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Microarray Study of Single Nucleotide Polymorphisms and Expression of ATP-Binding Cassette Genes in Breast Tumors

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Abstract. Our previous research establishes that changes of expression of the ATP-binding cassette genes family is connected with the neoadjuvant chemotherapy effect. However, the mechanism of regulation of resistance gene expression remains unclear. As many researchers believe, single nucleotide polymorphisms can be involved in this process. Thereupon, microarray analysis is used to study polymorphisms in ATP-binding cassette genes. It is thus found that MDR gene expression is connected with 5 polymorphisms, i.e. rs241432, rs241429, rs241430, rs3784867, rs59409230, which participate in the regulation of expression of own genes.

INTRODUCTION

Multidrug resistance (MDR) is considered to be one of the main cancer cell properties responsible for resistance to chemotherapy drugs and a leading factor in the malignant neoplasm treatment inefficiency. A molecular mechanism of MDR is associated with overexpression of ATP-binding cassette (ABC) transporter genes in cancer cells. Products of the ABC gene family are energy-dependent pumps capable of transporting drugs from the cell against their concentration gradient [1–4].

Our findings show that initial levels of MDR gene expression weakly correlate with the neoadjuvant chemotherapy (NAC) effectiveness. On the other hand, the NAC effectiveness is related to MDR expression when chemotherapy is being performed. If under NAC the MDR gene expression is decreased, clinical response is observed in patients. In the reversed situation, when the MDR expression is increased, there was no clinical response to chemotherapy (stabilization or progression) [5, 6]. Furthermore, in the majority of cases the same changes in ABC gene (ABCB1, ABCC1, ABCC2, ABCG1, ABCG2) expression is noted [7].

That is why it would be interesting to explore regulatory mechanisms of MDR gene expression, which includes methylation of promoter regions [8, 9], microRNAs [10], various signaling pathways, and intracellular messengers [11, 12].

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Individual characteristics of a tumor-bearing organism, such as single nucleotide polymorphisms (SNP) of ABC genes, could also be related to MDR regulation. A significant number of SNP in ABC genes (ABCG2 R482T or R482G, ABCB1 rs1045642, ABCC1 rs35605, GSTP1 rs1695, ABCG2 rs2725264, ABCC2 (rs1885301, rs717620 and rs3740066 etc.) were recently shown to play a role in expression and function of efflux transporters as well as to be related to chemotherapy effectiveness [13–21].

That is why the aim of this research is evaluation of the SNP effect on ABC gene expression in breast cancer during NAC chemotherapy.

MATERIAL AND METHODS

The study group. 84 patients with breast cancer (BC) were enrolled, aged 28–68 (Tab. 1).

TABLE 1 – Demographics of the breast cancer patients.

Trait	Value	Number of Patients, (%)
Age (year)	≤45	31 (36.9)
	>45	53 (63.1)
Menstrual status	Premenopausal	47 (56.0)
	Postmenopausal	36 (42.9)
Histological type	Invasive ductal carcinoma	63 (75.0)
	Invasive lobular carcinoma	4 (4.8)
	Medullary carcinoma	2 (2.4)
	Others	14 (16.7)
Tumor size	T_1	10 (11.9)
	T_2	63 (75.0)
	T_3	3 (3.6)
	T_4	8 (9.5)
Lymph node status	N_0	29 (34.5)
	N_1	32 (38.1)
	N_2	5 (6.0)
	N_3	7 (8.3)
Estrogen receptor	+	48 (57.1)
	–	32 (38.1)
	No data	4 (4.8)
Progesterone receptor	+	48 (57.1)
	–	32 (38.1)
	No data	4 (4.8)
HER2	0/+	63 (75.0)
	++	10 (11.9)
	+++	6 (7.1)
	No data	5 (6.0)
Histological form	Unicentric	53 (63.1)
	Multicentric	31 (36.9)
NAC regimen	CAX	24 (28.6)
	FAC	42 (50.0)
	Taxoter	18 (21.4)

The diagnosis of BC was verified morphologically. The tumor stages were IIA-IIIC. In accordance with the "Consensus Conference on Neoadjuvant Chemotherapy in Carcinoma of the Breast, April 26–28, 2003, Philadelphia, Pennsylvania" [22], all patients underwent 2–4 courses of NAC by the FAC scheme (fluorouracil, doxorubicin, cyclophosphamide), CAX scheme (cyclophosphamide, doxorubicin, xeloda), or taxoter monotherapy. Surgical operation was carried out in 3–5 weeks after NAC followed by two courses of adjuvant chemotherapy by the FAC scheme. Radiotherapy and/or hormonal treatment were prescribed if required.

The study was carried out in accordance with Helsinki Declaration of 1964 (amended in 1975 and 1983) and was approved by the Ethical Committee of the Institute of Oncology. A signed informed consent was received from all participants. Tumor tissues (~10 mm^3) were obtained before treatment by ultrasound controlled biopsy as well as from surgically resected specimens (~60–70 mm3) after NAC. The tissues were placed in RNAlater (Ambion, USA), incubated for 24 h at room temperature and stored at –80ºC until DNA and RNA extraction.

RNA and DNA extraction. RNA was extracted from 68 matched tissue specimens (before and after NAC) using RNeasy Plus mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was extracted from 68 biopsy specimens of tumor tissues using QIAamp DNA mini Kit (Qiagen, Germany). RNA and DNA concentration and purity were assessed using a NanoDrop 2000 instrument (Thermo Scientific, USA). The DNA concentration varied from 50 to 150 ng/μl and the A260/A280 and A260/A230 ratios were 1.95–2.05 and 2.15–2.40, respectively. The RNA concentration varied between 80 and 250 ng/μl and the A260/A280 and A260/A230 ratios were 1.75–1.95 and 1.90–2.31, respectively. The RNA and DNA integrity was assessed using a TapeStation instrument. The DNA fragments were as little as 48 kbp, thus suggesting its high integrity. The RIN for RNA values were 5.6–7.8.

Expression profiling of ABC genes. Expression profiling of the ABCB1, ABCB3, ABCC1, ABCC2, ABCC5, ABCG1 and ABCG2 genes was carried out using quantitative real-time PCR (qPCR) with the use of TaqMan probes and a RotorGene-6000 instrument (Corbett Research, Australia). The RNA was reverse transcribed to cDNA using RevertAid™ kit (Fermentas, Lithuania) according to the manufacturer's instructions. The qPCR was done in three replicates as described earlier [5]. Custom oligonucleotide primers and TaqMan probes were used. The GAPDH gene was used as the reference and the relative gene expression was assessed by the Pfaffl method [23]. Pooled RNA from normal breast tissues of 10 BC patients who did not get NAC was used as a calibrator.

Microarray analysis with the high density CytoScan array (Affymetrix, USA) was used for tumor genotyping. A cytoscan array contains more than 750 thousand SNPs, including for ABC genes (ABCB1 - 21, ABCB3 – 5 SNP; ABCC1 – 49; ABCC2 – 7; ABCC5 – 8; ABCG1 – 16 and for ABCG2 – 28 SNP). All procedures for sample preparation, hybridization, and scanning were performed according to the manufacturer's protocol for run on a Affymetrix GeneChip® Scanner 3000 7G (Affymetrix, USA). Microarray results were processed using the Chromosome Analysis Suite 2.0 (Affymetrix, USA) developed specifically for the CytoScan HD Array.

Statistical analysis was performed using the STATISTICA 8.0 application package (StatSoft Inc., USA). For each sample the mean and mean square error is calculated. The Wilcoxon-Mann-Whitney test is used to test the hypothesis on the significance of differences between treatment groups.

RESULTS AND DISCUSSION

The SNP effect on ABC gene auto-expression was evaluated in this paper. Statistical analysis of all mutant SNPs consisted of the comparison against the wild SNP type.

The SNP distribution of ABC genes is shown in Tab. 2.

TABLE 2. The studied SNP of ATP-binding cassette genes presented in the CytoScan HD Array.

Genes	SNP
ABCB1	*rs1045642, rs4437575, rs6949448, rs35280822, rs7787082, rs4148735, rs2235035, rs10276036, rs13237132, rs6950978, rs10260862, rs10280623, rs10264990, rs1202172, rs1202171, rs17327442, rs1202181, rs17327624, rs13233308, rs10267099, rs28381744*
ABCB3	*rs1044043, rs241432, rs241430, rs4148871, rs241429*
ABCC1	*rs215101, rs215095, rs215094, rs12922404, rs215088, rs129116, rs16967227, rs7195962, rs6498594, rs215052, rs215063, rs246220, rs4781712, rs7198766, rs3784863, rs246214, rs246240, rs11642957, rs3784864, rs924136, rs2062541, rs246230, rs17205838, rs35589, rs35592, rs3743526, rs35595, rs35596, rs35597, rs35599, rs35600, rs35610, rs35625, rs35626, rs35627, rs35628, rs4148353, rs4148358, rs4148366, rs2074085, rs4148371, rs11864374, rs3784867, rs3887893, rs212081, rs212083, rs212085, rs212086, rs212087*
ABCC2	*rs4919395, rs2756103, rs4148389, rs2804400, rs6584327, rs2273697, rs4148396*
ABCC5	*rs7646621, rs1402001, rs1402002, rs1402003, rs4912515, rs1464322, rs3792582, rs6775518*
ABCG1	*rs4148082, rs9975740, rs4148087, rs4148102, rs9976024, rs4148104, rs225443, rs225444, rs225445, rs225396, rs225398, rs2234718, rs2839482, rs7277991, rs4148137, rs3788010*
ABCG2	*rs7681519, rs34472643, rs12505410, rs2622621, rs1871744, rs1564481, rs2725252, rs4148149, rs6857600, rs34633905, rs2622604, rs3114019, rs2622608, rs59409230, rs2622609, rs7657531, rs7682521, rs7658584, rs1481017, rs13147650, rs2127863, rs6854688, rs1481011, rs6819328, rs4693936, rs4693205, rs4491984, rs10032109*

Five SNPs were found to influence ABC expression. Three SNPs of ABCB3 gene (rs241432, rs241429 and rs241430) were shown to influence the post-NAC expression level of this gene in breast cancer. In comparison with wild-type breast cancer, mutant tumors had significantly lower levels of gene auto-expression ($p=0.049$ for rs241432; $p=0.01$ for rs241429 and $p=0.05$ for rs241430). Based on the open SNP database (http://compbio.cs.queensu.ca/F-SNP), these polymorphisms function as transcription regulators, which gives them an ability to significantly influence their own expression. Although the majority of SNPs were present in ABCC1

genes, only rs3784867 was shown to influence its own expression. Tumors with the mutant TT genotype had a significantly higher post-NAC auto-expression level when compared to the wild type (p=0.045). Rs59409230 of the ABCG2 gene influences both the pre- and post-NAC auto-expression levels. In mutant tumors with the AA SNP genotype, the pre- and post-NAC level of ABCG2 expression was significantly different (p<0.02), when compared to the wild type (Tab. 3).

TABLE 3. Effect of SNPs on the expression level in the pre- and post-NAC breast tumor samples.

SNP (rs)		Pre-NAC expression	P-level	Post-NAC expression	P-level
ABCB3 rs241432	*TT*	1.110±0.198	0.073	1.194±0.383	**0.049**
	GG	0.575±0.225		0.307±0.109	
ABCB3 rs241429	*GG*	1.115±0.213	0.059	1.227±0.406	**0.019**
	AA	0.533±0.269		0.294±0.138	
ABCB3 rs241430	*CC*	1.075±0.194	0,076	1.171±0.371	**0,050**
	TT	0.547±0.229		0.344±0.125	
ABCC1 rs3784867	*CC*	0.887±0.228	0.291	1.200±0.368	**0.046**
	TT	1.013±0.446		1.513±0.326	
ABCG2 rs59409230	*CC*	1.656±0.401	**0.024**	1.811±0.494	**0.012**
	AA	3.315±0.816		3.496±0.939	

It is worth mentioning that all 11 SNPs (*ABCB1 rs35280822* (p=0.09), *ABCB1 rs4148735* (p=0.06); *ABCB3 rs1044043* (p=0.07), *ABCB3 rs241430* (p=0.07); *ABCC1 rs35596* (p=0.07), *ABCC1 rs35600* (p=0.07), *ABCC1 rs4148371* (p=0.06), *ABCC1 rs11864374* (p=0.06); *ABCC5 rs4912515* (p=0.07); *ABCG1 rs225398* (p=0.09); *ABCG2 rs6857600* (p=0.06)) tend to influence the gene expression. With increasing size of samples, these cases would probably become statistically significant.

Therefore, this work shows that the majority of mutations and polymorphisms of ABC genes have a substantial impact on the ATP-binding cassette transporter regulation.

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