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RESEARCH ARTICLE

Assessment of Multidrug Resistance Gene (MDR1) Expression in Iraqi Acute Myeloid Leukemic patients

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Abstract

MDR1gene expression upregulation has been shown to be associated with resistance to chemotherapy treatment in acute leukaemia. The aim of this study is to investigate the levels of MDR1 transcript by RTqPCR at different phases of AML, and correlate these levels with patients' response to chemotherapy treatment. We investigated mRNA levels of MDR1 in 31 newly diagnosis patients at presentation, 1st induction, 2nd induction and consolidation. The mean fold of 10 healthy voluntary blood controls was defined as cutoff value (1.1±0.03), with samples showing higher levels considered positive while those showing lower levels considered negative for MDR1 expression. At presentation, 6/31 (19.4%) and 25/31 (80.6%) were positive and negative for MDR1 expression respectively. After first induction 9/15 (60%) were MDR1 positive. 4/8 (50%) of patients tested positive for MDR1 upregulation after 2nd induction. After consolidation 1/3 (33.3%) showed upregulation of MDR1 transcription. Statistical analysis showed significant effect of mean fold for MDR1 expression in non-responder (NR) AML patients at presentation (2.74 fold ±0.12, $P<0.01$) compared to (0.34 fold ±0.02; 1.1 fold ±0.03) for complete remission (CR) and control respectively. However, there were no significant differences between CR and NR ($p=0.768$) after 1st induction. Early relapse cases showed highly significant effect for MDR1 expression in consolidation as compared to CR patients (1.66±0.05, $p=0.033$). There was progressively increased MDR1 expression in individual patients after 1st induction. Uneven distribution of MDR1 expression among FAB AML subtypes was detected, with highest level MDR1 expression detected in M2. In Conclusion our findings suggest that detection of positive MDR1 expression in newly diagnosis AML patients was associated with poor clinical outcome and with M2 subtype. MDR1 positivity in AML patients with CR at consolidation was associated with early relapse.

INTRODUCTION

Acute myeloid leukemia (AML) is clinically, cytogenetically and molecularly a heterogeneous disease (Zou, 2007). High risk AML constitutes a biologically distinct subset of diseases Comprised sizeable percentage with adults (Estey,2012). Unfortunately, Iraq continues to be the largest contributor to cancer related mortality (Ministry of health, Iraqi cancer Board, 2012). Resent study in Iraq indicate that genetic alteration with leukemia caused by

polluted environment (AlFaisal *et al.,* 2014). Due to the lack of any nationwide leukemia screening program, the majority of the population of Iraq is still unaware of this blood disorder. Lack of awareness also plays a role in underlying late presentation and noncompliance with screening guidelines. Until recently, the morphologic classification was according to the French-American-British group which distinguishes AML into distinct subtypes (Yan *et al.,* 2013). Leukemia was the third most common cancer in Iraq accounting for 7% of all cancers (Saleh *et al.,* 2009). Greater part of patients with AML achieve complete remission (CR) after standard induction chemotherapy with an anthracycline as doxorubicin (Adriamycin) and cytarabine (3+7), most will relapse (Elliott *et al.,* 2007). Patients when treated with such above regimens, which are modulated in their intracellular retention by the p- glycoprotein 170(p-gp), encoded by the human multidrug-resistance (MDR1/ABCB1) gene (Baguley, 2010). In addition to reduction of intracellular drug concentration, redistributions of the drug from the nucleus to the cytoplasm have been associated with overexpression of MDR1 of cancer cells (Larsen *et al*., 2000). Many authors observed one of the major mechanisms for drug resistance, in vitro and in vivo, is associated with altered the ability of P-glycoprotein to recognize target substrates (Shen *et al*., 1986; Mahjoubi *et al.,* 2008). Recent study showed there is indication that MDR1 mRNA expression may be considered as a potential marker for response to chemotherapy in AML patients (Doxani *et al.,* 2013). Our aim was to analysis MDR1 gene expression at transcriptional level during follow up patients and connected with clinical outcomes among Iraqi acute myeloid leukemic patients. The relative expression levels were investigated by quantitative real-time reverse transcription-PCR (qRT-PCR) and then studied in relation to the type of response to chemotherapy.

Patients and Methods

Peripheral blood samples were collected from 31 newly diagnosed AML patients, were provided by the major hospitals in Iraq (Hematology Unit of Baghdad Teaching Hospital and DAR-ALTamrredh 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 year (rang, 16-72). 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 non-responder (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 (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 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 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) (Table 1). 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-flurescein) reporter dye and a 3' TAMRA (6 carboxy-tetramethylrodamine) quencher dye for all reactions.

Plate Setup

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.

Standard Curve and Constructs Preparation

MDR1 construct was synthesised using primers shown in table 2. PCR Parameters were: one cycle at 95C° for 10 min. for enzyme activation, 50 cycles of 95C° for 15sec., 55C° for 20sec., and 72C° for 30 sec for denaturation, annealing and extension respectively, followed by final extension at 72C° for 7 min.

Data Analysis

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.

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. Least significant difference –LSD test was used to significant compare between means in this study.

Results

Calibration curve of real-time quantitative RT-PCR

The calibration curve showed a strong correlation between RNA input amounts and Ct for ABL and MDR1 genes respectively (R^2= 0.999; R^2=0.999) Fig.2-A and Fig.2-B. The efficiency of the MDR1 gene amplification and ABL gene amplification showed similar values for MDR1 and ABL (1.97; 1.98) respectively.

Table 1.Primers and Probes Sequences

Primer	Sequence	Melting Tm
MDR1- F	5'-TGCTCAGACAGGATGTGAGTTG-3'	49.7C°
MDR1- R	5'-TTACAGCAAGCCTGGAACCTAT-3'	47.9C°
MDR1- P	5'-AGCATTGACTACCAGGCTCGC-3'	54C°
ABL-F	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'	49.9 Cº
ABL-R	5'- GATGTAGTTGCTTGGGACCCA-3'	47.3 Cº
ABL-P	5'-CCATTTTTGGTTTGGGCTTCACACCATT-3'	52.5 Cº

Table 2.Sequences of Primers Constructs

Primer	Sequences	Melting Tm
MDR1 CF-Construct F	5'-ATTTCTTTTATTACATTTTTCCTTCAG-3'	44C°
MDR1 CR-Construct R	5'-ATTGCTTCAGTAGCGATCTTCC-3'	47.9C°

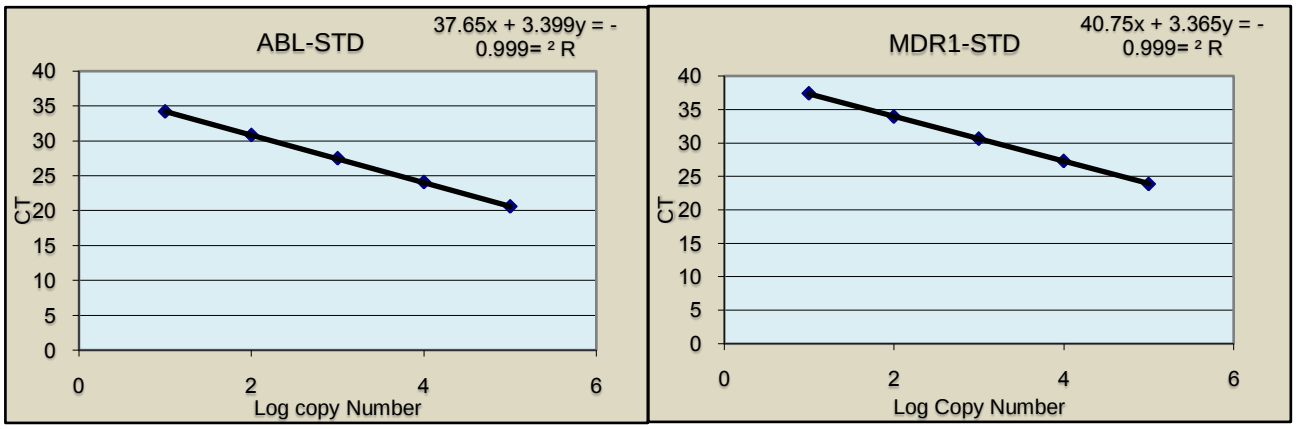

Figure 2: STD of ABL gene and MDR1 gene

Expression of MDR1 in Control Samples

The amplification accuracy of MDR1 and ABL product was shown by identical or very close Cts for the duplicate reactions for both transcripts. The level of MDR1 expression estimated in healthy blood cells was used as the basis of expression values. We defined the cut-off for MDR1 as (1.1 fold ± 0.03; mean±SE), and the samples above this cut-off value were considered positive.

Expressions of MDR1 and Correlation with Response

The expression of MDR1 gene in serial samples was analyzed before chemotherapy (at presentation) and after 3 courses of treatments for the same patients according to clinical outcome. The 4 groups categorized as Group A consisted of 31 patients at presentation; Group B included 15 patients after first induction; Group C comprised 8 patients after second induction and Group D included 3 patients in consolidation. Overexpression of MDR1 gene occurs in 4(66.7 %) of patients > 40 years, compared to only 2(33.3 %) of younger patients < 40 years (Table 3). Out of the 31 AML patients in group A, 19.4% (6/31) of them were MDR1 positives and 80.6% (25/31) of them were negative. All of positive MDR1 was showed NR to first induction, while 45.1% (14/25) of negative MDR1 showed CR and 35.5% (11/25) was NR (Table 4).

Table 3: Age Analysis with MDR1 Overexpression

Age	MDR1 overexpression
>40	2(33.3%)
<40	4(66.7%)
Results represent number (%)	

Table 4: Distribution of Negative to Positive Ratio of MDR1 Expression According to Cut-off Value Related with Responses

AML Clinical Outcome	**Group A n=31**		**Group B n=15**		**Group C n=8**		**Group D n=3**	
	-	+	-	+	-	+	-	+
	n=25(80.6)	n=6(19.4)	n=6(40)	n=9(60)	n=4(50)	n=4(50)	n=2(66.66)	n=1(33.33)
NR	11(35.5)	6(19.4)	4(26.7)	4(26.7)	2(25)	1(12.5)	1(33.33)	--
CR	14(45.1)	--	2(13.3)	5(33.3)	2(25)	3(37.5)	1(33.33)	ER 1(33.33)
Results represent n= number (%)								

This investigation showed that MDR1 gene was expressed in non-responding AML patients at a significantly higher mean fold (2.74±0.12, mean± SE; p=0.0037<0.01) compared to patients who achieved complete remission (CR) and control (0.34 fold ±0.02; 1.1 fold ±0.03, mean± SE) respectively (Fig. 4)

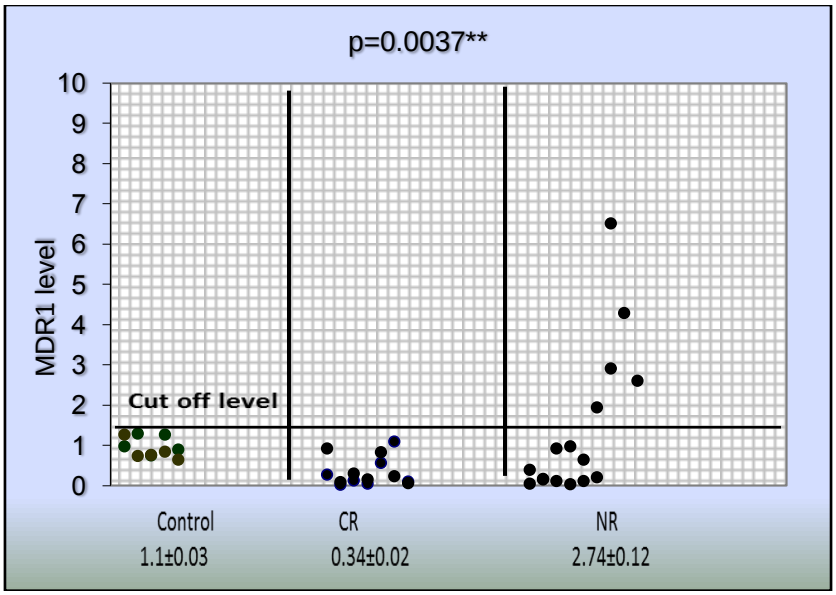

Figure 4: Level Expression of MDR1 in Control; CR and NR Groups at presentation

Out of 31 AML patients in Group A, 15 of them were following up as Group B. High positivity 60% (9/15) for MDR1 was detected after completion of one course of treatment as compared to levels at presentation. The percentage of positive MDR1 expression among CR patients increased to 33.3%, as compared to group A (Table.4). Follow up samples (after first induction) from CR patients showed higher mean fold of MDR1 gene as compared to levels at presentation (1.42 ±0.04). However, there was no statistically significant difference between levels of MDR1 transcript in CR and NR patients (p=0.783) in the same group (Table.5). Third group (group C) consisted of 8 patients were samples taken after 2 courses of treatments was completed. Results from group C showed that MDR1 expression to have no statistically significant effect on patient's outcome (p=0.316) (Table.5), and equal number of positive and negative samples (Table. 4). The last group (group D) consisted of 3 patients who received consolidation treatment. RT-qPCR results showed that positivity of MDR1 expression in this group is associated with early relapse. Statistical analysis showed significant differences between early relapse ER patient (1.66±0.05 p=0.033<0.05) and those who NR (0.5±0.01) in the same group (Table .5)

Our results observed the mean fold of MDR1 gene expression at diagnosis to be significantly higher in NR patients as compared to CR patients. However, MDR1 mean level dropped in NR patients as the treatment progressed (2.74-1.26-0.74-0.56) while mean levels in CR patients increased as the treatment progressed (0.34-1.42-1.01-1.66). In fact MDR1 mean level after consolidation was higher in CR patients than in NR ones (table 5). The number of samples tested at this phase was small, and this finding requires further investigation on a large cohort of patients to verify it.

Table 5: Expression of MDR1 Gene in Acute Myeloid Leukemia in Follow up Samples

Clinical outcome		Mean ± SE				P-value	LSD value
		Group A	Group B	Group C	Group D		
AML	NR	2.74 ± 0.12	1.26 ± 0.03	0.74 ± 0.02	0.56 ± 0.01	0.0027 **	0.577
	CR	0.34 ± 0.02	1.42 ± 0.04	1.01 ± 0.02	1.66 ± 0.05	0.026 *	0.491
P-value	---	0.0128 **	0.783 NS	0.316 NS	0.033 *	----	
*Results represent mean ± SE, *(P<0.05),**(P<0.01) and NS non-significant*							

Alterations of MDR1 Fold Change during Follow up

Out of 31 of AML patients, 15 were analyzed individually over the duration of treatment. Results show variable level for MDR1 fold change individually through 4 follow up study groups with respect to response and AML subtype. Most of AML patients showed progressively increased levels of MDR1 after first induction (Fig. 5)

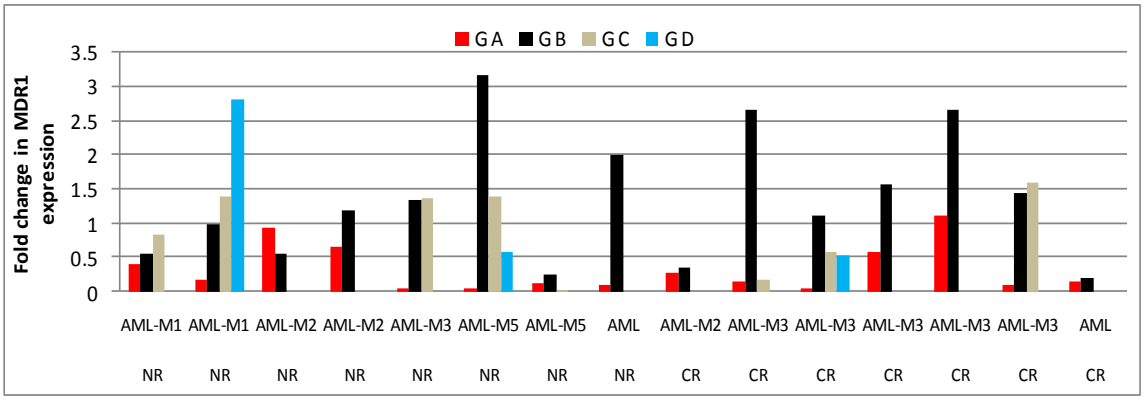

Figure 5: Alterations of MDR1 Fold Change during Follow up study

Expression of MDR1 and Relation with FAB Subtype

The study of relationship between MDR1 gene expression and AML FAB subtypes showed MDR1 levels to be significantly higher in M2 at presentation (3.81±0.12, p=0.00317 <0.01) as compared to other subtypes (Table 6). After the first induction mean level of MDR1 was a raised significantly with M3 and M5 subtype (1.792±0.05 and 1.7±0.02, p=0.0417<0.05) respectively, but regressed with M2 and un-classified AML (0.683±0.02 and 0.642±0.01) respectively. Patients with C group were showed significant decreasing mean fold (0.705±0.02, p=0.0488<0.05) with M5 and constantly with M1; M3 (1.095; 1.075) respectively. Our analysis reported one early relapse case in post-remission consolidation that significantly higher fold MDR1 gene expression in M1 subtype (2.806±0.02, p=0.0147<0.01) compare with very low mean fold of MDR1 in M3 and M5(0.508±0.02 and 0.563±0.01) respectively (Table 6).

Table 5: The relationship between MDR1 expression and FAB AML subtypes

Subtype	Mean ± SE				P-value	LSD
	A	B	C	D		
M1	0.275 ±0.03	0.759 ± 0.05	1.095±0.02	2.806±0.02	0.0316 *	0.683
M2	3.871 ±0.12	0.683 ± 0.02	---	---	0.0052 **	0.802
M3	0.352 ±0.02	1.792 ± 0.05	1.075±0.04	0.508±0.02	0.0271 *	0.426
M5	0.071±0.004	1.700 ± 0.02	0.705±0.02	0.563±0.01	0.0266 *	0.673
AML-undiagnosed	1.408 ± 0.02	0.648 ± 0.01	--	---	0.0419 *	0.392
P- value	0.00317 **	0.0417 *	0.0488 *	0.0147 **	---	---
Results represent mean ± SE, *(P<0.05),**(P<0.01) and NS non-significant						

Discussion

The goal of AML treatment is to achieve complete remission and then to prevent relapse in post-remission therapy. Unfortunately, chemotherapy treatment is ineffective in poor risk patients for whom bone marrow transplantation is more effective. In the last few years, there have developing changing in the diagnosis and treatment of AML based on molecular genetic assessment of MDR1 expression for designing novel curative regimens that reversing regulation of drug resistant phenotype of AML cells (Cianfriglia, 2013). Response to treatments is affected by many factors according to risk categories associated with morphological features, genetic criteria and age (Buchner and Heinecke, 1996; Kern *et al*., 2000; Ismail and Hosny, 2011; Yanada and Naoe, 2012). Evidence accumulated shows MDR1 gene expression to be a poor prognostic marker in AML patients (Estey *et al.*, 2000; van den Heuvel-Eibrink, 2000; Ismail and Hosny, 2011; Yanada and Naoe, 2012). Our study findings agreed with previous studies and suggest that molecular assessment of MDR1 gene expression at presentation provide important prognostic determination for AML patient's response to chemotherapy. Huh *et al.*, (2006) reported 23.1 %(9/39) positive and 76.9%(30/39) negative, for MDR1 expression. They classified negative as (0), positive as (weakly 0-0.07=+1; moderately 0.7-1=+2; strongly >1=+3). Fujimaki *et al.*, (2002) determined positive MDR1 expression according to determined cut-off point in BM (0.0054) and observed 37.5% (6/16) of MDR1 expressed higher than cut-off point. In current study we found 19.4% (6/31) of MDR1 expression was positive and 80.6% (25/31) negative based on the

cut-off value of mean fold MDR1 expression in healthy blood (1.1 fold ± 0.03). These differences in ratio of positive patients detected by these studies could be due to the cut-off point value or the way that positive MDR1 expression was calculated. Xu *et al*., (1999) estimated normal MDR1 mRNA level in mononuclear cells from nine healthy volunteers were (0.2 range 0.01-0.5). Giraud *et al.,* (2009) showed that NK cells in adults had high level ratio (3.31 ± 0.74), and significantly different from those of T and B lymphocytes, which have levels (1.48 ± 0.19 and 1.78 ±0.38, respectively). Therefore, the determination of cut-off value is very important for estimation MDR1 expression. Statistical analysis indicate all positive MDR1 19.4% were non-responding for induction chemotherapy and showing high level of MDR1 (2.74 fold ±0.12, p=0.0128 <0.01), while 80.6% were (0.341 vs 0.316) negative was showed low level MDR1 with 14/25 negative patients achieving CR. Then mean level of MDR1 in negative patients who achieved CR was slightly, but not significantly, lower than in those who did not achieve CR (0.316 vs 0.341). Our finding is in agreement with Fujimaki *et al*., (2002) and Huh *et al* (2006) who showed both AML and ALL patients with high MDR1 expression at presentation have low remission rate. Such conclusion was also reported by others (Karaszi *et al.,* 2001; Schaich *et al*., 2001). Recently the investigation of the mRNA expression above cut-off points for panel of MDR genes among acute leukemia patients showed that MDR1 was arise risk in patients (Rahgozar *et al*., 2014). Our result showed all patients with MDR1 range (6-24) fold and age >50 died before first induction. Investigators have considered leukemia as intrinsic resistant disease to chemotherapy occurred with older AML (Do *et al*., 2007; Yanada and Naoe, 2012). Our data support such idea. Data analysis during follow up study with group B showed up regulation with mean fold MDR1 expression in most patients, especially in those who showed response to chemotherapy. These finding correlate with the concept of AML being an intrinsically resistant disease, and that such upregulation could be acquired during induction treatment (Longley and Johnston, 2005). Up regulation of MDR1 after induction has been reported by others. Baran *et al.,* (2007) showed in vitro study, the MDR1 and MRP1 (drug resistance proteins) mediated in multidrug resistance of human leukemic cells through the mechanism of resistance to doxorubicin-induce cell death in human HL60 AML cells, they indicated the continuous exposure of leukemic cells to stepwise increasing concentration of doxorubicin resulted in the selection of HL60/DOX cells, which expressed about 10.7 fold resistance as compared to parental sensitive. Furthermore, Tallman and Altman (2009) showed evaluation of molecular response after initial induction is not useful because many patients remain complete remission because of delayed maturation of the leukemic cells, and there is no prognostic value in the result. In contrast, molecular remission after consolidation has important prognostic value. Cornelissen and colleagues 2007 revealed almost 80% of AML patients (18-60 years) will achieve complete remission (CR). But approximately 50% of these patients will experience a relapse. This implies that despite CR, in these patients a number of cancer cells survive treatment and can grow out to cause a relapse. Solali *et al.,* (2013) observed the increased MDR1 expression after induction may cause early relapse and progression disease. These studies agreed with our finding that revealed in one case showed complete remission after induction then relapsed occurred after consolidation. The heterogeneic distribution of MDR1 expression among FAB AML subtype was reported by many studies. Paietta *et al.,* (1994) conducted low level of MDR1 expression in M3 were assessed in 11 AML-M3 patients at the molecular level and functional level in comparison to 48 non-M3 cases. The MDR1-specific transcript levels, determined by qRT-PCR, were significantly lower in mononuclear cells from M3 than the other AML cases (p=0.013). Steinbach *et al.,* (2003) who reported that expression in 58 children with de novo AML was at high level in M1 and M2 (Kruskal–Wallis test P = 0.004) and at low level in M4 and M5 at diagnosis. (Yang *et al*., 2012) was corroborated with Steinbach that MDR1 was highly increased FAB M1 and M2 types of AML and in B-precursor ALL also. On the other hand no association between MDR1 phenotype and AML FAB classification was reported by Nikougoftar *et al*., (2003). In our study, MDR1 expression was expressed unevenly among FAB subtypes. Our results showed mean fold MDR1 expression in M2 subtype of (3.871 ±0.12) which is highly significant from the low levels detected in M1, M3 and M5 (p= 0.00317 <0.01). After first induction, mean fold of MDR1 gene expression increased in M3 and M5, but decreased in M2. The result showed that all FAB subtypes showed decreased levels after consolidation apart from M1 which showed an increase in expression level with significant mean fold of MDR1 gene expression (2.806±0.02), this mean that increased level of MDR1 after complete remission suggested to be associated with relapse in M1 subtype. These variations in expression level of MDR1 have been reported to be of prognostic importance. M2 and M1 have far worse because 7/9 (77.7%) of patients in M2 subtype with high mean fold of MDR1 gene expression ranged (0.9-24.5 fold) at presentation were NR. These results were similar to that obtained by Balamurugan *et al*., (2007) who found that half of patients died within first induction. It is interesting to note that all patients with M3 subtype, which us a good prognostic group, showed a drop in MDR1 expression in consolidation. Although M5 patients showed similar drop MDR1 gene expression in consolidation but didn't achieve remission. In conclusion, our findings suggest that detection of positive MDR1 expression in de novo AML patients was associated with poor clinical outcome.

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