



Effect of *MDR1* Gene Expression Related with C1236T Polymorphism in Iraqi Acute Myeloid Leukemia patients

Abdul Hussein M. AL-Faisal¹

Kifah Jabbar Alyaqubi²

¹Molecular Oncology Unit, Guys Hospital, Kings College, London, UK .

²Alfurat Alawast Cancer Unit, College of Medicine, Kufa University, Najaf, Iraq.

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 acute myeloid leukemia (AML) patients to detect the susceptibility of C1236T genotype carrier to develop AML. Also the study aimed to correlate the expression level of *MDR1* mRNA with *MDR1* C1236T polymorphism in newly diagnosed AML patients to predict clinical outcomes at initial diagnosis. The results showed there was a significant difference in genotype and allele frequency with heterozygous CT in AML while appeared significant with CC wild type in healthy control. Both AML and control showed non-significant in allele frequency. In regard with gene expression the healthy control showed significantly high level of *MDR1* mRNAs expression in CC genotype at position 1236 compared with CT and TT. Whereas *MDR1* heterozygous 1236CT genotype was showed highly significant difference in *MDR1* mRNA expression among AML patients. According to the clinical outcome status *MDR1* C1236T genotype showed statistically high significant differences with low level of CC genotype compared to CT/TT genotype at initial diagnosis in NR patients, while CR group was showed non-significantly with *MDR1* C1236T polymorphism. In conclusion healthy Iraq populations have predominantly CC genotype and appeared a protective genotype, while *MDR1* 1236CT/TT genotype were indicated affected genotype and associated with poor prognosis in de novo AML patients, while CC appeared good prognosis.

Key words: C1236T, *MDR1*, AML, Genotype, SNP, Iraq.

Corresponding address: Prof.Dr. Abdul Hussein M. AlFaisal, alfais2013@yahoo.com.

تأثير تعبير جين المقاومة المتعددة *MDR1* وعلاقته مع تعدد طرز الموقع C1236T في مرضى ابيضاض الدم النخاعي الحاد العراقيون

كفاح جبار اليعقوبي²

عبد الحسين مويث الفيصل¹

¹معهد الهندسة الوراثية والتقانات الأحيائية للدراسات العليا-جامعة بغداد

²كلية الطب - جامعة الكوفة

الخلاصة: ان تعدد طرز النيوكليوتيدة المفردة SNP في جين المقاومة المتعددة *MDR1* قد يؤدي الى تغيير تعبير الجين *MDR1* مما له تأثير على الاستجابة للعلاج الكيميائي وعلى حساسية المرض. أن هدف هذه الدراسة هو تحديد تكرار اليلات الجين في مرضى ابيضاض الدم النخاعي الحاد العراقيون ولمعرفة حساسية الطراز الوراثي للموقع C1236T وعلاقته بالإصابة بالمرض. كما هدفت الدراسة الى ايجاد العلاقة بين مستوى تعبير جين المقاومة المتعددة و طرزه الوراثية في المرضى المشخصين حديثا للاستفادة منها في التشخيص والتوقع الطبي للمرض. بينت الدراسة بوجود اختلاف معنوي في المظاهر الوراثية و تكرار اليلات مع الزيجة المغايرة للطراز الوراثي *MDR1* C1236CT في المرضى مقارنة مع معنويه في الطراز الوراثي *MDR1* C1236CC في الاصحاء. كما بينت النتائج بأن كلا المرضى والاصحاء بينوا عدم معنوية تكرار اليلات. بينما كان تعبير الجين *MDR1* معنوي عالي في طرازه الوراثي *MDR1* C1236CC مقارنة مع طرزه الوراثية *MDR1* C1236CT و *MDR1* C1236TT عند الاصحاء بينما كان المظهر الوراثي مغاير الزيجة *MDR1* C1236CT عالي المعنوية عند المرضى. واستنادا للتوقع الطبي للمرض فأن الطراز الوراثي *MDR1* C1236CT كان مختلف معنويا وعاليا مع انخفاض الطرز الوراثية *MDR1* C1236CC و *MDR1* C1236CC\TT عند التشخيص الاول للمرض. بينما كانت مجموعة ذات تعدد طرز وراثية غير معنوي. وكخلاصة فأن تكرار الطراز الوراثي *MDR1* C1236CC شائع لدى الاصحاء العراقيون ويبدو انه مرتبط مع الحماية بينما تمثل الطرز *MDR1* C1236CT\TT الطرز المتأثرة والتي ترتبط مع تشخيص ضعيف للمرض مقارنة مع الطراز *MDR1* C1236CC الذي يمثل طراز جيد لتشخيص المرض.

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 (Green et al., 2012). 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, thereby 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). However, rs1128503 (1236T>C) appears larger interethnic allele frequency differences, ranging from 30 to 93% depending upon the ethnic population, with C being the minor allele in Asians, and T being the minor allele in Africans (Hodges et al., 2011). Here, we focused on MDR1 expression with C1236T variant among Iraq population affected with AML because several studies have shown effect of this variant in acute leukemia outcomes (Illmer et al., 2002; Urayama et al., 2007; Green et al., 2012).

Patients and Methods

Peripheral blood samples were collected from 31 de novo AML patients, were provided by major hospital in Iraq (hematology unit of Baghdad Teaching Hospital and DAR-ALTamred Privet Hospital in medical city), and 10 healthy donors for MDR1 investigation. The mean of blast cells in bone marrow and peripheral blood was 77.7% and 67% respectively. There

were 15 males and 16 females, (1:1.1) male to female ratio for de novo AML patients and (M:F-1:1) male to female ratio for control. Patients and healthy subjects were equally distributed in respect to gender. The mean age of the patients was 36.8 ± 15.99 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) 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). 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 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

Total RNA (15 μl) reverse transcription to cDNA was achieved with random primers using High Capacity cDNA Reverse Transcription Kit, Applied Biosystem. After initial denaturation of RNA at 65°C 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'-TGGAGATAACACTCTAAGCATAA CTAAAGGT-3' ABL reversed 5'-GATGTAGTTGCTTGGGACCCA-3' ABL probe 5'-CCATTTTGGTTTGGGCTTCACAC-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). PCR products were detected using a 5' FAM (6-carboxy-fluorescein) 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 °C 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 $\Delta\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{untreated}}$ for calibration, and normalized by $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{endogenous reference}}$ (Bustin et al., 2009, Bolha, et al., 2012).

DNA Extraction and Genotyping

Interphase layer of 41 blood samples with TRIzol (31 patients and 10 controls) were isolated for genomic DNA extraction with QIAamp DNA Mini Kit (Qiagen, UK) which was designed for purifying genomic DNA. MDR1 C1236T 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'.

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.

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 C1236T 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 frequency with wild type CC (40%: $p=0.046^* < 0.05$) and heterozygous CT (54.83%: $p=0.0028^{**} < 0.01$) in healthy and AML patients respectively (Table 1). There was no significant difference in allele frequency in both healthy control and AML patients (Table 1).

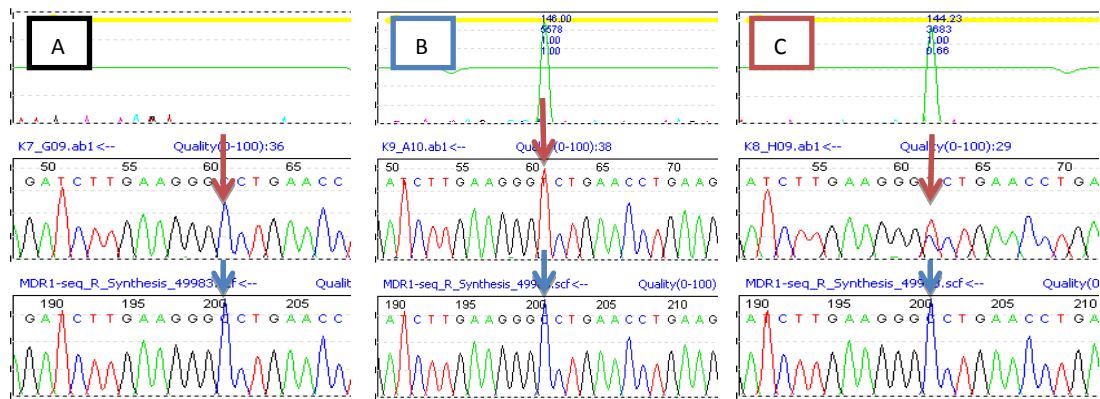


Figure 1: Electrograph show DNA sequencing for (A) wild type C1236T wt/wt(C/C) (B) homozygous mt/mt (T/T) (C) heterozygous wt/mt (C/T). Upper arrow represented sample and lower arrow reference MDR1 (wild type)

Table 1: Genotype and Allele Frequency of MDR1 C1236T Polymorphism among Healthy and AML Patients

Genotype C1236T	Control n=10	AML n=31
CC	4(40)	6(19.35)
CT	3(30)	17(54.83)
TT	3(30)	8(25.8)
P-value(χ^2)	0.046*(4.681)	0.0028**(10.418)
Allele Frequency		
C	11(55)	29(46.77)
T	9(45)	33(53.22)
P-value(χ^2)	0.046*(4.048)	0.319 NS
The results expressed as n(%) * (P<0.05), ** (P<0.01), NS (no significant)		

Odds ratio and their 95% confidence interval (ORs and 95% CIs) analysis was used to detect the MDR1 C1236T genotype risk factors carriers to developing AML between populations. Odds ratio test listed in (Table 2) revealed that the AML patients observed increased risk to developing AML related with 1236CT carriers in

2.15 fold than TT carriers (ORs: 2.15; 95% CI 0.15-29.93). Odds ratio analysis indicates that the MDR1 1236CT genotype is possible to be a risk factor for the development of AML, but we suggest increasing the samples size to obtain more accuracy in confidence interval.

Table 2: Analysis of MDR1C1236T Genotype Risk Factors of AML and Control

Genotype	AML Cases	Controls	Odd Ratios	ORs (95%CI)
CC	6(19.35)	4(40)	CC vs CT	0.26 (0.002-28.26)
CT	17(54.83)	3(30)	CT vs TT	2.15 (0.15-29.93)
TT	8(25.8)	3(30)	CC vs TT	0.56 (0.074-4.245)

MDR1 C1236T Genotype-Phenotype Association

In order to investigate the susceptibility of MDR1 phenotype-dependent C1236T genotype to developing acute leukemia, mean fold change of MDR1 gene expression in controls and acute leukemia patients at initial diagnosis was categorized according to MDR1 C1236T SNPs. The statistical analysis showed in (Table 3) indicate that the MDR1 1236CC genotype was significantly express among healthy controls (1.47 ± 0.04 ; $p=0.033$) compared to CT and TT (0.88

± 0.02 and 0.86 ± 0.02) respectively, as well as showed significant differences with high genotype frequency (40%: $p=0.046^*$). In regard to acute leukemia types, AML patients showed highly significant differences ($p=0.0139^{**}$) between the mean fold change of MDR1 gene expression and MDR1 C1236T SNPs. The MDR1 1236CT showed high mean fold change (2.1 ± 0.03) compared to CC and TT (0.32 ± 0.02 and 1.71 ± 0.02) respectively, moreover MDR1 1236CT showed high significant differences in genotype frequency (54.83%: $p=0.0028^{**}$).

Table 3: MDR1 C1236T Genotype-Phenotype Association in Control and AML Patients

Genotype C1236T	Control Frequency n=10	Control MDR1 Expression	AML Frequency n=31	AML MDR1 Expression
CC	4(40)	1.47 ± 0.04	6(19.35)	0.32 ± 0.02
CT	3(30)	0.88 ± 0.02	17(54.83)	2.1 ± 0.03
TT	3(30)	0.86 ± 0.02	8(25.8)	1.71 ± 0.02
p-value	0.046 *	0.033*	0.0028 **	0.0139 **

MDR1 C1236T Genotype-Phenotype Association with Clinical Outcomes of AML

Results listed in (Table 4) showed out of 31 AML newly diagnosed patients, 17(54.83%) of them were NR indicated high significant differences between MDR1 fold change and MDR1 C1236T genotype ($p=0.013^{**}$). The low mean fold change of MDR1 was appeared with 1236CC genotype (0.45 ± 0.02), compare to high mean fold of MDR1 1236CT and TT (3.32 ± 0.11 and 3.01 ± 0.08) respectively, at presentation. In contrast,

14(45.16%) of AML CR patients did not show a significant influence between MDR1 gene expression and MDR1 1236 variants at presentation. We can conclude that the MDR1 1236CT/TT genotypes probably contribute to the MDR1 mediated drug resistant phenotype in AML patients due to significantly occurred at presentation with high MDR1 fold change in NR patients. While MDR1 1236CC genotype appeared good prognosis with AML treatment outcomes due to showed low level of MDR1 gene expression in NR and CR patients.

Table 4: Relationship between MDR1 Gene Expression and MDR1 C1236T Genotype with AML Clinical Outcomes

Genotype C1236T	MDR1 Fold Change of NR AML	MDR1 Fold Change of CR AML
	n=17	n=14
CC	0.45 ± 0.02 (3)	0.37 ± 0.02 (3)
CT	3.32 ± 0.11 (10)	0.30 ± 0.02 (7)
TT	3.01 ± 0.08 (4)	0.41 ± 0.01 (4)
p-value	0.013 **	0.317 NS

Discussion

The development of multidrug resistance during cancer chemotherapy is one of the major obstacles to successful treatment of AML clinically. 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 association 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). It is observed that healthy population in Iraq was significant with CC genotype frequency in the position 1236 MDR1 SNPs. Other populations such Turkish, Serbian, Russian, French, German, and Jordanian showed CT was with a high

frequency compared to CC and TT (Gümüş-Akay et al., 2008; Milojkovic et al., 2011; Goreva et al., 2004; Jeannesson et al., 2007; Cascorbi et al., 2001; Khabour et al., 2013). Some population showed TT was more frequent than CC and CT such as Indian, Chains (Chowbay et al., 2003, Yu et al., 2011). Few studies have agreed with the high frequency of 1236CC wild type which was detected by the current work was also detected in Poland and Istanbul populations, (Dudarewicz et al., 2013; Karatoprak et al., 2013). As regards AML the significant frequency of MDR1 1236CT genotype among AML patients was reported by (Illmer et al., 2002) who reported 131 case (32.3%) MDR1 1236CT among AML patients compared to CC and TT, 92(22.7%) and 57(14.1%) respectively. Green et al., (2012) also found the 1236CT high frequency in AML patients but non-significantly with control. The differences of MDR1 gene polymorphism between populations may be attributed to the increased incidence of cancer in population than other.

Our genetic and expression data analysis showed that the healthy individuals with CC genotype MDR1 C1236T 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. Kim et al., (2014) found that the wild type of MDR1 gene at position C1236T, G2677T/A, and C3435T was a protective genotype. In contrast heterozygous mutant genotype was associated with the risk for developing cancer. Through many studies related to risk factors associated to developing diseases with MDR1 C1236T polymorphism, (Dudarewicz et al., 2013) it has been shown the 1236CT/TT genotypes have a higher predisposition to develop the diseases. In a study by (Hemauer et al., 2010), it was indicated that in human placenta the MDR1 1236CT/TT were related to decrease P-GP expression in comparison with wild type 1236CC genotype. This suggest that MDR1 1236CT/TT genotypes were more risk to developing cancer than wild type the fact that low expression of MDR1 protein lead to accumulation of a variety of natural cytotoxic drugs and toxic xenobiotics inside the cells (Gervasini et al., 2006).

However, AML patients showed low level of MDR1 expression for MDR1 C1236T CC carriers compare with 1236CT/TT genotype. Furthermore, the high expression of MDR1 1236CT/TT genotypes were associated with non-responding AML give an interesting finding that these

genotypes were poorer outcomes while 1236CC showed favorable response. (Illmer et al., 2002) was agreed with our finding, they found 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. 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 have predominantly CC genotype and appeared a protective genotype, while MDR1 1236CT/TT genotype were indicated affected genotype and associated with poor prognosis in de novo AML patients while CC appeared good prognosis.

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