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Relationship Between the Drug Responsiveness of Acute Myeloid Leukemia Iraqi Patients and Gene Expression of Drug Resistance *ABCB1* and *ABCG2* Genes

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Abstract: Resistance of the acute myeloid leukemia (AML) to chemotherapy is a major clinical problem resulting in poor patients' prognosis. One of the known multidrug resistance (MDR) mechanisms in AML is the overexpression of efflux pumps belonging to the superfamily of ATP- Binding Cassette (ABC) transporters such as *ABCB1* and *ABCG2*. For this purpose, the present study investigated the gene expression of these genes using real-time PCR. Blood samples of AML Iraqi patients were collected at diagnosis and after induction therapy to investigate the correlation of ABC genes expression and response to chemotherapy. Results reveled low expression of *ABCB1* and *ABCG2* at diagnosis when compared with those after treatment and healthy subjects suggesting that the gene expression of these genes could be considered as a marker of AML regression.

Keywords: Acute myeloid leukemia; *ABCB1*; *ABCG2*; Drug resistance.

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Introduction

Acute myeloid leukemia (AML), is hematological disease; it is characterized by the clonal expansion of immature myeloid hematopoietic cells (myeloblast) which accumulate (myeloblasts > 20%) in the bone marrow (BM) and blood (1).

The displacement of functional hematopoietic cells in the BM causes marrow failure with a consequent shortage in the production of the cellular components of blood as erythrocytes, leukocytes and platelets (1).

The AML can be diagnosed at any age, but it is most occur in adults. The average age of diagnosis is 68 years old. However, AML makes up 32% of all adult leukemia cases, thus, older patients (over 60) do not suffer as well as younger patients as they are more likely to have unfavorable chromosome abnormalities as well as having another medical conditions that can make it harder to use intense chemotherapy regimens (2). Chemotherapy is the use of drugs to destroy cancer cells (blast cells), usually by ending the cancer cells ability to grow and divide, and decreasing its count in bone marrow to below 5% (3). One of the major clinically significant challenges to successful treatment of AML is the development of multidrug resistance during cancer chemotherapy (4). AML is slightly more common among men than women (5). High expression of ABC transporter proteins contributes to multidrug resistance with

the ATP-dependent drug efflux, a process that serves to protect cells from cytotoxic drugs causing failure of chemotherapy. *ABCB1*, *ABCG2* represent the most common multidrug resistance genes that have been identified in tumor cells (6). ABC transporters besides being involved in drug resistance may contribute to the protection of undifferentiated human stem cells and of cells during initial tissue differentiation (7). The expression of *ABCG2* and *ABCB1* genes was detected in patients with AML with poor prognosis (8).

Materials and Methods

The present study was done on thirty AML Iraqi patients (18 males and 12 females; age 14-70 years) who were clinically diagnosed by a consultant medical staff in the Baghdad Teaching Hospital and thirty apparently healthy individuals who had been randomly selected to be matched with the patients regarding to age and gender. Informed consent was obtained from all the study participants.

Blood samples were collected from thirty AML Iraqi patients before treatment and after treatment and from the apparently healthy (control) group. Blood samples from all cases and control were collected. All the samples subjected to molecular study. The samples preservation with TRIzol was done before RNA extraction. The total RNA extraction was achieved by steps depends on Guy's protocol for total RNA isolation using the kit protocol from Wes Bio Company/Korea (9).

Reverse transcription of the total RNA (7µl) to cDNA was achieved using WizScript RT FD mix (Hexamer) cDNA Reverse Transcription Kit (Wes Bio Company/ Korea). The kit is an RNA-depndant DNA polymerase that was used in cDNA synthesis with long RNA templates. To confirm the expression of target gene, quantitative real time qRT-PCR and Top Green master mix was used. Specific primers for ABCG1 and ABCG2 and for housekeeping gene (GAPDH) (Alpha DNA Ltd / Canada) was used (10)**.** Primers were stored as lyophilized until used. Primers sequences are listed in table (1).

Table (1): Primers sequence used in gene expression

Primer	Sequence (5'-3'direction)
ABCG2	**F**-CCCGCGACAGCTTCCAATGA
	R-GGCGTTGAGACCAGGTTTCA
ABCB1	**F**-GTGGTGGGAACTTTGGCTG
	R-TACCTGGTCATGTCTTCCTCC
GAPDH	**F**-AGCCGAGCCACATCGCT
	R-CAGCCCTGGTGACCAGGC

Statistical Analysis

The Statistical Analysis System SAS (2010) was used to evaluate the effect of different factors in study parameters. Chi-square test was used to significant compare between percentage and least significant difference –LSD test was used to significant compare between means in this study. Values less than (0.05)* and (0.01)** was considered to be statistically significant andhigh significant respectively.

Results and Discussion

Treatment Outcomes

Response to treatment was categorized according to the clinical data of patients like white blood cells (WBCs) count, blast cells percentage in bone marrow (BM) and Peripheral blood, platelet count and Hb.

According to the blast account, AML patients classified after each induction cycles into complete remission (CR); preserving complete remission according to established conditions for about 2 weeks with less than 5% blast cells in the bone marrow aspirate after the induction of chemotherapy and absence of leukemia in other sites and Non-responder (NR); as more than 5% blast cells in the bone marrow or evidence of leukemia in other sites (11, 12).

The CR and NR evaluation was taken from the tumor registry files of patients with the help of medical hematologists during patients follow up. Results of this study showed a different rate of response in newly diagnosed AML patients during treatment follow up.

An overall CR rate of AML patients was 14(46.66%), induction failure was 9 (30%) represented by NR and 7 (23.33%) unclassified patients due to loss of follow up (Table 2).

Failure of induction attributed mostly to multidrug resistance genes up-regulation that presented either as inherited or acquired in the leukemic cells (13,14). Also Local experience in Iraq with AML treatment shows poor outcome-related mortality of 34% (15).

Table (2): AML Subtypes and Response

AML subtype	Response patients' number	Non-response patients
M0	1	2
M1	1	0
M2	5	1
M4	3	1
M5	4	5
M6	0	0
Total*	14 (46.66%)	9 (30%)

***7 (23.33%) unclassified patients due to loss of follow up

Gene Expression Profile Study by qRT- PCR

Assessment of the Extracted RNA Purity and Concentration

The results of this study showed high purity of RNA (1.8 - 2.00) for both patients, and control samples. Total RNA was successfully extracted from all samples, the concentration of total RNA ranged from 20 to 30 ng/ µl in patient's samples, and 40 to 50 in control's sample.

This differences in RNA concentrations was depend on the blood values mainly WBCs count which was different between patients and control group and between different stages of patient's treatment. In addition, it may be belong to somewhat degrade of RNA samples or sample handling and preparation technique (16).

The cDNA Reverse Transcription

As cDNA was needed for gene expression of *ABCB1* and *ABCG2* in addition to housekeeping gene (*GAPDH*), cDNA reverse transcription was conducted on the same day of RNA extraction to get a good yield of cDNA.

Quantitative Real Time PCR

The quantification real time PCR was applied utilizing the Top green "a fluorescent dye" which recognizes any double stranded DNA including cDNA, the amplification was recorded as a cycle threshold value (Ct value).

The Quantification Real time PCR of *GAPDH* Expression

The results of fold gene expression for *GAPDH* depending on Ct values between the study groups revealed no differences ensuring that *GAPDH* gene is a useful internal control gene.

Gene Expression of *ABCG2*

According to the fold of *ABCB1* expression depending on $2^{-\Delta\Delta Ct}$**,** the expression of *ABCB1* gene (2.02 ±0.17) after treatment administration, in the present study was significantly higher (P≤0.01) than in before treatment (1.56 ±0.08), and in control group (1.00 ±0.00) as shown in tables (3).

Table (3): Fold of *ABCB1* Expression Depending on $2^{-\Delta\Delta Ct}$ Method (Livak method)

Groups	Means Ct of *ABCB1*	Means Ct of GAPDH	ΔCt (Means Ct of *ABCB1* - Means Ct of GAPDH)	Mean ΔCt Calibrator (13.76)- Mean ct *GAPDH*	ΔΔCt	$2^{-\Delta\Delta Ct}$	experimental group/ Control group	Fold of gene expression ± SE
After	12.43	19.24	-6.81	-5.46	-1.35	2.549	2.54/1.25	2.02 ±0.17 a
Before	12.8	19.241	-6.441	-5.46	-0.981	1.973	1.97/1.25	1.56 ±0.08 b
Healthy	13.43	19.221	-5.791	-5.46	-0.331	1.257	1.25/1.25	1.00 ±0.00 c
LSD value	-	-	-	-	-	-	-	0.319 **
P-value	-	-	-	-	-	-	-	0.0001 **
Means having with the different letters in same column differed significantly. ** (P≤0.01).								

It is evident from the present results of high *ABCB1* expression for the patients group (After treatment) when it compared with this in before treatment is associated with the highest copy number of mRNAs. Williams *et al.* (17) suggested that the drug efflux pump *ABCB1* is a key driver of chemo resistance, and high expression predicts for treatment failure in AML. The administration of drug mainly daunorubicin chemotherapy used in standard induction and consolidation

regimens is the most common cause of treatment failure in AML (17).

And this is can be explained by many factors, one of the most important them is that constitutive *ABCB1* expression is predominantly regulated transcriptionally by binding of transcription factors (18). So, after the exposure of leukemia cells to chemotherapeutic drugs activated an integrated stress response-like transcriptional program to induce *ABCB1* through remodeling and activation of an ATF4, stress-responsive enhancer (19). The transcription factor ATF4 is a critical effecter and is highly expressed in many cancers as a result of extrinsic stress or direct activation by constitutive oncogene expression. And this leads to induce up regulation of a drug-resistance mechanism in AML blast cells that may contribute to therapeutic failure and disease relapse (17).

ATP-binding Cassette Super-family G member 2 Gene Expression

The table (4) shows the expression of *ABCG2* gene in this study groups. The *ABCG2* gene expression gave highly significant ($P \leq 0.01$) increase in Ct fold in after drug administration group (1.752±0.27) compared with before drug administration (1.035 ±0.08) and control group (1.00 ±0.00).

Seedhouse *et al*. (20) reported the relationship between drug-induced upregulation of *ABCB1* expression with non-response AML patients.

The amplification plots of *ABCB1* ranged from 21.97 to 23.75 cycles. The dissociation curves by qPCR samples included all study groups, and the melting temperature ranged from 78.0°C to 79.0.

This is explained by Shaffer *et al.* (21) who justify such results for the effect of daunorubicin exposure associated with chemo resistance in AML. These results came in parallel to the results of *ABCB1* over expression as they are from the same family (ABC family), in other term they have co-expression pattern, and *ABCB1* and *ABCG2*, when co-expressed, can function independently to transport substrates *ABCB1* and *ABCG2* are found to be co-expressed in some cancers, particularly leukemia (22).

Wilson *et al.* (23) reported gene expression profiles for 170 AML patients who characterized by the highest levels of drug resistant showed increased in *ABCB1* and *ABCG2* expression.

Table (4): Fold of *ABCG2* Expression Using Livak method on $2^{-\Delta\Delta Ct}$ Method

Groups	Means Ct of *ABCG2*	Means Ct of GAPDH	ΔCt (Means Ct of *ABCG2* - Means Ct of GAPDH)	Mean ΔCt Calibrator (ct*ABCG2*- ct *GAPDH*	ΔΔCt	$2^{-\Delta\Delta Ct}$	experimental group/ Control group	Fold of gene expression
After Treatment	17.42	19.24	-1.82	-0.45	-1.37	2.584	2.58/1.47	1.752 ±0.27 a
Before Treatment	18.18	19.241	-1.061	-0.45	-0.611	1.527	1.52/1.47	1.035 ±0.08 b
Control	18.21	19.221	-1.011	-0.45	-0.561	1.475	1.47/1.47	1.00 ±0.00 b
LSD value	-	-	-	-	-	-	-	0.294 **
P-value	-	-	-	-	-	-	-	0.0006
Means having with the different letters in same column differed significantly. ** ($P \leq 0.01$).								

This is in line with Damiani *et al.* (24) who reported that the *ABCG2* gene was found overexpressed in patients with AML and it was associated with resistance to therapy.

Coexpression and coexistence of *ABCB1* and other transporters such as *ABCG2* has also been reported in normal and leukemic stem cells, is a default stem cell program in AML, and perhaps in other cancers (25).The same relationship between drug-induced upregulation of *ABCB1* expression with non-response AML patients was noted with *ABCG2* in the present study.

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