% of advanced patients relapse [5,6].

Ovarian cancer patients are usually treated with initial cytoreductive surgery followed by platinum-based combination therapy. Relapsed patients are treated in most cases with further systemic chemotherapy. Nevertheless, generally only limited and temporary response rates are observed in this palliative setting.

The development of multiple drug resistance (MDR) is the crucial step in tumor progression in recurrent ovarian cancer. Cancer cells are able to develop MDR by a number of mechanisms. One of them is increased excretion of the chemotherapeutic agents.

ATP binding cassette (ABC) transporters are known to be responsible for this mechanism in cancer cell lines. The ABC transporter superfamily contains membrane proteins that translocate a wide variety of substrates, including metabolic products, lipids, sterols, and drugs across extra- and intracellular membranes. Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds either within the cell as a part of a metabolic process or outside the cell for transport to other organs or for secretion from the body [7]. The transporters are either organized as full or half transporters, the half transporters can form both homodimers and heterodimers. Phylogenetic analysis places the 48 known human ABC transporters in 7 distinct subfamilies of proteins (ABCA–ABCG). The ABC pumps are mostly unidirectional. ABC genes play an important role in MDR, at least 4 genes are reported to be associated with drug transport [8].

We studied gene expression of 9 ABC transporters (ABCB1, ABCB3; ABCC1, ABCC2, ABCC3, ABCC5, ABCC7; ABCF2, and ABCG2) in benign ovarian tissue and recurrent ovarian cancer lesions from patients after chemotherapy. Transporters showing a statistical upregulation of

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gene expression in recurrent lesions, gene expression was further examined in primary tumor samples. The aim of this study was to get further insight if these 9 ABC transporters can play a role in MDR of ovarian cancer, and to get a more detailed overview if gene expression is already elevated in primary tumors prior to chemotherapy or is upregulated through the chemotherapy itself.

Materials and methods

Patients

50 cystadenomas and benign ovarian tissue samples, 50 primary, and 50 recurrent ovarian carcinoma samples were analyzed. All samples were snap frozen in liquid nitrogen and stored at −80 °C.

The 50 benign samples, consisting of 9 normal tissue samples and 41 cystadenomas of the ovary, were collected at the Department of Obstetrics and Gynecology, Medical University Vienna between 1989 and 2006.

The 100 tumor samples originate from patients seen between 2001 and 2004 at the Department of Obstetrics and Gynecology, Charité University Hospital, Campus Virchow, Berlin within the tumor bank ovarian cancer (www.toc.network.de). For baseline data of ovarian tumor samples see Table 1.

Treatment of patients

In the group of patients with primary ovarian carcinoma all patients were treated with platinum-based combination chemotherapy. 34 patients received carboplatin combined with paclitaxel. 11 patients were treated with paclitaxel, gemcitabine and carboplatin. One patient received a combination of carboplatin and topotecan, and one patient was administered cyclophosphamide and carboplatin after initial surgery.

In the group of patients with recurrent carcinoma all patients were administered at least one chemotherapeutic treatment circle. First line chemotherapy for 38 patients consisted of carboplatin and paclitaxel. 7 patients obtained a platinum containing compound combination chemotherapy as first line medication. 5 patients were treated with other miscellaneous chemotherapies. Most of the patients have been treated with multiple different chemotherapies of various combinations prior to the excision of the examined tumors.

All patients gave their written informed consent and the study was approved by the local institutional review boards.

RNA extraction

Approximately 100 mg nitrogen frozen tissue was powdered with a Mikro-Dissmembrator U (B. Braun Biotech International, Melsungen, Germany) and suspended in 3 mL buffer RLT (Qiagen, Hilden, Germany) containing beta mercaptoethanol.

400 μL of the suspension was used for RNA extraction. RNA was purified using the RNeasy minikit (Qiagen, Hilden, Germany) including an on column DNA digestion according to manufacturer's protocol.

RNA was quantified with a biophotometer (Eppendorf Ag, Hamburg, Germany) and the quality of the RNA was evaluated with the Agilent 2100 Bioanalyzer using the RNA nano kit (Agilent, Santa Clara, USA). Reverse transcription was conducted using the Omniscript RT kit (Qiagen, Hilden, Germany) with random hexamers (GE healthcare, Little Chalfont, UK).

Table 1
Baseline data of primary and recurrent ovarian tumor samples.

	Primary	Recurrent	Total
Total	50	50	100
Histotype			
Serous	40	44	84
Endometroid	7	2	9
Mucinous	1	1	2
Others/unknown	2	3	5
Grading			
1	0	5	5
2	18	25	43
3	32	20	52
FIGO stage			
1	7	5	12
2	7	5	12
3	26	29	55
4	10	11	21

Real-time PCR

The 9 ABC transporters (ABCB1, ABCB3; ABCC1, ABCC2, ABCC3, ABCC5, ABCC7; ABCF2, and ABCG2) and beta-2-microglobulin, which was used as reference gene, were analysed with commercially available "Assay on Demand" systems. Assays on Demand and Universal PCR Master Mix, No AmpErase UNG 7500 were purchased from Applied Biosystems (Foster City, USA). For each reaction an equivalent of 25 ng RNA was used, Assays on Demand and Universal Master Mix were utilized according to manufacturer's protocol.

All real-time PCRs were conducted on the Applied Biosystems 7500 Real-time PCR system, software version 1.3.1 (Applied Biosystems, Foster City, USA). Following run settings were used: initial 2 min at 50 °C and 10 min at 95 °C, thereafter 50 cycles consisting of denaturation at 95 °C for 15 s, and annealing/extension at 69 °C for 1 min. All expression levels were evaluated in duplicates.

All real-time PCR data were analyzed with the delta Ct method. Expression levels were first analyzed in recurrent carcinoma and benign ovarian tissue. Genes with significant difference in expression between these groups were further evaluated in the primary ovarian carcinoma samples.

Statistics

Results were analyzed using receiver operating characteristic (ROC) curves. A ROC curve is a graphical plot of the sensitivity vs. (1–specificity) for a binary classifier system as its discrimination threshold is varied. ROC analysis provides tools to select possibly optimal models. A perfectly discriminating test would result in a curve coinciding with the left and top sides of the graph, a completely useless test would yield a diagonal from the left lower to the right upper corner. The area under the curve (AUC) of a ROC curve is calculated to display the performance of a classifier. The AUC can take a value from 0 to 1. An area of 0.5 corresponds to mere chance, an AUC with a value greater than 0.8 is normally used as indicator of usefulness of the test [9].

Data from more than two groups was evaluated with Kruskal–Wallis test and multiple Wilcoxon–Mann–Whitney *U*-tests with Shaffer correction [10]. The Shaffer procedure is an improved Bonferroni–Holm procedure and should be used if all possible pairs are compared. All statistical calculations were conducted with SPSS 15.0 (SPSS Inc; Chicago, IL, USA).

Results

ROC curves

To test for differences in expression levels between benign tissue and recurrent carcinoma ROC-curves were used. 7 genes were expressed with a significant difference between the two groups. An AUC of 0.8 was set as cut-off value for usefulness of the test. 4 genes, *ABCB3*, *ABCC1*, *ABCC2*, and *ABCC3* fulfilled this criterion and were