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RESEARCH ARTICLE

EFFECT OF MDR1 GENE EXPRESSION RELATED TO C3435T POLYMORPHISM IN IRAQI ACUTE MYELOID LEUKEMIA PATIENTS

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ABSTRACT

Single nucleotide polymorphism (SNP) in multidrug resistance gene1 (MDR1) could alter the gene expression level and may have effect role in responses to drug therapy and diseases susceptibility. The aim of the present study is to investigate allele frequency in Iraqi healthy and AML patients to detect the susceptibility of C3435T genotype carrier to develop acute myeloid leukemia. Also the study aimed to correlate the expression level of MDR1 mRNA with MDR1 gene and C3435T polymorphism in de novo AML patients. The results showed there was a significant difference in genotype and allele frequency with heterozygous CT and mutant T-allele. The results also showed that there was no significant difference between genotype and allele frequency in healthy control and AML patients. According to the clinical outcome status MDR1 3435CT showed statistically high significant differences, while CR group was showed significantly with homozygous TT. Both NR and CR group in AML patients showed high mutant-T allele frequency. In regard with gene expression, the healthy control showed significantly high level of MDR1 mRNAs expression in CC genotype at position 3435 compared with CT and TT. Whereas MDR1 heterozygous 3435CT genotype showed a highly significant difference in MDR1 mRNA expression among AML patients. In conclusion healthy Iraq populations and AML patients have predominantly CT genotype and mutant-T allele frequency for MDR1 C3435T polymorphism. MDR1 3435CT/TT genotype in regard with MDR1gene expression in de novo AML patients associated with poor prognosis, while CC genotype was protective Carrier.

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INTRODUCTION

Acute myeloid leukemia (AML) is a clonal disease resulting from genetic mutations and transformation of a single early progenitor myeloid cell (Chessells, 2000). Despite broadly research the causes of acute leukemia remain largely unknown (Deschler and Lübbert, 2006). One of the major clinically relevant obstacles to successful treatment of acute myeloid leukemia is the development of multidrug resistance during cancer chemotherapy (Miladpour *et al*., 2010; Green *et al*., 2012; Zhang *et al*., 2013). 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. Whereas the reduced of expression leads to higher intracellular concentration of toxins leading to diseases progression (Rao *et al*., 2010). MDR1 expression and P-glycoprotein (P-gp) function has been affected by Allelic variants in healthy volunteers (Sipeky *et al*., 2011). Many studies reported that MDR1 gene polymorphism association with susceptibility to diseases (Tan *et al*., 2005; Rao *et al*., 2010) and impact on response to chemotherapy (Jamroziak *et al*., 2004; Jamroziak *et al*., 2005; Zhai *et al*., 2012). The MDR1 gene localized in chromosome 7q21.12 contain 29 exons, ranging in size from 49 to 587 bp, in a genomic region spanning 209.6 kb (Bodor *et al*., 2005). According to the SNP databases mentioned by National Center for Biotechnology Information (NCBI) there are 50 SNPs in the human MDR1 coding region and one in the start codon (Hodges *et al*., 2011). Several single nucleotide polymorphisms (SNPs) in the MDR1 gene have been identified, of which C1236T (silent), G2677T/A (Ala893Ser/Thr) and C3435T (silent) have been associated with altered MDR1 expression and consequently drug resistance of cells (Hoffmeyer *et al*., 2000). Recently, rs1045642 (3435T>C) appears larger interethnic allele frequency differences, with the 3435C allele ranging between 34 and 90% across populations.

An important characteristic of this SNP is that its allele frequency varies in different human populations (Sakaeda *et al*., 2003). Here, we focused on the correlation between MDR1 expression with C3435T variant among Iraq AML patients.

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MATERIALS AND METHODS

Peripheral blood samples were collected from 31 de novo AML patients and 10 healthy donors were provided by major hospital in Iraq (Hematology unit of Baghdad Teaching Hospital and DAR-ALTamred Privet Hospital in medical city). The mean of blast cells in bone marrow and peripheral blood was 77.7% and 67% respectively. Patients and healthy subjects were equally distributed in respect to gender. The mean age of the patients was 36.8 yr (rang, 16-72 yr). The study was performed on adults AML 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 lack of response (NR) were took from the tumour 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). The induction chemotherapy regimens were, combined cytarabine plus adriamycin or combined vincristine plus doxorubicin or daunorubicin and ATRA (All-trans retinoic acid) plus induction chemotherapy, depended on the subtype of AML. All patients underwent 2 induction cycles followed by consolidation. Early death (within 2weeks of induction and after complete induction) appeared in 5(16%) and 2(6.45%) patients, respectively.

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 (Huh *et al*., 2006), and early relapse within 6 month from remission (Michieli *et al*., 1999). CR and NR was evaluated after each induction cycles.

RNA Isolation

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

cDNA Synthesis

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 transrition (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 samples were stored at -20C° then 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'-TGGAGATAACACTCTAAGCATAACTAA AGGT-3' ABL reversed5'- GATGTAGTTGCTTGGGACC CA-3' ABL probe 5'-CCATTTTTGGTTTGGGCTTCACAC-CATT-3'. All RT-qPCR quantifications were performed in duplicate reaction. Duplicate reactions showing differences of more than 0.3 CT were repeated. Two non-template controls were also included in each run. The mRNA levels of endogenous control gene, 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, primers and probe were used according to Van Dongen *et al*., 1999. 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 ΔCt= Ct target gene- Ct endogenous reference.

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 Min Kit (Qiagen, UK) which was designed for purifying of genomic DNA. MDR1 C3435T 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 X26-Forward 5'-GAT-CTG-TGA-ACT-CTT-GTT-TTC-A-3' and MDR1 X26-Reversed 5'-GAA-GAG-AGA-CTT-ACA-TTA-GGC-3'.

The genotyping was performed in tow 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.

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

Genotypes and allele frequency of MDR1 C3435T polymorphism

Genotype was successfully 100% by direct sequencing analysis (Fig 1). Resulting in a total of 31 AML cases matched with 10 samples healthy control form Iraqi population. Genotype results showed there was significant difference in genotype and allele frequency with heterozygous CT (50%: p=0.0038<0.01) and mutant T-allele (55%: p=0.044<0.05) respectively for MDR1 SNP C3435T in normal Iraqi population. AML patients was showed same distribution with predominant heterozygous CT (48%: p=0.014<0.01) and mutant T-allele (56%: p=0.052<0.05). There was no significant difference between genotype and allele frequency associated with control (Table 1). Odds ratio and their 95% confidence interval (ORs and 95%CIs) analysis was used to detect the MDR1 C3435T genotype risk factors carriers to developing AML between populations. ORs and (95%CI) revealed no relative risk associated with MDR1 C3435T polymorphism to development AML (Table 2). We suggest to increase the number scales between case controls and patients to confirm our finding.

Table 1. Genotype and allele frequency of MDR1 C3435T polymorphism among acute leukemia and control

Genotype C3435T	Control n=10	AML n=31	P-value
CC	2(20)	6(19.35)	0.844 NS
CT	5(50)	15(48.38)	0.749 NS
TT	3(30)	10(32.25)	0.802 NS
P-value	0.0038 **	0.014 **	
	Alleles frequency		
C	9(45)	27(43.5)	0.752 NS
T	11(55)	35(56.5)	0.955 NS
P-value	0.044 *	0.052 *	

No.(%) (P<0.05), (P<0.01)**, NS (no significant)*

Genotype and allele frequency of MDR1 C3435T related with clinical outcome of acute myeloid leukemia

According to the clinical outcome, there were 17(54.83%) patients showed NR to chemotherapy at presentation, 11

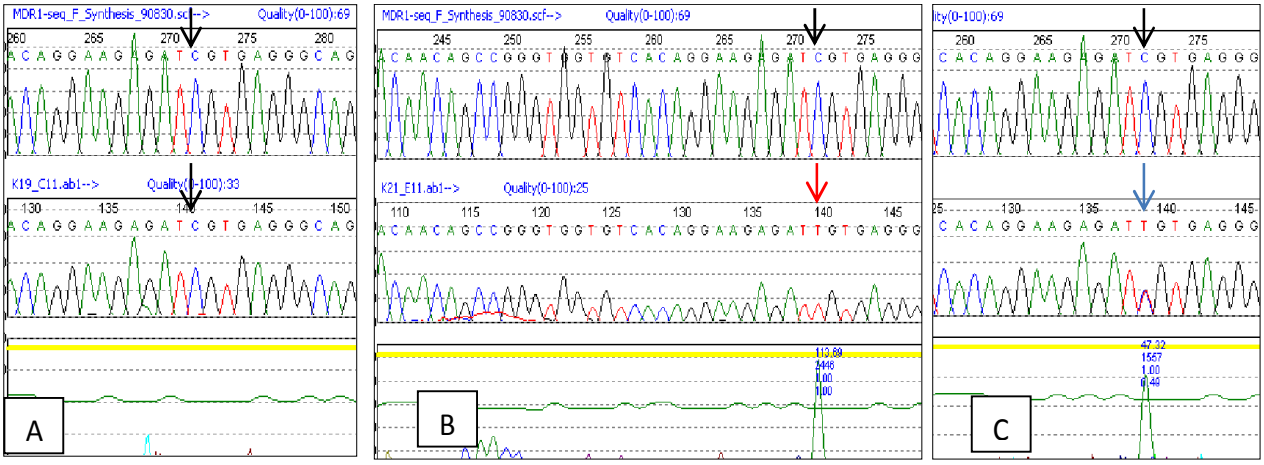

Figure 1. Electrograph show DNA sequencing for (A) wild type C3435T wt/wt(C/C) (B) homozygous mt/mt (T/T) (C) heterozygous wt/mt (C/T). upper arrow represented references MDR1 (wild type) and lower row the sample

Table 2. Estimation of risk developing in AML association with MDR1C3435T Genotype

Genotype C3435T	AML n=31	Control =10	χ^2	OR	(95%CI)
CC	6(19.35)	2(20)	0.991	CC vs CT	1.0
CT	15(48.38)	5(50)		CT vs TT	0.90.73-1.101
TT	10(32.25)	3(30)		CC vs TT	0.90.73-1.101
	Allele frequency				
C	9(45)	27(43.5)	0.909		
T	11(55)	35(56.5)			

(35.48%) of them were MDR1 heterozygous 3435CT genotype, 4(12.9%) were homozygous mutant TT and 2(6.45%) were wild type CC. While 14(45.16%) patients were showed CR, 6(19.35%) of them were MDR1 homozygous mutant and 4 (12.9%) for both heterozygous and wild type (Figure 2). According to the clinical outcome status, the percentage of patients with MDR1 3435CT was higher than those with 3435CC/TT among NR AML, while in CR group was showed high with homozygous TT. The observation didn't shown statistical analysis due to small size numbers.

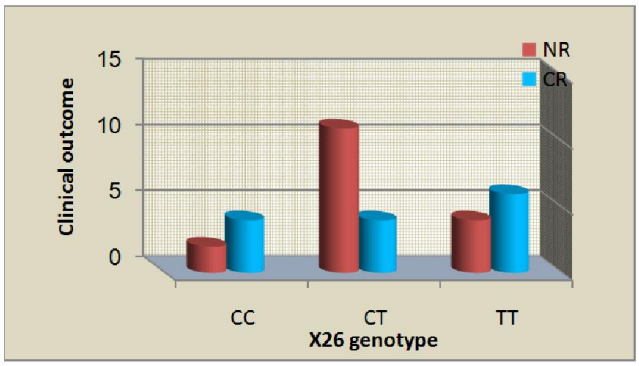

Figure 2. Genotype frequency of MDR1 C3435T related with AML clinical outcome

MDR1 C3435T SNP association with MDR1 gene expression in normal and acute myeloid leukemia

In regard to MDR1 gene expression, MDR1 polymorphism at position C3435 was categorized according to the normal and AML patient's mean fold change of MDR1 mRNA expression. The statistical analysis showed significant difference in MDR1 C3435T polymorphism associated with MDR1 gene expression (p=0.0238* <0.05) with control, it's showed high MDR1 mean fold change in CC wild type compare with CT and TT (1.91 ± 0.02; 0.9 ± 0.02 and 0.96 ± 0.03) respectively (Figure- 3). On the opposite side heterozygous CT genotype was occurred highly significant MDR1 expression with AML patients (p=0.0018 **<0.01) compared with CC and TT (2.32 ± 0.06, 0.26 ± 0.02 and 1.50 ± 0.01) respectively (Figure- 3). Furthermore, according to the AML clinical outcome the statistical analysis showed highly significant differences in MDR1 gene expression dependent in 3435CT/TT genotype in non-responding patients (3.10 p=0.0025**<0.01) (3.01 p=0.126**<0.01) respectively, while CC genotype appeared non-significant with clinical outcome (Table-3)

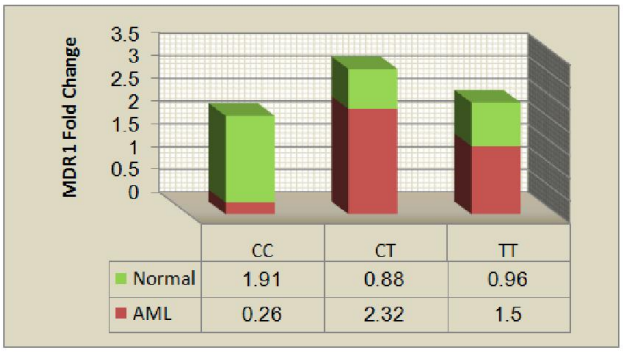

Figure 3. Comparison analysis between normal and AML patients in MDR1 gene expression dependent MDR1 C3435T polymorphism

Table 3. MDR1 expression related with C3435T SNP in AML clinical outcome

Genotype C3435T	AML NR	AML CR	p-value
CC	0.21	0.29	0.439 NS
CT	3.10	0.17	0.0025 **
TT	3.01	0.50	0.0126 **

DISCUSSION

The development of multidrug resistance during cancer chemotherapy is one of the major obstacles to successful treatment of AML. MDR1 expression and P-glycoprotein (P-gp) function have been affected by Allelic variants in healthy volunteers (Sipeky *et al*., 2011). Many studies reported that MDR1 gene polymorphism associated with susceptibility to diseases (Tan *et al*., 2005; Rao *et al*., 2010; Mhaidat *et al*., 2011) and impact on response to chemotherapy (Jamroziak *et al*., 2004; Jamroziak *et al*., 2005; Sharom, 2008; Zhai *et al*., 2012). Our results showed that the heterozygous CT (50%) and mutant-T allele (55%) for MDR1 C3435T SNP are more frequent in healthy Iraqi population. Same incidence with AML patients occurred, the genotype frequency were (48%) for heterozygous CT and (56%) for mutant-T allele and no significant difference between the two groups. Many studies agreed with our finding with similar proportion of genotype and allele frequency in healthy control and AML patients. Rao *et al*., (2010) showed that the frequency in Indian healthy were 51.7% for CT and 55 for T allele, which closed to AML patients values (51% CT and 52% T allele). In healthy Iranian population, Miladpour *et al*., (2009) found similar results to our results in CT genotype frequency but with non-significant for T and C allele frequency. Also Urayama *et al*. (2007) in their

study on healthy non-Hispanic White population found (48.2%) of people had CT and (27.1 %, 24.7%) for CC and TT respectively. Highly frequency with CT showed by Miladpour *et al*. (2010) in Iranian population compared with homozygous genotype. They reported that (71.9%) of healthy people had CT genotype with mutant-T allele frequency 51.8%. On the other hand, in German and Portuguese Caucasian these percentage ranging from 42% to 57% (Ameyaw *et al*., 2001; Cascorbi *et al*., 2001; Siegsmund *et al*., 2002). Many other studies reported similar genotype frequency with heterozygous CT but related with wild type-C (Hoffmeyer *et al*., 2000; Ameyaw *et al*., 2001; Tanabe *et al*., 2001; Mhaidat *et al*., 2011). Our finding revealed that C3435T MDR1 SNP generally common and genetically stable with heterozygous CT genotype, but allele frequency distribution showed significant ethnic variation. These variations in allele frequency made some population susceptible to diseases while other not. The presence mutant-T with CT genotype may be increased risk of susceptibility for diseases to half, because the mutant-T allele more disposed to environmental toxins and carcinogens. The individuals who have a TT haplotype for 3 common MDR1 SNPs (C3435T, C1236T and G2677T) are with more risk to developing diseases and cancer (Mhaidat *et al*., 2011). The similarities between incidence distribution of AML patients and controls in genotype which related with non-significant difference to estimate the risk of AML developing associated with MDR1 C3435T genotype carriers. Our results are in agreement with Miladpour *et al*. (2009) who found that MDR1 X26 C3435T SNP has no significant effect with incidence of diseases or cancers. Although no significant differences in MDR1 C3435T with risk of developing AML was detected in the current study. The degree of MDR1gene expression and activity are significantly different in individuals with variety constitutional genotypes of the MDR1 (Hoffmeyer *et al*., 2000). Our genetic and expression data analysis showed that the healthy individuals with CC genotype MDR1 C3435T had significantly higher levels MDR1 gene expression than individuals with CT heterozygous and TT homozygous. This finding indicated that the individual who had CC genotype was more protective than those with CT and TT which due to high expression of MDR1 in normal cells that decrease the risk of accumulation intracellular concentration of toxins. While the low expression of MDR1 leading to increase DNA damage and Cytogenetic aberrations that considered important prognostic factors in AML. These results are in agreement with the results detected by Fellay *et al*. (2002) who found that CC genotype had high significant level of MDR1 gene expression with MDR1 C3435T than TT homozygous or CT heterozygous. However, AML patients showed low level of MDR1 expression for MDR1 C3435T CC carriers compare with 3435CT/TT genotype. Furthermore, the significant associated of MDR1 3435CT/TT genotypes with non-responding AML give an interesting finding that these genotypes were associated with non-responding outcome.

Our finding is agreed with Illmer *et al.* (2002) who found that the genotype-phenotype association shown a clear correlation between the homozygote *CC* variant(s) and a lower MDR1 expression in blast samples and Kim *et al.* (2014) showed MDR1 wild-type genotype may be associated with a more favorable response to therapy. In corroborate with Illmer 2002, study were obtained by Vivona *et al*. (2014) that showed a tendency to higher MDR1 expression rates in heterozygotes. Other studies indicate that C allele with high expression in acute leukemia might eliminating the antileukemic drugs (Anthracyclines, Daunorubicin and Vincristeine) (Roa *et al*., 2010). Doxani *et al*. (2010) reported that gene variants (C3435T, G2677A and C1236T) were not associated with response to treatment, but there is a potential marker for response to chemotherapy in AML patients. SNPs in ABC drug-efflux pumps may play a role in responses to drug therapy and diseases susceptibility. The effect of various genotype and haplotypes on the expression and function of these proteins is not clear, and their true remains controversial (Sharom 2003). In conclusion healthy Iraq populations and AML patients have predominantly CT genotype and mutant-T allele frequency for MDR1 C3435T polymorphism. MDR1 3435CT/TT genotype in regard with MDR1gene expression in de novo AML patients associated with poor prognosis, while CC genotype was protective Carrier.

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