Whole Exome Sequencing Analysis of *ABCC8* and *ABCD2* Genes Associating With Clinical Course of Breast Carcinoma

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Summary

The aim of the present study was to introduce methods for exome sequencing of two ATP-binding cassette (ABC) transporters *ABCC8* and *ABCD2* recently suggested to play a putative role in breast cancer progression and prognosis of patients. We performed next generation sequencing targeted at analysis of all exons in *ABCC8* and *ABCD2* genes and surrounding noncoding sequences in blood DNA samples from 24 patients with breast cancer. The revealed alterations were characterized by *in silico* tools. We then compared the most frequent functionally relevant polymorphism rs757110 in *ABCC8* with clinical data of patients. In total, the study identified 113 genetic alterations (>70 % novel ones) in both genes. Of these alterations, 83 were noncoding, 13 synonymous, 10 frameshifts and 7 were missense alterations. Four *in silico* programs predicted pathogenicity of two polymorphisms and four newly identified alterations. Rs757110 polymorphism in *ABCC8* did not significantly associate with clinical data of the patients. In conclusion, exome sequencing identified several functionally relevant alterations in *ABCC8* and *ABCD2* genes that may further be used for a larger follow-up study aiming to assess their clinical significance.

Key words

ABC transporter • Breast • Cancer • Therapy • Next generation sequencing

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Introduction

Breast cancer is the most common cancer in women and caused 471,000 deaths worldwide in 2013 (Global Burden of Disease Cancer Collaboration 2015). A number of cellular processes that in some cases lead to the tumor resistance limits efficacy of breast cancer therapy. Multidrug resistance (MDR) to a variety of chemotherapy drugs presents one of these processes (Baguley 2010).

MDR is often caused by the decreased cellular uptake or increased efflux of drugs and by alterations in DNA repair and apoptotic pathways. Drug efflux is mediated by membrane-bound ATP-binding cassette (ABC) transporters (Sakacs *et al.* 2006). ABC transporter family in humans consists of 48 genes and one pseudogene (Dean *et al.* 2001). ABCs translocate a wide variety of substrates, including lipids, sterols and drugs across extra- and intracellular membranes (Klaassen and Aleksunes 2010). Therefore, the most prominent ABCs as ABCB1/P-glycoprotein (Wolf *et al.* 2011), ABCC1/MRP1 (Kunicka and Soucek 2014), and ABCG2/BCRP (Natarajan *et al.* 2012), known to transport plethora of anticancer drugs belong to the most studied in cancer pathophysiology.

Our recent studies have shown that gene expression levels of a number of ABCs significantly differ

between the tumor and paired non-malignant tissues from patients with solid tumors (colorectal – Hlavata *et al.* 2012, breast – Hlavac *et al.* 2013, pancreas – Mohelnikova-Duchonova *et al.* 2013) suggesting their potential role in cancer progression. Moreover, tumor levels of some ABCs were associated with clinical characteristics of the patients including prognostic factors (e.g. the expression of estrogen receptor with ABCC1 and ABCC8 in breast cancer or grade with ABCC10 in colorectal cancer). Most interestingly, intratumoral ABCs levels were associated with the response to neoadjuvant chemotherapy in breast cancer (Hlavac *et al.* 2013) and survival of the patients in colorectal cancer (Hlavata *et al.* 2012). Particularly, observed significant overexpression of ABCD2 in tumor tissues of breast cancer patients with partial or complete response (responders) after the neoadjuvant chemotherapy in comparison to patients with stable or progressive disease (non-responders) attracts our attention (Hlavac *et al.* 2013). The revealed existence of a broad variability in protein expression of ABCC8 (OMIM: 600509) and ABCD2 (OMIM: 601081) between tumor samples (Hlavac *et al.* 2013) further suggests that expression changes of ABCs levels could be biologically relevant for breast cancer.

Additionally, our most recent data showed that genetic variability in candidate marker *ABCC1* associates with its gene expression in tumor tissue and with survival of breast cancer patients (Kunicka *et al.* 2014) providing a proof-of-principle for further explorations.

Thus, recent studies demonstrate that phenotype and genotype of genes associated with MDR, namely ABCs, could be useful for individualization of cancer therapy. The aim of our present study is to expand the present knowledge about genotype of the most interesting ABCs for breast cancer prognosis and therapy outcome. Thus, here we explored genetic variability of candidate ABC transporters (namely *ABCC8* and *ABCD2*) in peripheral blood DNA samples from breast cancer patients by exome sequencing and predicted functional consequences of the identified alterations on their phenotypes. The most interesting alterations may now be used for a large scale follow-up study targeted at evaluation of their functional, prognostic, and predictive potential.

Patients and Methods

Patients

The study included a total of 24 breast cancer patients (C50 according to the International Classification

of Diseases, the 10th revision) of Caucasian origin diagnosed in Institute for the Care for Mother and Child and the Department of Oncosurgery, Medicon, in Prague during 2006-2012. Patients underwent preoperative neoadjuvant chemotherapy regimens based on 5-fluorouracil/anthracyclines/cyclophosphamide (FAC or FEC) and/or taxanes. The collection of blood samples, and the retrieval of clinical data were performed as described before (Vaclavikova *et al.* 2012). Following data on patients were retrieved from medical records: age at diagnosis, menopausal status, personal medical history, family history (number of relatives affected by breast/ovarian carcinoma or other malignant diseases), stage, tumor size, presence of lymph node metastasis, histological type and grade of the tumor, expression of estrogen, progesterone and ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) receptors, expression of Ki67, response to the therapy and progression-free survival. All patients after primary chemotherapy and surgery were followed for local or distant relapse or in the case of palliative setting for disease progression by regular medical visits every 3 months during the first 3 years, twice a year during the next 2 years and yearly then after. During the visits, mammography, chest X ray, skeletal survey, and abdominal ultrasound were performed yearly. Clinical examination together with tumor markers (CEA and CA 15-3) was performed during every visit. In the case of clinical uncertainty, additional tests and examinations were performed to rule out possible disease relapse or progression. Clinical characteristics of patients are presented in Table 1.

All subjects were informed and gave their written consent to participate in the study. The design of the study was approved by the Ethical Committee of the National Institute of Public Health in Prague.

DNA extraction

Blood samples were collected during the diagnostic procedures using tubes with K₃EDTA anticoagulant. Peripheral blood lymphocytes were prepared by using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) from fresh blood samples of patients. Genomic DNA was isolated from human peripheral blood lymphocytes by the standard phenol/chloroform extraction and ethanol precipitation method (Topic and Gluhak 1991). DNA samples were stored in aliquots at –20 °C prior to analysis.

Table 1. Clinical characteristics of patients.

Clinical characteristics		N	%
Age at diagnosis (years)		48.9 ± 10.8	
Menopausal status	Premenopausal	13	54
	Postmenopausal	11	46
Tumor size (mm)		22.8 ± 13.4	
Lymph node metastasis	Absent (pN0)	16	67
	Present (pN1-3)	8	33
Pathological stage	I	9	41
	II	11	50
	III	2	9
	IV	0	-
	Not determined	2	-
Histological type	Invasive ductal carcinoma	19	79
	Other type	5	21
Pathological grade	1	3	13
	2	9	39
	3	11	48
	Gx	1	-
Estrogen receptor status	Positive	18	75
	Negative	6	25
Progesterone receptor status	Positive	18	75
	Negative	6	25
Expression of HER2	Positive	8	65
	Negative	15	35
	Not determined	1	-
Expression of Ki-67 (%)		37.2 ± 24.5	
Response to neoadjuvant chemotherapy	Complete or partial response	11	48
	Stable disease or progression	12	52
	Not determined	1	-
Distribution of ABCC8 rs757110 allele frequencies	CC	4	17
	CA	9	38
	AA	11	46

Data are mean ± SD.

Exome sequencing

Libraries encompassing all exons of *ABCC8* (39 exons) and *ABCD2* (10 exons) genes were prepared according to the manufacturer (Roche, Prague, Czech Republic). Based on the character of probe design, i.e. tiling; the exons were surrounded by approximately 30 bp regions of intronic sequences which were also sequenced in both directions. Target enrichment was

performed using SeqCap EZ Choice by Nimblegen. Libraries were prepared using Rapid Library Preparation Kit (Roche). Samples were sequenced on 454 GS Junior system (Roche).

Data analysis

Raw data were processed by pipeline software Sequence Pilot (JSI Medical Systems, Ettenheim,

Fig. 1. Distribution of alterations in *ABCC8* and *ABCD2* genes. **Top:** The frequency of genetic alterations in *ABCC8* (left) and *ABCD2* (right) analyzed by the USCS server (<http://snp-nexus.org>). Numbers of alterations are on y-axis. **Bottom:** The positions of coding SNPs with the predicted pathogenic effects in *ABCC8* (upper image) and *ABCD2* (lower image) genes are depicted by triangles. The schematic presentation of ABC domains is adopted from NCBI's Conserved Domain Database (Marchler-Bauer *et al.* 2011).

Table 2. Overview of identified alterations in *ABCC8* and *ABCD2* genes in breast cancer patients.

Type	<i>ABCC8</i> ^a	<i>ABCD2</i> ^a	Total ^a
Noncoding	21 (1)	62 (2)	83 (3)
Frameshift	6	4	10
Missense	5 (2)	2 (1)	7 (3)
Synonymous	10	3	13
All	41 (3)	72 (3)	113 (6)

Numbers of alterations with numbers of pathogenic ones in parentheses, ^a Pathogenic by Regulome DB, SIFT, PolyPhen or HaploReg (see Table 3).

Germany) and variant calling was performed with the following settings: minimal absolute coverage, 15-combined; both directions minimal absolute coverage, off; minimal % coverage, 10 % per direction.

Associations of single nucleotide polymorphisms (SNPs) and novel pathogenic alterations with prognostic

clinical data (tumor size and grade, presence of lymph node metastasis, expression of hormonal receptors, ERBB2 and Ki67, progression-free survival, and response to the therapy) were evaluated by the two-sided Pearson chi square and the Spearman tests. A p-value of less than 0.05 was considered statistically significant. Analyses were conducted by the statistical program SPSS v15.0 (SPSS, Chicago, IL).

The functional relevance of the examined SNPs was analyzed *in silico* by Regulome DB (<http://regulome.stanford.edu>) (Boyle *et al.* 2012), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>), SIFT (<http://sift.jcvi.org>) and HaploReg v2 and v3 (Ward and Kellis 2012) programs.

Results

Exome sequencing of *ABCC8* and *ABCD2* in breast cancer patients

The 24 samples were sequenced with a mean coverage 87. Of the total number 49 regions, 100 % base

pairs were called in 42 regions, three regions were covered by >99 %, four by <80 % and one region (exon 1 in *ABCC8*) was covered by less than 50 %. In the two genes (*ABCC8* and *ABCD2*), 113 genetic alterations were identified, of which 83 alterations were in noncoding regions and the rest in the coding regions. Thirteen coding alterations were synonymous, seven missense amino acid changes and the rest of the identified alterations (n=10) were frameshift mutations present in three patients of the 24. Together, 81 (72 %) novel alterations (20 of 41, i.e. 50 % in *ABCC8* and 61 of 72, i.e. 85 % in *ABCD2*) were discovered. The observed genetic alterations are summarized in Table 2. Figure 1 (top part) shows the distribution of genetic alterations by type.

Functional aspects

In silico analysis by four different programs was used to predict functional relevance of all identified alterations. SIFT and/or PolyPhen and Regulome DB predicted probably deleterious effect of polymorphisms rs757110 in *ABCC8* and rs117275340 in *ABCD2*. The analysis by HaploReg suggested that these SNPs change binding sites for a number of transcription factors. One novel SNP (V17M) and one insertion in *ABCC8* and two insertion/deletions in *ABCD2* were predicted by the Regulome DB, SIFT and PolyPhen as deleterious (Table 3). Figure 1 (bottom part) depicts positions of coding alterations with the predicted pathogenic effects.

Table 3. *In silico* functional analysis of alterations in *ABCC8* and *ABCD2* genes revealed by the study.

Coordinate	SNP ID	Amino acid change	Regulome DB	SIFT ^a	PolyPhen ^b	HaploReg ^c
<i>ABCC8</i>						
chr11:17498225InsC	novel	none	Likely to affect binding	N/A	N/A	N/A
chr11:1749826C>T	novel	V17M	N/A	0.02	0.984	N/A
chr11:17418476C>A	rs757110	A197S	Likely to affect binding and linked to expression of a gene target	N/A	0.681	CCNT2 SZF1-1
<i>ABCD2</i>						
chr12:39947545Delins3	novel	none	Likely to affect binding	N/A	N/A	N/A
chr12:39947547DelA	novel	none	Likely to affect binding	N/A	N/A	N/A
chr12:40013392G>C	rs117275340	A9G	N/A	0.023	0.84	AP-4_1 AP-4_3 Ascl2 CTCF_disc10 E2A_3 GATA_disc4 AP-4_3 Ascl2 CTCF_disc10 E2A_3 GATA_disc4 HEN1_1 HEN1_2 LBP-1_2 Myf_2 Myf_3 SREBP_known4 TCF12_known1

^a score cutoff <0.05; ^b score cutoff >0.2; ^c motif changed according to HaploReg v3; *in silico* predictions for novel variants are not available (N/A). Missense alterations in grey.

Table 4. Distributions of pathogenic alterations in *ABCC8* and *ABCD2* genes in breast cancer patients.

Patient no.	<i>ABCC8</i>			<i>ABCD2</i>		
	chr11:174184 77C>A	chr11:174982 75C>T	chr11:174982 25InsC	chr12:399475 45Delins3	chr12:399475 47DelA	chr12:400133 92G>C
P3	CA	CC	-/-	-/-	-/-	GG
P8	AA	CC	-/-	-/-	-/-	GG
P9	AA	CC	-/-	-/-	-/-	GG
P13	AA	CC	-/-	-/-	-/-	GG
P31	AA	CC	-/-	-/-	-/-	GG
P32	AA	CC	-/-	-/-	-/-	GG
P40	CA	CC	-/-	-/-	-/-	GG
P45	CA	CC	-/-	-/-	-/-	GG
P52	CA	CC	-/-	-/-	-/-	GG
P55	AA	CC	-/-	-/-	-/-	GG
P67	AA	CT	insC/-	-/-	-/-	GG
P69	CA	CC	-/-	-/-	-/-	GG
P71	CC	CC	-/-	-/-	-/-	GG
P84	CA	CC	-/-	del/-	-/-	GG
P85	CA	CC	-/-	-/-	-/-	GG
P95	CC	CC	-/-	-/-	-/-	GG
P96	CC	CC	-/-	-/-	delA/-	GC
P97	AA	CC	-/-	-/-	-/-	GG
P102	CC	CC	-/-	-/-	-/-	GG
P117	CA	CC	-/-	-/-	-/-	GG
P120	AA	CC	-/-	-/-	-/-	GG
P122	AA	CC	-/-	-/-	-/-	GG
P133	AA	CC	-/-	-/-	-/-	GG
P143	CA	CC	-/-	-/-	-/-	GG

Table presents identified genotypes with minor allele in bold; -/- means no deletion or insertion was found.

Clinical aspects

Table 4 shows the list of patients carrying functionally relevant genetic alterations. Due to the very low frequency of alterations with the predicted pathogenic effects, we evaluated just associations of the rs757110 SNP in *ABCC8* with clinical data of the patients. None of the analyzed characteristics (with age, menopausal status, tumor size, presence of lymph node metastasis, grade, expression of hormonal receptors, ERBB2 and Ki-67, progression-free survival, and response to the neoadjuvant therapy) associated significantly with the carriage of rare alleles in this polymorphism (data not shown).

Discussion

The present study shows that exome sequencing of both target genes is feasible and can be used for further

studies on their relevance for prognosis and prediction of therapy outcome of cancer and eventually other serious diseases.

The role of *ABCC8* and *ABCD2* in human cancer is underexplored. Despite the earlier reports on their expression in human breast, colorectal and pancreatic carcinomas and associations of their intratumoral expression with clinical data of patients (Hlavata *et al.* 2012, Hlavac *et al.* 2013, Mohelnikova-Duchonova *et al.* 2013) further information and functional aspects are missing. However, the recently reported link between the *ABCC8* overexpression in animal model of brain metastasis and blood-tumor barrier permeability together with the demonstrated anti-tumor potential of its inhibition by glyburide (Thompson *et al.* 2013) raise further interest. Evaluation of functional connections between genotype and phenotype of *ABCC8* may become major pharmacogenetic tool for

stratification of patients for such therapy.

ABCC8 also known as sulfonylurea receptor (SUR1, Miki *et al.* 1999) belongs to the most often analyzed genes in both neonatal and maturity-onset of the young (MODY) forms of diabetes mellitus and previous studies suggested its potential use as pharmacogenetic marker for decision between therapy by oral sulfonylureas or insulin (Gloyn *et al.* 2004, Pearson *et al.* 2006). Its gene product forms together with the product of *KCNJ11* gene, the Kir6.2 subunit (OMIM: 600937), the ATP-dependent potassium channel playing a critical role in glucose homeostasis (Bennett *et al.* 2010).

The present study identified three alterations in ABCC8 with the *in silico* predicted functional relevance for its phenotype. Two alterations were newly discovered compared with the previously published results of whole exome sequencing of ABCC8 (Bonnefond *et al.* 2010, Johansson *et al.* 2012, Proverbio *et al.* 2013) and dbSNP database (<http://www.ncbi.nlm.nih.gov/snp>). The rs757110 polymorphism leading to the substitution of alanine at 1369 residue to serine inside the nucleotide binding domain (NBD2) is suspected to modify proper function of the ABCC8 transporter (Krugluger *et al.* 2000). Indeed, PolyPhen-2 used in the present study predicted damaging effects particularly of its truncated isoform with substitution of alanine at 197 residue to serine in NBD2.

However, in the stratified analysis of the patient set the rs757110 polymorphism did not significantly associate with any of the followed clinical characteristics of breast carcinoma patients. Despite this result, due to the small size of the patient set, the possibility of clinical relevance of the examined polymorphism cannot be ruled out.

Whole exome sequencing study of the *ABCD2* gene coding ALDRP (adrenoleukodystrophy-related protein) was not published so far and thus, the presently reported data is novel as well as the method for its assessment. Therefore, in contrary to *ABCC8*, the *ABCD2* analysis revealed 62 (85 % of total) novel alterations with the predicted functional effect in three of them.

ABCD2 is active in peroxisomal transport of

very long fatty acids, saturated fatty acids, monounsaturated acids and polyunsaturated fatty acids (Hlavac and Soucek 2015). The reported overexpression of ABCD2 in white adipose tissue during adipogenesis (Liu *et al.* 2010) demonstrates the key role of ABCD2 in lipid metabolism and together with the current epidemiological evidence supporting the role of obesity as a major cancer risk factor (Park *et al.* 2011) implicates a potential importance of ABCD2 for cancer development and progression.

Interestingly, ABCD2 knock-down was reported to stimulate apoptosis in ovarian cancer cell line SKOV3 after cisplatin treatment *in vitro* suggesting a possible link between ABCD2 and platinum resistance (LaCroix *et al.* 2014).

The small sample size of the analyzed patient group may be considered a major limitation of this study. However, the study was designed as the first step to enable large-scale screening targeted either at alterations with the predicted functional effect or at assessment of overall impact of exome alterations for the disease burden.

In conclusion, the present study provides new methods for the testing of genetic variability of ABCC8 and ABCD2 transporters with implications for screening of genetic background of diabetes, impairment of lipid homeostasis, and potentially also further research of their link to cancer.

Conflict of Interest

There is no conflict of interest.

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