CHEMISTRY SECTION

Assessment of gene expression level of ATP binding cassette G member 2 (ABCG2) transporter in non-metastatic ductal breast carcinoma patients receiving adjuvant chemotherapy

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Abstract: The efficacy of cancer chemotherapy is limited by cellular mechanisms of resistance that result in increased drug efflux of chemotherapeutic agents thereby reducing intracellular drug levels and causing drug resistance. Overexpression of some members of ATP binding cassette transporter superfamily, including ATP binding cassette G member 2 (ABCG2), which mediates energy-dependent transport of drugs out of the cells against concentration gradient, is one of the major mechanisms responsible for multidrug resistance in the treatment of breast cancer. In the current study, the expression of ABCG2 mRNA gene was evaluated in the peripheral blood of newly diagnosed non-metastatic breast cancer (NMBC) patients immediately before surgical resection of the breast and in extirpated breast tumors, then sequentially in the blood of patients after receiving adjuvant chemotherapy consisting of 5-flurouracil, doxorubicin and cyclophosphamide (FAC). Compared with non-tumor regions, breast tumor specimens expressed higher levels of ABCG2 gene ($p<0.001$). In addition, a gradual significant increase in the expression of peripheral blood ABCG2 gene of NMBC patients among different treatment periods was recorded ($p<0.001$). Furthermore, a significant positive correlation between peripheral blood ABCG2 gene expression of NMBC patients receiving chemotherapy and disease progression was found. In conclusion, initial assessment of ABCG2 gene expression may be a prerequisite in evaluating the effectiveness of chemotherapy-treated breast cancer patients.

Keywords: *ATP binding cassette G member 2; Breast cancer; Adjuvant chemotherapy.*

Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related deaths among women worldwide (Siegel et al., 2018). In Egypt, the incidence of breast cancer ranks second after liver cancer, with 23,081 newly diagnosed cases in 2018, representing 35.1% of all female cancers (23,081/65,693), and 9,254 death that represent 23.8% of all female cancer death (9,254/38,814) (Globocan, 2018).

The acquisition of resistance to multiple structurally unrelated compounds, known as multidrug resistance (MDR), is considered a frequent problem in the treatment of cancer and most patients with metastatic cancer die from multidrug resistant disease (Gottesman et al., 2016). Furthermore, resistance to chemotherapeutic drugs by cancer cells may also be acquired over the course of therapy (Zahreddine and Borden, 2013).

One of three human multidrug pumps that transport diverse chemicals out of cells is the ATP binding cassette G member 2 (ABCG2), also known as breast cancer resistance protein (BCRP) (Cox et al., 2018). ABCG2 is the principal ABC transporter involved in the multidrug resistance of breast cancer (Bhardwaj et al., 2019). Overexpression of ABCG2 can render the cancer cells resistant to the ABCG2 substrate chemotherapy agents, including mitoxantrone, doxorubicin and several tyrosine kinase inhibitors (Toyoda et al., 2019). The aim of the current study aimed at evaluating the sequential expression of peripheral blood ABCG2 gene expression following adjuvant chemotherapy in non-metastatic breast cancer patients, in order to assess the effectiveness of ABCG2 substrate chemotherapeutic drugs and to highlight the need for alternative local protocols for the treatment of chemotherapy-resistant breast cancer.

Subjects and Methods

Subjects

Thirty newly diagnosed female patients, aged 25-60 years, with non-metastatic breast cancer, morphological and cytogenetic evidence of breast tumors, and normal complete blood picture, hepatic and renal functions were selected from El-Salam Oncology Center (Specialized Medical Center, Ministry of Health and Population, Cairo, Egypt). Diagnosis of all patients was based on standard clinical criteria including the tumor marker CA15.3, histopathology of biopsy samples from breast tissues, mammogram and computerized tomography (CT) scans. The time interval of sample collection was between March 2017 and January 2018. The TNM cancer staging was taken from the pathological reports of patients.

Study design

The study included 60 women; 30 patients diagnosed with non-metastatic breast cancer (NMBC) and 30 healthy women volunteers (Control) matched for age. All subjects gave written informed consent to participate in the study. Breast cancer patients were treated according to the National Cancer Treatment Guidelines of the Higher Committee of Cancer (Egyptian Ministry of Health and Population) following National Comprehensive Cancer Network (NCCN) Guidelines for the treatment of invasive breast cancer. Patients received a 6-cycle adjuvant chemotherapy regimen (one cycle/3 weeks) that started immediately after mastectomy. The intravenous FAC regimen consists of 5-flurouracil (500 mg/m^2), doxorubicin (50 mg/m^2, an adriamycin derivative) and cyclophosphamide (500 mg/m^2). In case of heart diseases, patients received intravenous epirubcin (100 mg/m^2, an adriamycin derivative) instead of

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doxorubicin. The baseline characteristics of NMBC patients regarding hormonal statuses are shown in Table 1.

Tissue and blood sample collections

Tissues from tumor and non-tumor regions were separately dissected from extirpated specimens at surgery and immediately placed in liquid nitrogen then stored at -80°C. Peripheral blood samples were collected with EDTA one day before surgical operation (First-NMBC), and after receiving 3 and 6 cycles of chemotherapy (Mid and Last-NMBC, respectively). All blood samples were stored at -80°C.

RNA extraction and real-time quantitative PCR

Total cellular RNA was extracted from peripheral whole blood and frozen tissues using RNeasy® Mini kits (Qiagen, Hilden, Germany). The concentration and purity of total RNA were then assessed by measuring absorbance at 260 and 280 nm, respectively, in a spectrophotometer (Nano Drop 2000, Thermo Scientific, USA). Reverse transcription of the extracted RNA to synthesize first strand complementary DNA (cDNA) was performed using AMV Reverse Transcriptase kit (Promega, WI, USA). Real-time PCR amplification and analysis were performed in ABI PRISM 7500 Fast Sequence Detection System Thermal Cycler (Applied Biosystems, California, USA), using Power SYBR®Green PCR Master Mix (Applied Biosystems, USA). PCR conditions were 15 s at 95ºC (denaturation step), 60 s at 60ºC (annealing and extension) for 40 cycles. Primers used for ABCG2 gene were 5′-CAATGGGATCATGAAACCTG-3′ (forward primer) and 5′-GAGGCTGATGAATGGAGAA-3′ (reverse primer) and GAPDH (housekeeping gene) were 5′- ACCACAGTCCATGCCATCAC-3′ (forward primer) and 5′- TCCACCACCCTGTTGCTGTA-3′ (reverse primer). The expression of GAPDH was normalized between samples in each set, so that the relative expression of genes of interest could be established. For each experimental sample, the amount of investigated genes and endogenous reference (GAPDH) was determined. The relative expression of the RT-PCR amplified products and the fold change in the target genes were determined by the ΔΔCt method (Livak and Schmittgen, 2001). This method calculates the relative expression rate of the gene of interest by calculating the difference in expression, expressed as cycle threshold (Ct) cycle, between the test gene and the reference gene (ΔCt) compared to that of the control samples (calibrator) (ΔΔCt) then calculating the fold induction using the formula $2^{-(\Delta\Delta Ct)}$.

Statistical methods

The Shapiro-Wilks test for normality ($p>0.05$) showed that all data were non-parametric. The quantitative results were expressed as median, 25th and 75th percentile (quartiles) values. The non-parametric Mann-Whitney U test was used for comparing results between two independent groups. Wilcoxon test for multiple comparisons of non-parametric data was applied followed by Friedman's test to compare repeated measurements. Spearman's correlation was used to measure statistical dependence between two variables. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was calculated to assess the diagnostic accuracy of ABCG2 gene expression. Crosstabulation analysis was carried out and the significance χ^2 and likelihood ratio (LR) were calculated from the chosen cut-off values. The results were computed and all statistics were analyzed using SPSS statistical software version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

The baseline characteristics of NMBC patients included in the study were shown in Table 1.

Table 1. Baseline characteristics of the 30 non-metastatic breast cancer (NMBC) patients included in the study.

Clinical features (n=30)		
Age (years)		(25-60)
Grade		II (n=30)
Type		Invasive ductal carcinoma
Menopause	**Menopause**	n=17
	Post menopause	n=13
Tumor size		(0.5-10) 3.86±0.7
Lymph nodes involvement		(0-26) 5.8±1.05
Estrogen receptor	**+ve**	n=21
	-ve	n=9
Progesterone receptor	**+ve**	n=17
	-ve	n=13
HER2 receptor	**+ve**	n=16
	-ve	n=14
Triple negative		n=3

Data presented in Table 2 demonstrate a sequential significant increase in the expression of peripheral blood ABCG2 gene in NMBC starting at baseline and by the end of the third and sixth cycles of chemotherapy, compared to healthy subjects ($p<0.001$, Mann-Whitney U test), and among different periods ($p<0.001$, Friedman test followed by Wilcoxon test for repeated measurements).

Table 2. Change in blood ABCG2 mRNA gene expression between different treatment stages of non-metastatic breast cancer (NMBC) patients and healthy subjects, as well as among different treatment periods.

	Control (n=30)	First- NMBC (n=30)	Mid-NMBC (n=30)	Last-NMBC (n=30)
Median	1.00	2.04*,≠	3.45*,+	5.60*,±
IQR	(1.00-1.04)	(1.70-2.53)	(2.45-4.35)	(4.25-6.80)
p<	0.001			

* Significant versus control (Mann-Whitney U test).
# Significant versus Mid- and last-NMBC (Friedman test followed by Wilcoxon test for repeated measurements).
+ Significant versus First- and Last-NMBC (Friedman test followed by Wilcoxon test for repeated measurements).
± Significant versus First- and Mid-NMBC (Friedman test followed by Wilcoxon test for repeated measurements).

In addition, the expression of ABCG2 gene in tumor tissues of NMBC patients was significantly increased, compared to non-tumor samples of the same patients ($p<0.001$, Student's T test) (Table 3).

Table 3. Statistical significance of tissue ABCG2 mRNA gene expression of NMBC patients in breast tumor versus non-tumor samples.

	Non-tumor (n=30)	Tumor (n=30)
Mean ± SD	1.01 ± 0.04	7.01* ± 1.60
Range	(0.93-1.08)	(4.02-10.3)
Change%	-----	96%
p<	0.001	

Student's T test for parametric data was applied (*p<0.001).

Receiving operating curves (ROC) were generated for blood ABCG2 gene expression of NMBC patients at baseline (First-NMBC), as well as tissue ABCG2 gene expression, and the sensitivity, specificity, area under the curve (AUC) and the best cut-off values that discriminate between NMBC patients and healthy subjects were detected (Table 4).

Table 4. Sensitivity, specificity and cut-off values of blood and tissue ABCG2 mRNA gene expression.

	AUC	SE	p<	95%CI		Cut-off value	Sens.%	Spec.%
				LB	UB			
Blood ABCG2	0.998	0.003	0.001	0.992	1.000	1.25	93.3%	100%
Tissue ABCG2	1.000	0.001	0.001	1.000	1.000	2.56	100%	100%

According to the calculated cut-off values, cross-tabulation demonstrates that blood and tissue, ABCG2 gene expression was able to significantly differentiate between non-metastatic breast cancer patients and control subjects according to the likelihood ratio presented (Table 5).

Table 5. Cross-tabulation showing the reliability of blood and tissue ABCG2 mRNA gene expression between NMBC patients and healthy subjects.

			Groups			
Parameter	**Cut-off values**	**Counts**	**Control**	**Patient**	χ^2	**LR**
Blood ABCG2 gene expression (First- NMBC)	**≤1.25**	n	30	2	0.001	68.22
		%	100%	6.7%		
	>1.25	n	0	28		
		%	0%	93.3%		
Blood ABCG2 gene expression (Mid- NMBC)	**≤1.25**	n	30	0	0.001	83.18
		%	100%	0%		
	>1.25	n	0	30		
		%	0%	100%		
Blood ABCG2 gene expression (Last- NMBC)	**≤1.25**	n	30	0	0.001	83.18
		%	100%	0%		
	>1.25	n	0	30		
		%	0%	100%		
Tissue ABCG2 gene expression	**≤2.56**	n	30	0	0.001	83.18
		%	100%	0%		
	>2.56	n	0	30		
		%	0%	100%		

χ^2: Pearson chi-square; LR: Likelihood ratio

Progression of disease (recurrence or metastasis) in breast cancer patients was positively correlated with lymph node involvement and numbers, expression of human epidermal growth factor 2 (HER2), as well as Mid- and Last-NMBC blood ABCG2 gene expression. By contrast, the disease progression was negatively correlated with estrogen and progesterone receptors expression. On the other hand, progesterone receptor expression was positively correlated with estrogen receptor expression and negatively correlated with triple negative cases (Table 6).

Table 6. Significant correlation (Spearman's rho) between chosen markers.

	Parameters	r*	p<
Progression	Lymph node involvement	0.394	0.031
	Number of Lymph nodes	0.502	0.005
	Estrogen receptor expression	-0.36	0.048
	Progesterone receptor expression	-0.471	0.009
	HER2 expression	0.401	0.028
	Mid-NMBC ABCG2 gene expression	0.540	0.002
	Last-NMBC ABCG2 gene expression	0.652	0.001
Progesterone receptor expression	Estrogen receptor expression	0.602	0.001
	Triple negative	-0.381	0.038

r*: Correlation coefficient

The selected significant parameters in NMBC patients show that patients with positive lymph node involvement and HER2 expression are more susceptible for disease progression (recurrence or metastasis), whereas patients with positive estrogen or progesterone hormone receptor expression are less susceptible for disease progression (Table 7).

Table 7. Crosstabulation showing the reliability of some markers at initial diagnosis and the progression of the disease in NMBC patients.

			Progression			
Parameter			**Free**	**Recurrence and metastasis**	χ^2	**LR**
Lymph node involvement	Negative	(n)	6	1	0.03	5.06
		%	85.7%	14.3%	1	
	Positive	(n)	9	14		
		%	39.1%	60.9%		
Estrogen receptor expression	Negative	(n)	2	7	0.04	4.14
		%	22.2%	77.8%	6	
	Positive	(n)	13	8		
		%	61.9%	38.1%		
Progesterone receptor expression	Negative	(n)	3	10	0.01	6.95
		%	23.1%	76.9%		
	Positive	(n)	12	5		
		%	70.6%	29.4%		
HER2 expression	Negative	(n)	10	4	0.02	4.96
		%	71.4%	28.6%	8	
	Positive	(n)	5	11		
		%	31.3%	68.8%		

χ^2: Pearson chi square; LR: Likelihood ratio

Discussion

Chemotherapy is a major form of treatment for various cancers. However, a major obstacle for the effective chemotherapy is multi-drug resistance (MDR), where cells resist numerous structurally and functionally unrelated drugs via increasing drug effluxing that reduces their concentration in the cells (Liang et al., 2014; Kalimutho et al., 2015). It has been reported that clinical resistance to chemotherapy in a series of cancers may come with poor prognosis and high risk of death (Bartholomae et al., 2016).

A large number of hematological malignancies and solid tumors have been detected as exhibiting ABCG2 (Omran, 2012). Also, ABCG2 is overexpressed in highly aggressive breast cancers (Collina et al., 2015) and is considered critically responsible for drug resistance in mammalian cells (Zhang et al., 2018). Increased ABCG2 transporter expression and activity have been implicated in unresponsive breast cancer patients, while its inhibition has been shown to restore cell sensitivity to chemotherapeutic drugs (Palasuberniam et al., 2015). In the current study, assaying peripheral blood ABCG2 gene expression in NMBC patients receiving adjuvant therapy was fulfilled in order to evaluate the effectiveness of treatment. Initially, the expression of ABCG2 gene in tumor tissues of NMBC patients was significantly increased compared to non-tumor samples. Furthermore, a sequential significant increase was recorded in the expression of peripheral blood ABCG2 gene in NMBC patients at baseline and by the end of the both cycles of chemotherapy, compared to healthy subjects, and among different treatment periods. ABCG2 transporter is known to confer resistance to anthracycline anticancer drugs (Gerk and Vore, 2002), and it is therefore clear that an MDR phenotype and a non-response effect occurred in NMBC patients receiving adjuvant chemotherapy under the current treatment protocol.

ABCG2 overexpression has been associated with cancer progression by promoting proliferation and antiapoptosis via MAPK signaling pathway in laryngeal squamous cell carcinoma (Chen et al., 2016). We recorded a positive correlation between disease progression (recurrence or metastasis) in NMBC patients receiving chemotherapy and peripheral blood ABCG2 gene expression. Thus far, our results reveal that ABCG2 gene overexpression following chemotherapy treatment of NMBC patients may be correlated with poor outcome. More interestingly, hormone sensitive NMBC patients (ER+ve or PR+ve) were found to be less susceptible for disease progression (recurrence or metastasis), whereas NMBC patients with positive lymph node involvement and HER2 expression are more susceptible for disease progression. ABCG2 was reported to be expressed in different intensities and distributions in tumor cells of the breast invasive ductal carcinoma patients and a statistically significant correlation was demonstrated between ABCG2 expression and HER2 expression ($p=0.001$), lymph node metastasis ($p=0.049$), and clinical stage ($p=0.015$) (Xiang et al., 2011). Similarly, a positive correlation was recorded in the present study between disease progression in NMBC patients and both HER2 expression ($p<0.028$) and lymph nodes (involvement and number) ($p<0.031$ and 0.005, respectively).

ABCG2 is considered critically responsible for drug resistance in mammalian cells (Zhang et al., 2018). It is well documented that the expression of ABCG2 transporters on cancer cell membrane is responsible for MDR and is associated with the level of response of chemotherapy, which finally leads to the failure of chemotherapy and the progression of malignancy (Yang et al., 2015; Kartal Yandim et al., 2016).

Conclusions

In conclusion, ABCG2 gene expression does not only evaluate chemotherapy effectiveness in NMBC patients, but also may be a biomarker predictor for clinical prognosis and disease progression. More interestingly, blockage of ABCG2 could be considered as a beneficial strategy for breast cancer chemotherapy. We therefore recommend the evaluate ABCG2 gene expression in breast cancer patients at the beginning of anthracyclines chemotherapy in order to ascertain the degree of response to treatment.

There is hope that further elucidation of the cellular and molecular processes in future perspective that allow tumor cells to develop resistance and the use of new agents to prevent these processes and improve outcome for patients with breast cancer.

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