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Polymorphisms and haplotypes in multidrug resistance 1 (*MDR*1) gene and their association with clinical outcome of some Iraqi patients with acute leukemia.

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Abstract: The human multidrug resistance (*MDR*1, *ABCB*1) gene encodes P-glycoprotein (P-gp), which affects the pharmacokinetics of many drugs. Polymorphisms in *ABCB*1 might contribute to cancer risk and therapeutic response. Here, we investigated whether common *MDR*1 single nucleotide polymorphisms (SNPs) (C1236T, C3435T, and G2677T/A) and their expressed levels associated with clinical outcome of acute leukemia. Genotyping was performed in 46 patients with acute leukemia (AL) by direct sequencing analysis resulting in a total of 31 AML and 15 ALL cases matched with 10 samples healthy control. There was a high significant difference in the heterozygous haplotype B (CTGTCT) of *MDR*1 associated with high level of *MDR1* mRNA expression (p=0.0017**: 3.21 ± 0.24) in non-responder (NR) patients with AML patients. While a mutant homozygous C (TTT) haplotype was found to be associated in both NR and CR ALL patients (p=0.0428* and 0.0336*) respectively, with high level of *MDR1* gene expression (1.14 ± 0.05 and 1.25 ± 0.03). We conclude that B haplotype of *MDR*1 associated with poor prognosis of AML, while the C mutant haplotype of *MDR*1 was associated with ALL but there was no significant differences with clinical outcomes.

Key Words: Polymorphism , *MDR*1 gene , acute leukemia.

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Introduction:

One of the major clinically relevant obstacles to successful treatment of acute leukemia is the development of multidrug resistance during cancer chemotherapy (1, 2,3). Overexpression of P-glycoprotein encoded by the *ABCB1* gene in cancer cells is one of the causes related to resistant disease and failure of therapy due to decrease in drug accumulation, there by mediating cellular resistance to many of chemotherapy(3,4). Whereas the reduced of expression leads to higher intracellular concentration of toxins leading to diseases progression (5). MDR1 expression and P-glycoprotein (P-gp) function has been affected by Allelic variants in healthy volunteers(6). Many studies reported that MDR1 gene polymorphism association with susceptibility to diseases (7,8) and impact on response to chemotherapy (9,10,11). Among these, *MDR1* single nucleotide polymorphisms (SNPs) were investigated for somatic genotypes in exon 12 (C1236T), exon 21 (G2677TA), and exon 26 (C3435T) are the most reported repeatedly shown to

predict changes in the function of P-GP. The term haplotypes is used for genetic variants in linkage disequilibration. These genetic variants can be located at the same chromosome range in locus number from 3 to 55 (12,13). Or, on different genes and chromosomes with the same effect (14). Three single nucleotide polymorphisms (SNPs) occur frequently and have strong linkage, creating a common haplotype at positions 1236C>T (G412G), 2677G>T (A893S) and 3435C>T (I1145I) (15,16,17,18). Recently, many pharmacogenomics studies aim to elucidate the genetic bases for inter individual differences and to use such genetic information to predict the safety, toxicity, and/or efficacy of drugs (19,20). So the identification of patient subpopulations most likely to respond to therapy is a central goal of modern molecular medicine which plays an important role in mediating multidrug resistance to chemotherapeutic agents (21,22,23). Also it's a common genotypes affect predisposition to leukemia (24). The aim of this work is to evaluate the role of polymorphism of the SNPs C1236T, C3435T, and G2677T/A of the MDR1 gene in acute leukemia in Iraqi patients.

Patients and Methods:

Peripheral blood samples were collected from 46 cases of newly diagnosed acute leukemia and distributed by one third acute lymphoblastic leukemia 15(32.6%) and two thirds acute myeloblastic leukemia 31(67.4%) match with10 healthy donors for MDR1 investigation. The samples provided by major hospital in Iraq (hematology unit of Baghdad Teaching Hospital and DAR-ALTamred Private Hospital in medical city). The mean of blast cells in bone marrow and peripheral blood was 77.7% and 67% respectively. The acute leukemic patients were equally distributed in respect to gender M:F-1:1 beside the healthy volunteers, and there were no differences between healthy subjects 34.4±8.5 and patients 34.2±15.7 (Mean ±SD) in regarding age. The study was performed on adults AL patients with follow-up of 10 months during July 2011 to May 2012. Patient's clinical data like WBC count, blast% in BM and peripheral blood, platelet count, HB, complete remission (CR) and non response (NR) was noted from the tumor registry files with the help of medical hematologists during follow up. All patients were treated according to the chemotherapy protocols of (Hematology Unit-Baghdad Teaching Hospital-Iraq).

Samples Preservation:

Trizol was used to lyse blood cells shortly after collection of samples. This helps to stabilize RNA in these samples.

Assessment of Therapy:

Response to treatment was categorized as complete remission (CR); preserving complete remission according to established conditions for >6 months: cellularity of more than 20% with less than 5% blast cells in the bone marrow aspirate after induction chemotherapy and absence of leukemia in other sites; non-responder (NR) as more than 5% blast cells in the bone marrow or evidence of leukemia in other sites, after at least two courses of chemotherapy (25).

RNA Isolation:

Total RNA Isolation performed in Molecular Oncology diagnostic Unit/ Guys and ST Thomas's Hospital /London/UK based on the method of Chomczynski and Mackey (1995)(26). The concentration and purity of the RNA samples were determined by Nano drop, and they were stored at -80 °C until use.

cDNA Synthesis:

Total RNA (15µl) reverse transcription to cDNA was achieved with random primers using High Capacity cDNA Reverse Transcrioption Kit, Applied Biosystem. After initial denaturation of RNA at 65C for 5 minutes, reverse transcription (RT) reactions were performed with the following parameters: 25C° for 10 min, at 37C° 10 min, 60min. at 42C° followed by 75C° for 5min. cDNA was stored at -20C° and used as a template for PCR amplification for MDR1.

Real Time Quantification Polymerase Chain Reaction (RT-qPCR):

The expression levels of MDR1 transcript in blood samples were estimated by RT-qPCR using a TaqMan probe assay and an ABI PRISM 7900HT (Applied Biosystems). Primers and probes were designed by computer program Primer Express (ABI, USA) as following: MDR1 forward 5'-TGCTCAGACAGGATGTGAGTTG-3' MDR1 reversed 5'-TTACAGCAAGCCTGGAACCTAT-3' MDR1 probe 5'-AGCATTGACTACCAGGCTCGC-3'. ABL gene was selected as endogenous housekeeping gene for normalization MDR1: ABL forward 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3' ABL reversed5'-GATGTAGTTGCTTGGGACCCA-3' ABL probe 5'-CCATTTTTGGTTTGGGCTTCACAC-CATT-3'. All RT-qPCR quantifications were performed in duplicate reaction. Duplicate reactions showing differences of more than 0.3CT were repeated. Two non-template controls were also included in each run. The mRNA levels of endogenous control gene, i.e., ABL, were amplified and used to normalize the mRNA levels of the MDR1 gene and correct synthesis of cDNA as well as the calculations descriptions. For ABL quantification we used primers and probe designed and published by Van Dongen *et al*., 1999 (27). PCR products were detected using a 5' FAM (6-carboxy-flurescein) reporter dye and a 3' TAMRA (6 carboxy-tetramethylrodamine) quencher dye for all reactions.

Real time TaqMan assay was performed in a 20µl retraction volume containing 10µl of master mix (TaqMan® Universal PCR Master Mix), 0.093µl for each primer, 0.1µl of probe, 4.71µl of RNase free water and 5µl of cDNA template. For accurate quantification, calibration curves were generated by the quantification of serial dilutions of a construct synthesised from an MDR1 positive leukaemia sample, and serial dilutions of a leukaemia sample for ABL standard curve. RT-qPCR reaction parameters were: stage 1: 2min at 50C°, then stage 2: 95C° for 10 min and in a stage 3: Two step cycles achieved (denaturation 95 C° for 15 Sec. and annealing 60 ºC for 1 min) repeated for 50 cycles. The amount of target MDR1 gene, normalized to an endogenous reference ABL gene and relative to a calibrator untreated normal control, is given by: $2^{-\Delta\Delta Ct}$. The gene expression fold change calculated by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ target- ΔCt untreated for calibration, and normalized by $\Delta Ct = Ct$ target gene- Ct endogenous reference (28,29).

DNA Extraction and Genotyping:

Interphase layer of 56 blood samples with TRIzol (46 patients and 10 controls) were isolated for genomic DNA extraction with QIAamp DNA Mini Kit (Qiagen, UK) which was designed for purifying ogenomic DNA. MDR1 in three selected single-nucleotide polymorphism (SNPs) C1236T exon 12, G2677T exon 21 and C3435T exon 26, polymorphism was detected using Automated DNA Sequencer 3730 (Applied BioSystem, USA) using ABI PRISM Big Dye® Terminator v 3.1 Cycle Sequencing kit. Primers were designed by computer program Primer Express (ABI, USA) as following: MDR1 X12-Forward 5'-CCTGTGTCTGTGAATTGCCTTG-3' and MDR1 X12-Reversed 5'-ATCAGAAAGATGTGCAATGTGA-3'; MDR1 G2677T X21-F 5'-GTTTTCCAGGCTATAGGTTCC-3' and MDR1 G2677 X21R 5'-TTTAGTTTGACTCACCTT-3' and MDR1 C3435T X26F 5'-GATCTGTGAACTCTTGTTTTC-A-3' and MDR1 C3435T X26R 5'-GAAGAGAGACTTACATTAGGC-3'.

Ethical use of data:

Informed consent was obtained from all the study participants and the guidelines set by the ethics committee of our institute and hospitals were applied.

Statistical analysis:

The Statistical Analysis System-SAS (2010) was used to 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. Odds ratio and 95% confidence interval (CI) was calculated to estimation risk development. A p value less than (0.05)* and (0.01) ** was considered to be statistically significant and high significant respectively.

Results:

MDR1 Haplotype (C1236T-G2677T/A-C3435T):

Genotype was successfully 100% by direct sequencing analysis (Fig 1). In all three loci, homozygous wild-type alleles were classified as genotype A (CGC), heterozygous as B (CTGTCT), and homozygous mutant (alternative) allele as C (TTT). Thus, these three haplotype combinations cover 35(76.08%) of all investigated cases and other combination 11(23.78%).

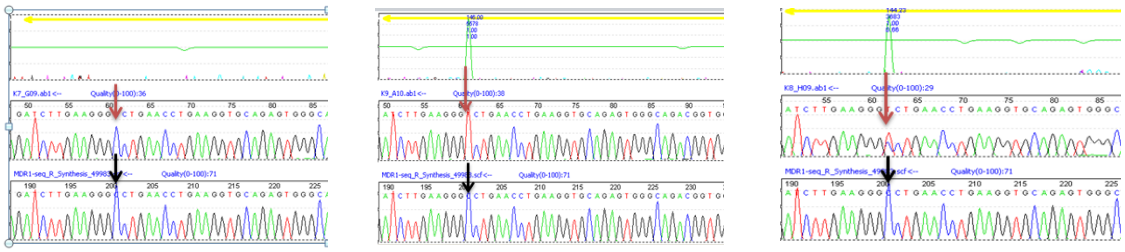

Figure (1): Electrograph shows DNA Sequencing for left wild type; middle Homozygous mt/mt and right Heterozygous wt/mt. Black arrow represented references *MDR1* (wild type) and red arrow was the sample.

The genotyping was performed in two PCR amplification and cleanup process. First PCR was performed by HotStarTag® Master Mix Kit using MDR1 X26 F and R, 0.7µl of primer mix (pmol/l), 3µl of DNA, and 8.8µl of RNase free water in total volume of 25µl. The first PCR cycle including one cycle at 94C° for 10 min. for enzyme activation, 10 cycles of 94C° for 30sec., 60C° for 30sec., and 72C° for 30sec., followed 40 cycles of 94C° for 30sec., 54C° for 30sec., and 72C° for 30sec for denaturation, annealing and extension respectively, followed by final extension at 72C° for 5 min. Purifying PCR products by Charge Switch PCR Clean-up kit according to the manufacturer's instructions. Successful amplification was confirmed by detection band on a 2% agarose gel using a 100 bp DNA ladder. The purified products were used as a templates sequence cycle using ABI BigDye terminator ready reactions Kit (Applied Biosystems, USA). Then post cleanup was performed after the sequencing PCR completed. The second PCR products were proceed to the next purification using Agencourt® CleanSEQ® dye-terminator Removal Kit. Sequencing reaction with HI DI (deionizedformamide) was achieved. The plate was placed in the ABI Automated DNA Sequencer 3730 and then data analysis achieved by Mutation Surveyor Software of reading sequencing Version 3.24.

MDR1 Phenotype-Haplotype Association with Acute Leukemia Clinical Outcomes:

According to the clinical outcomes, *MDR1* haplotype variants frequency showed non-significant differences with clinical outcomes in AML and ALL patients, accept B heterozygous haplotype was showed significant difference (p=0.043*) in NR AML patients, table (1).

Table (1): *MDR1* Haplotype Frequency According to Acute Leukemia Clinical Outcomes

Clinical Outcomes	Heterozygous BBB	Homozygous CCC	Wild type AAA
	n (%)	n (%)	n (%)
AML NR	(9)19.56	(3)6.25	(2)4.34
AML CR	(4)8.69	(3)6.25	(2)4.34
p-value	0.043 *	1.00 NS	1.00 NS
ALL NR	(2)4.34	(2)4.34	(3)6.25
ALL CR	(2)4.34	(2)4.34	(1)2.17
P-value	1.00 NS	1.00 NS	0.628 NS

In regards to *MDR1* gene expression, statistical data analysis explained in table (2) showed high significant differences (p=0.0017**) in AML NR patients with high level of *MDR1* mRNA expression in B and C (3.21 ± 0.24 and 1.84 ± 0.05) respectively, compared to low level of A wild type haplotype (0.21 ± 0.01). The AML CR patients have no significant differences in haplotype and *MDR1* gene expression. On the other hand, *MDR1* C haplotype was showed significant differences in both NR and CR ALL patients (p=0.0428* and 0.0336*) respectively, with high level of *MDR1* gene expression (1.14 ± 0.05 and 1.25 ± 0.03) compared to B and A

variants that showed low level of *MDR1* gene expression, table (2). Because there is no difference between the NR and CR in the level of *MDR1* gene expression, it was concluded that C haplotype did not associated with clinical outcomes among ALL patients.

Table (2): Relationship between *MDR1* Gene Expression and Haplotype According to Acute Leukemia Outcomes

Clinical Outcomes	BBB Haplotype mean± SE	CCC Haplotype mean± SE	AAA Haplotype mean± SE	P-value
AML NR	3.21 ± 0.24	1.84 ± 0.05	0.21 ± 0.01	0.0017 **
AML CR	0.17 ± 0.02	0.5 ± 0.04	0.54 ± 0.03	0.217 NS
ALL NR	0.44 ± 0.05	1.14 ± 0.05	0.57 ± 0.03	0.0428 *
ALL CR	0.57 ± 0.05	1.25 ± 0.03	0.02 ± 0.00	0.0336 *

Discussion:

It is clear that *MDR1* polymorphism analysis can provide important information to optimize the individualized therapeutic approach, significant interethnic variations have been identified worldwide. *MDR1* phenotype relevant polymorphisms in the *MDR1* gene are likely to be present among acute leukemic patients and may have an impact on response to anti leukemic drug treatment. Kim *et al.*, 2001, Brambila-Tapia 2013(30,31) showed the haplotype containing the three wild-type or reference variants (CGC) is the most frequent in African or African- American populations (43.6-79.3%) followed by South Americans (25-65%) and Caucasians (32.5-45%). Asians showed the lowest frequency of the wild-type haplotype (16-25.9%) and the highest frequency of the TTT haplotype (32.2-45.3%). The study found that the 3 SNPs in exon 12 , 21 and 26 were in linkage disequilibrium in up to 76% of Iraqi population in acute leukemia with a high significant frequency of B heterozygous haplotype (CTGTCT) 36.95%, while healthy individuals showed only 10% for TTT haplotype was linkage disequilibrium and 90% were different allelic combination. The current study suggests the linkage disequilibrium of these 3 *MDR1* SNPs associated with patients more than with healthy. Many studies agreed with findings of this study, Illmer *et al*. (2002)(32) showed that the high frequency of high level *MDR1* gene expression associated with B variants in AML patients. Urayama *et al.* (2007)(33) reported equal distribution of A and C haplotype among ALL patients, but they found the C(TTT) variants carriers was significantly associated with increased risk of ALL compared to A variants (CGC) carriers. Semsei (2011)(34) studied the role of *MDR1* haplotype in the development of childhood ALL and he showed the heterozygous haplotype was more frequent in cases than controls (9.4% vs 3.9% p=0.002). Many studies were agreed with findings of this study, Hung *et al.* (2008)(35) showed the *MDR1* in the double SNPs haplotype (1236T-3435T) or triple haplotype (1236T-2677A/T 3435T) have diminished effect of some drugs. The study by Vivona *et al.* (2014)(36) showed the *MDR1* 1236CT/3435CT/2677GT and 1236TT/3435TT/2677TT haplotypes are associated with reduced P-GP activity in CML patients treated with a

standard dose of imatinib (IM). Kim *et al.* (2014) (37) showed the C-G-C haplotype was associated with a decreased risk for NHL and Diffuse Large B-Cell Lymphoma (DLBCL).

Together these results suggest that *MDR1* SNPs may have a slight influence on some drug pharmacokinetics and response in specific populations; nevertheless, it seems to be substrate-dependent because different alleles have been associated with different drugs. Many other transporters have been identified that also may ultimately be found to be important for determining individual therapeutic response. Also, many more SNPs for transport proteins have been identified studied *in vivo*. The advent of inexpensive broad genetic screening for transport protein polymorphisms will no doubt be instrumental in a new era of truly personalized therapy.

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